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L-arginine-dependent nitric oxide production of a murine macrophage-like RAW 264.7 cell line stimulated with *Porphyromonas gingivalis* lipopolysaccharide

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The aim of this study was to determine nitric oxide (NO) production of a murine macrophage cell line (RAW 264.7 cells) when stimulated with *Porphyromonas gingivalis* lipopolysaccharides (Pg-LPS). RAW264.7 cells were incubated with i) various concentrations of Pg-LPS or *Salmonella typhosa* LPS (St-LPS), ii) Pg-LPS with or without L-arginine and/or N^G-monomethyl-L-arginine (NMMA), an arginine analog or iii) Pg-LPS and interferon-γ (IFN-γ) with or without anti-IFN-γ antibodies or interleukin-10 (IL-10). Tissue culture supernatants were assayed for NO levels after 24 h in culture. NO was not observed in tissue culture supernatants of RAW 264.7 cells following stimulation with Pg-LPS, but was observed after stimulation with St-LPS. Exogenous L-arginine restored the ability of Pg-LPS to induce NO production; however, the increase in NO levels of cells stimulated with Pg-LPS with exogenous L-arginine was abolished by NMMA. IFN-γ induced independent NO production by Pg-LPS-stimulated macrophages and this stimulatory effect of IFN-γ could be completely suppressed by anti-IFN-γ antibodies and IL-10. These results suggest that Pg-LPS is able to stimulate NO production in the RAW264.7 macrophage cell model in an L-arginine-dependent mechanism which is itself independent of the action of IFN-γ.

Key words: lipopolysaccharide; nitric oxide; macrophages; *Porphyromonas gingivalis*; RAW264.7 cells

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Nitric oxide (NO) is a highly reactive free radical, produced as a result of the metabolism of L-arginine catalyzed by nitric oxide synthase (NOS) (22). NO is an essential element of antimicrobial and tumor immunity as well as autoimmunity (17, 20). So far, three NOS isoforms – the neural form (nNOS), endothelial form (eNOS) and inducible form (iNOS) – have been reported (22). Bacterial lipopolysaccharides (LPS) and interferon (IFN)-γ are known to be potent inducers of NO production by murine macrophages. Tumor necrosis factor (TNF)-α and interleukin (IL)-12 may upregulate the production of NO by murine macrophages only in the presence of IFN-γ (15, 29), indicating that both TNF-α and IL-12 may amplify IFN-γ-induced signal transduction of iNOS activation. On the other hand, IL-10, IL-4 and tumor growth factor (TGF)-β have been reported, under certain experimental conditions, to inhibit the production of macrophage NO (6, 7, 29). These reports suggest that the production of NO is controlled by cytokines in the surrounding milieu (17, 20, 22).

Lipopolysaccharide (LPS) isolated from the major periodontal pathogen *Porphyromonas gingivalis* (Pg-LPS) is known to be one of its most virulent factors. LPS is capable of inducing bone resorption (25), producing connective tissue-degrading matrix metalloproteinases (30), and inducing cytokine production by gingival cells (1, 4, 39) and apoptosis (16), all of which may contribute significantly to both the host defense and periodontal tissue destruction. The lipid A component of Pg-LPS is structurally different to that of *Escherichia coli* LPS (Ec-LPS) and is less potent in inducing a lethal endotoxic shock in animal models. Pg-LPS and Ec-LPS are able to induce cell proliferation, cytokine production and CD14 expression of mononuclear phagocytes in a similar fashion (28, 31, 36), indicating that they may have the same potential ability to activate monocytes. Previous reports have shown that NO could be produced by murine macrophages when stimulated with Pg-LPS; however, Pg-LPS was far less potent than LPS from enteric bacteria (9, 32, 36). We recently demonstrated that Pg-LPS failed to induce NO production by murine spleen cells (33). The aim of this study was to determine the regulation of NO production by RAW 264.7 cells, a murine macrophage cell line, stimulated with Pg-LPS.

