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Nicotine involved in periodontal disease through influence on cytokine levels

Keywords: animal models; nicotine; cytokines; periodontopathic bacteria; endotoxin

Running title: Influence of nicotine on cytokine levels
Abstract

Periodontal disease, for which smoking is a known risk factor, is infectious, and is associated with oral biofilm. Cytokines mediate and regulate immune and inflammatory responses. Lipopolysaccharide (LPS) produced by periodontopathic bacteria plays a role in the progression of periodontitis. The effect of nicotine on cytokine production in mice was evaluated in this study. Nicotine (10 or 200 µg/mouse) was administered intraperitoneally to 4-week-old female BALB/c mice, once a day, for 49 days. Control mice received injections of PBS. Blood was collected from all mice on the 42nd day and serum IL-6, IL-10, TNF-α and IFN-γ levels were measured by ELISA. IL-6, IL-10 and IFN-γ levels in the nicotine-treated mice were higher than those in the control mice. However, no differences were found in TNF-α levels between nicotine-treated and control mice. LPS (20 µg/mouse) purified from Aggregatibacter actinomycetemcomitans (formerly Actinobacillus actinomycetemcomitans) Y4 was administered intraperitoneally on the 49th day. We observed a rapid increase in TNF-α in the control mice at 2 h after administration of LPS. In contrast, no increase was noted in the nicotine-treated groups. Significantly higher levels of IFN-γ were seen in the 200 µg nicotine-treated mice at 2 h after administration of LPS (p<0.05). The results showed that cytokine levels were influenced by nicotine in mice.
Introduction

The main cause of periodontal disease is bacterial infection, the immune response to which can result in destruction of periodontal tissues if the ensuing inflammation persists. Periodontal disease is initiated by specific bacterial species. The local host response to these bacteria includes the recruitment of leukocytes and subsequent release of inflammatory mediators and cytokines such as interleukin (IL)-1, IL-6, IL-8, IL-10, IL-12 and tumor necrosis factor-α (TNF-α), which are thought to play an important role in the pathogenesis of this disease. Increased levels of several of these cytokines are involved in periodontal tissue destruction (Genco, 1992). Over the past two decades, smoking has been identified as a major environmental risk factor in periodontal disease (Bergström & Eliasson, 1987; Genco & Löe, 1993; Bergström & Preber, 1994; Grossi et al., 1994; 1995). Nicotine is a major component of cigarette smoke. However, there is no consensus on the mechanisms of nicotine as a risk factor. Further research is needed to elucidate the role of nicotine in periodontal disease and its influence on host response. Nicotine has been shown to increase the release of IL-6 by cultured murine osteoblasts (Kamer et al., 2006).

*Aggregatibacter actinomycetemcomitans* (formerly *Actinobacillus actinomycetemcomitans*) is a Gram-negative, non-motile capnophilic rod that has been
implicated as a causative microorganism in human periodontal disease (Slots et al., 1980). It has been demonstrated that stimulation by *A. actinomycetemcomitans* serotype b lipopolysaccharide (LPS) induces IL-4, IL-5 and IL-6 release from splenocytes in some strains of mice *in vitro* (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).

Although a number of studies have investigated the effect of smoking on inflammatory components in the periodontium in subjects diagnosed with periodontal disease (Bostrom et al., 1998; Erdemir et al., 2004; Kamma et al., 2004), in general, no conclusive results have been reported. The objective of this study was to investigate the effect of nicotine on cytokine induction by *A. actinomycetemcomitans* LPS in mice.
Materials and methods

LPS purification

*A. actinomycetemcomitans* Y4 bacterial cells were grown anaerobically at 37°C for 3 days in Trypticase soy broth (Becton Dickinson Microbiology System, Cockeysville, MD) with 0.4% yeast extract. Harvested cells were washed with phosphate-buffered saline (PBS; pH 7.2) and suspended in the same buffer. LPS was prepared by the hot phenol-water method (Westphal & Jann, 1965) and purified as described previously (Kato et al., 2006). LPS fractions were assessed for purity by SDS-polyacrylamide gel electrophoresis using silver staining. The LPS fractions revealed slow migrating and repeating ladder bands, forming a typical LPS pattern. The LPS fraction was dialyzed against pyrogen-free water and lyophilized.

Effects of nicotine on serum cytokine levels in mice

Nicotine (Product Number N5511) was obtained from Sigma Chemical Co. (St. Louis, MO). Four-week-old female BALB/c mice were divided into 2 nicotine-treated groups (10 µg/mouse group, 5 mice; 200 µg/mouse group, 6 mice) and a non-nicotine-treated control group (5 mice). Nicotine (10 or 200 µg/mouse) was administered intraperitoneally once a day for 49 days. Control mice received injections of PBS. Blood was collected from all mice on the 42nd day and serum was separated. Serum
IL-6, IL-10, TNF-α and IFN-γ levels were measured using the Endogen ELISA kit (Pierce Biotechnology, Inc., Rockford, IL). Next, *A. actinomycetemcomitans* Y4 LPS (20 µg/mouse) was administered on the 49th day in all groups including control and the effect of nicotine on LPS cytokine induction was determined. Blood was collected at 2 h and 24 h after LPS administration and serum was separated. All animals were treated in accordance with “The Guidelines for the Treatment of Experimental Animals at Tokyo Dental College”.