Materials and methods

Antigen preparations

P. gingivalis ATCC33277 was grown as previously described (13). The periodontopathic bacterium *P. gingivalis* ATCC 33277 (a gift from the late Dr Daria Love, Department of Veterinary Anatomy and Pathology, The University of Sydney) was revived from liquid nitrogen stocks and grown on trypticase soy agar (TSA) (30 g/l) supplemented with yeast extract (5 g/l), L-cysteine hydrochloride (0.5 g/l), sodium formate (2 g/l), sodium fumerate (3 g/l), menadione (1 mg/l), hemin (0.5 mg/l) and 5% defribrinated horse blood. All materials for bacterial growth were purchased from BBL (Becton Dickinson, Cockeysville, MD). Cultures were incubated at 37°C in an atmosphere of 80% N_2, 10% CO_2, and 10% H_2 in anaerobic jars for 4–5 days. Identification was confirmed by the black-pigmented colonies, gram-negative coccobacilli, positive hemagglutination of sheep red cells and a positive trypsin activity. *P. gingivalis* was expanded in 500 ml batch cultures of brain heart infusion broth (Difco Laboratories, Detroit, MI) supplemented with yeast extract (3 g/l), trypticase peptone (BBL) (10 g/l), sodium bicarbonate (2 g/l), hemin (1 mg/l) and menadione (0.5 mg/l). Bacteria were incubated at 37°C in an atmosphere of 80% N_2, 10% CO_2, and 10% H_2 in an anaerobic cabinet for 48–72 h. Purity was assessed by Gram stain and colonial morphology on TSA. Bacteria were harvested at late log phase by centrifugation and washed in 0.15 M sodium chloride. LPS was isolated and purified from *P. gingivalis* cells as previously described (13). Briefly, *P. gingivalis* cells were suspended in distilled water for 20 min and stored. The aqueous phase was obtained by centrifugation at $10{,}000 \times g$ for 30 min, dialyzed against distilled water and ultracentrifuged at $100{,}000 \times g$ at 4°C for 3 h. The pellet was resuspended in saline and subsequently treated with ribonuclease A (0.02 mg/ml) (Sigma Co., St. Louis, MO) for 2 h at room temperature, deoxyribonuclease 1 (0.005 mg/ml) (Sigma) for 6 h at room temperature, and pronase (0.05 mg/ml) (Calbiochem, San Diego, CA) overnight at 37°C. The material was then dialyzed against distilled water, centrifuged at $100{,}000 \times g$ for 3 h at 4°C, and the pellet resuspended in distilled water and lyophilized. Trace amounts of protein were detected by BCA Protein Assay (Pierce, Rockford, IL) and the typical ladder-like pattern on gel following polyacrylamide electrophoresis and silver staining (Bio-Rad Laboratories Inc. Hercules, CA). LPS of *Salmonella typhosa* (St-LPS) was obtained from a commercial supplier (Sigma).

Cell cultures

RAW 264.7 cells, a murine macrophage-like cell line, were grown in RPMI 1640 medium (Sigma) supplemented with 1% penicillin-streptomycin, 1% glutamine, 10% fetal calf serum (Sigma) and incubated at 37°C in a 5% CO_2 in an air humidified incubator. Cells were harvested when grown to confluence, and then washed in RPMI medium. Cell numbers were adjusted to 1×10^6 cells/ml and 200 μl of the cell suspension was added to wells of a sterile 96-well plate (Nuncl, Roskilde, Denmark) and incubated with various concentrations of Pg-LPS or St-LPS. After 24 h, culture supernatants were harvested and immediately analyzed for NO production. In other experiments, cells were incubated with Pg-LPS (100 ng) and various concentrations of L-arginine. To determine the role of exogenous L-arginine (Sigma), various concentrations of N^G-monomethyl-L-arginine (NMMA) (Sigma), an arginine analog, were added to the cells stimulated with Pg-LPS (100 ng) and L-arginine (500 μM).

To determine the effect of recombinant murine IFN-γ (rMuIFN-γ) (R&D System, MN) on NO production of Pg-LPS-stimulated RAW 264.7 cells, various concentrations of this cytokine were added to the cell cultures stimulated with 100 ng of Pg-LPS. RAW264.7 cells stimulated with 100 ng St-LPS served as a control. The stimulatory effect of IFN-γ on NO production was further examined by adding various dilutions of polyclonal antibodies anti-rMuIFN-γ to the cell cultures stimulated with 100 ng Pg-LPS and 200 units of rMuIFN-γ. Serum anti-rMuIFN-γ antibodies were generated by intraperitoneal injections of this cytokine (100–200 units/100 μl PBS) in rabbits, weekly for 3 weeks. One week after the final immunization, peripheral blood was collected from an ear vein and serum isolated. Anti-rMuIFN-γ antibody activity was assessed by an ELISA kit according to the manufacturer's instructions (R&D). RAW264.7 cells stimulated with 100 ng St-LPS, 100 ng Pg-LPS alone or 100 ng Pg-LPS and 200 units of rMuIFN-γ served as controls.

The effect of IL-10 on NO production was determined by adding various concentrations of recombinant mouse IL-10 (rMuIL-10) (R&D) to the cell cultures stimulated with 100 ng Pg-LPS and 200 units of rMuIFN-γ. RAW264.7 cells stimulated with either 100 ng St-LPS, 100 ng Pg-LPS alone or 100 ng Pg-LPS and 200 units of rMuIFN-γ served as controls.

Nitric oxide determination

All cultures were in triplicate and each experiment was repeated three times. After culture for 24 h, the levels of NO from the culture supernatants were assessed by the Griess reaction (33, 35). Culture supernatant 100 μl was mixed with an equal volume of the Griess reagent (1% sulfanilamide, 0.1% naphthlethylenediamine dihydrochloride in 2.5% phosphoric acid) and read in a microplate reader (Titertek Multiscan, Flowlab, North Ryde, Denmark) at 540 nm. Nitrite concentration was calculated from a standard curve prepared with sodium nitrite. All reagents for NO assay were purchased from Sigma.

Statistical analysis

An one-way analysis of variance to determine the differences between the groups within one experiment was carried out using a software package (Minitab Inc., State College, PA).

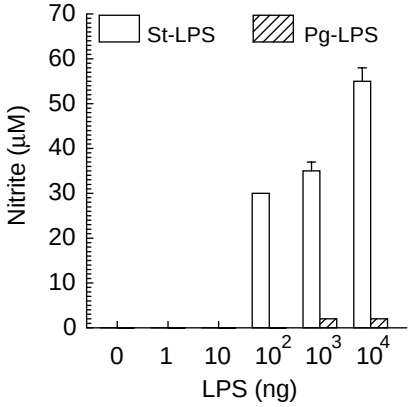

Fig. 1. Mean and standard deviation of NO production of RAW264.7 cells stimulated with *P. gingivalis* LPS. The cells were incubated with various concentrations of *P. gingivalis* LPS (Pg-LPS) or *S. typhosa* LPS (St-LPS) for 24 h. NO levels were determined in the culture supernatants. Where the bar is not shown, the error was so small as not to be graphically distinct.

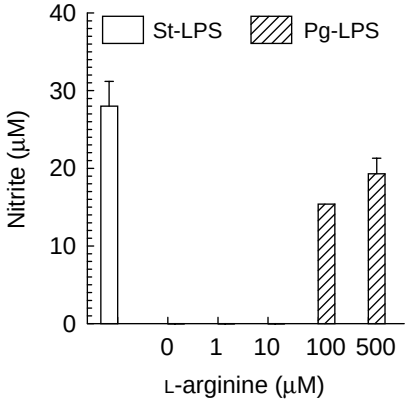

Fig. 2. Mean and standard deviation of NO production of RAW 264.7 cells stimulated with *P. gingivalis* LPS with additional L-arginine. The cells were incubated with 100 ng *S. typhosa* LPS (St-LPS) or 100 ng *P. gingivalis* LPS (Pg-LPS) plus L-arginine for 24 h. NO levels in the culture supernatants were determined and the levels of unstimulated cell cultures subtracted. Where the bar is not shown, the error was so small as not to be graphically distinct.

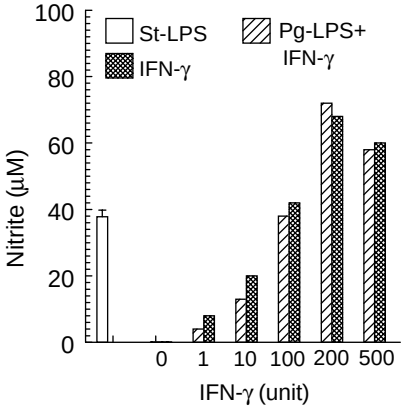

Fig. 4. Mean and standard deviation of NO production of RAW 264.7 cells stimulated with *P. gingivalis* LPS and additional IFN-γ. The cells were stimulated with 100 ng of *S. typhosa* LPS (St-LPS) or 100 ng of *P. gingivalis* LPS (Pg-LPS) with rIFN-γ or with rIFN-γ alone for 24 h. NO levels in the culture supernatants were determined and the levels of unstimulated cell cultures subtracted. Where the bar is not shown, the error was so small so as not to be graphically distinct.

Results

Macrophages stimulated with Pg-LPS

An increased production of NO (50–60 μM of nitrite concentration) could be seen in RAW264.7 cells stimulated with St-LPS in a dose-dependent fashion ($P < 0.05$) (Fig. 1). However, only very low levels of NO could be observed (1–2 μM of nitrite concentration) when the cells were stimulated with 10 μg Pg-LPS. Pg-LPS was therefore approximately 60-fold less potent than St-LPS in inducing NO production by RAW264.7 cells.

Effects of L-arginine

As Pg-LPS failed to induce NO production by RAW264.7 cells, the next experiment was carried out to confirm whether exogenous L-arginine is a prerequisite in Pg-LPS-induced NO production by RAW 264.7 cells. As seen in Fig. 2, NO was detected when 100 μM of L-arginine was added to the cultures containing cells stimulated with 100 ng Pg-LPS ($P > 0.01$). The production of NO was further elevated after adding 500 μM of L-arginine ($P > 0.01$), confirming that the ability of Pg-LPS to induce NO production by RAW 264.7 cells depends on the presence of L-arginine.