**Statistical analyses**

Data are expressed as means ± standard deviations. Statistical analysis was performed with Mann-Whitney *U*-test and where a probability of less than 0.05 was considered statistically significant.
Results

No mice died after nicotine injection during the course of the study. The body weight of each mouse was measured at 6 weeks of age. No significant difference was found between the body weights of the mice treated with nicotine and those of the control mice.

IL-6, IL-10 and IFN-γ levels in the 200 µg nicotine-treated mice were higher than those in the control mice, at p<0.01, p<0.05 and p<0.02, respectively (Fig. 1). However, no differences were found between the nicotine-treated and control mice in terms of TNF-α level.

Fig. 2 shows the effects of nicotine on A. actinomycetemcomitans LPS-induced cytokine levels. IL-6 levels showed a remarkable increase after stimulation with LPS. Although IL-6 levels were higher at 2 h and 24 h after LPS administration in 10 µg and 200 µg nicotine-treated mice, the differences were not significant. A rapid increase in TNF-α was observed in the control mice at 2 h after administration of LPS. In contrast, no increase was noted in the nicotine-treated groups. In addition, TNF-α levels in the nicotine-treated groups were significantly lower than those in the control group (nicotine-treated group, p<0.0001 in comparison with the controls). At 24 h after administration of LPS, TNF-α levels showed a decrease compared to at baseline in all
mice tested. No marked change was seen in IFN-γ levels in the controls or 10 µg nicotine-treated mice at 2 h after administration of LPS. However, significantly higher levels of IFN-γ were seen in the 200 µg nicotine-treated mice (p<0.05). IL-10 levels showed an increase after stimulation with LPS, although no significant difference was seen between the nicotine-treated and control mice.
Discussion

The results of the present study demonstrated that cytokine levels were influenced by nicotine. *A. actinomycetemcomitans* is a widely studied human periodontopathogen, and is believed to play a role in periodontal disease. *A. actinomycetemcomitans*-induced periodontal disease is an interesting model for the investigation of the mechanisms of both tissue destruction and control of infection in the peridontium, recommending this microorganism for use in this study. IL-6 levels in all groups showed a remarkable increase with *A. actinomycetemcomitans* LPS stimulation. At the physiological level, IL-6 plays an important role in the cytokine network. However, excessive production of IL-6 in response to exposure to LPS has an inflammatory effect, resulting in injury. Many cell types produce IL-6, but it is not clear whether the IL-6 produced by different cell types acts in the same way. LPS-induced IL-6 may be mainly derived from macrophages. Nicotine-induced IL-6 may be derived from different cells. The possibility of inhibition of TNF-α production by IL-6 has been reported in several studies (Fiers, 1991; Matthys et al., 1995; Mizuhara et al., 1994). In this study, significantly lower level of TNF-α in the nicotine-treated group with *A. actinomycetemcomitans* LPS stimulation may have been due to inhibition of TNF-α production by nicotine-induced IL-6.
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). Previous reports (Baker et al., 1999; Gorska et al., 2003; Kawai et al., 2000) have suggested that IFN-γ+ Th1 cells are strongly associated with enhanced alveolar bone loss during periodontal infections, and that high absolute levels of proinflammatory cytokines, including IFN-γ, are closely associated with degree of severity in periodontal disease. In addition, it has been reported that 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). In contrast, it has also been shown that IFN-γ may directly inhibit RANKL-mediated osteoclastogenesis in in vitro and animal studies (De Klerck et al., 2004; Takayanagi et al., 2000). Teng et al. (2005) clearly demonstrated a positive co-expression relationship between IFN-γ and RANKL-mediated osteoclastogenesis in a mouse periodontitis model. 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. In the present study, significantly higher levels of IFN-γ were seen in the 200 μg nicotine-treated mice (p<0.02), suggesting that this factor is
involved in periodontal tissue destruction.

We observed an increase in IL-10 levels in the nicotine-treated mice. The ability of IL-10 to inhibit cytokine synthesis by helper T cells was found to be due to its inhibitory effect on macrophage-monocytes (Fiorentino et al., 1991). IL-10 has been shown to reduce neutrophil-dependent bacterial killing (Laichalk et al., 1996). High levels of IL-10 may benefit periodontopathic bacteria survival.

This paper is the first to demonstrate changes in cytokine levels in mice continuously treated with nicotine for around 1 month. Nicotine may play an important role in the mechanism by which smoking induces periodontal diseases. Further studies are required to evaluate the impact of these findings on the understanding of the mechanisms by which smoking affects periodontal disease.

In conclusion, the results of the present study suggest that nicotine affects the immune response through disturbance of the cytokine network that plays a crucial role in the regulation of periodontal tissue inflammation.

Acknowledgements

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Figure legends

Fig. 1 Effects of nicotine on serum IL-6, IL-10, TNFα and IFNγ levels.

*, p<0.01 compared with control (Mann-Whitney U-test)

**, p<0.05 compared with control (Mann-Whitney U-test)

***, p<0.02 compared with control (Mann-Whitney U-test)

Fig. 2 Effects of nicotine on LPS-induced serum IL-6, IL-10, TNFα and IFNγ levels.

*, p<0.0001 compared with control (Mann-Whitney U-test)

**, p<0.05 compared with control (Mann-Whitney U-test)