To establish whether exogenous L-arginine was a prerequisite for NO synthesis, NMMA was added to the macrophage cultures containing Pg-LPS (100 ng) and L-arginine (500 μM). NO production was suppressed by the addition of 10 μM of NMMA and completely blocked by 100 μM of this arginine analog, suggesting that the L-arginine was indeed essential for NO production by Pg-LPS-stimulated RAW 164.7 cells (Fig. 3).

Effects of IFN-γ

As with bacterial LPS, IFN-γ is a potent macrophage activator capable to inducing NO production. The next experiment was carried out to determine whether this cytokine can upregulate NO production by Pg-LPS-stimulated RAW 264.7 cells. Figure 4 shows that NO production could be observed in the cells stimulated with 10 units of IFN-γ and 100 ng of Pg-LPS and was not observed in cells stimulated only with Pg-LPS ($P > 0.01$). The maximum stimulation was recorded with an additional 200 units of IFN-γ these levels were higher than those produced by cells stimulated only with St-LPS. When cells were stimulated with IFN-γ only, NO levels were comparable to those of cells stimulated with both Pg-LPS and IFN-γ ($P < 0.01$). This indicated that IFN-γ did not augment the ability of Pg-LPS to induce NO production by RAW 264.7 cells. To investigate this further, two experimental strategies were employed. Firstly, the cells were stimulated with Pg-LPS (100 ng) with or without 200 units of rMuIFN-γ and diluted rabbit anti-MuIFN-γ antibodies for 24 h. Culture supernatants were then collected and the NO levels determined. The effect of IFN-γ on NO production of Pg-LPS-stimulated RAW 264.7 cells could be completely abolished by rabbit anti-IFN-γ antibodies at a dilution of 1 : 10 and in a dose-dependent manner (Fig. 5). Secondly, it has been reported that IL-10 suppresses NO production of IFN-γ-activated murine macrophages (10). To elucidate the effect of IL-10 on NO production, RAW 264.7 cells were incubated with Pg-LPS (100 ng) plus IFN-γ (200 units) with as well as without rMuIL-10. The effect of IFN-γ on NO

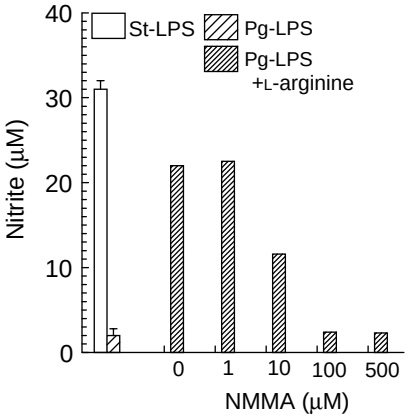

Fig. 3. Mean and standard deviation of NO production of RAW 264.7 cells stimulated with *P. gingivalis* LPS and additional L-arginine with or without NMMA. The cells were incubated with 100 ng of *S. typhosa* LPS (St-LPS) or 100 ng of *P. gingivalis* LPS (Pg-LPS) plus 500 μM of L-arginine with or without NMMA for 24 h. NO levels in the culture supernatants were determined and the levels of unstimulated cell cultures subtracted. Where the bar is not shown, the error was so small as not to be graphically distinct.

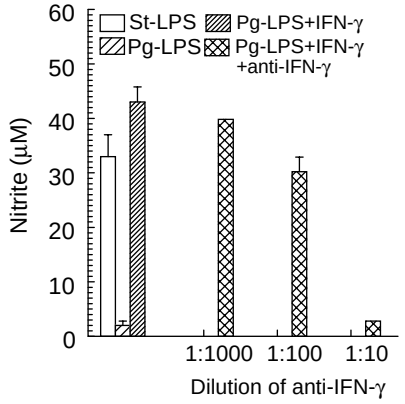

Fig. 5. Mean and standard deviation of NO production of RAW 264.7 cells stimulated with *P. gingivalis* LPS and IFN-γ with or without anti-IFN-γ antibodies. The cells were stimulated with 100 ng of *S. typhosa* LPS (St-LPS) or 100 ng of *P. gingivalis* LPS (Pg-LPS) with or without 200 units of rIFN-γ or with 100 ng of *P. gingivalis* LPS (Pg-LPS) plus 200 units of rIFN-γ and diluted anti-IFN-γ antibodies alone for 24 h. NO levels in the culture supernatants were determined and the levels of unstimulated cell cultures subtracted. Where the bar is not shown, the error was so small as not to be graphically distinct.

production of Pg-LPS-stimulated RAW 264.7 cells was completely suppressed by 100 ng of rMuIL-10 ($P < 0.05$) (Fig. 6). These results support the view that the stimulatory effects of IFN-γ-induced NO production by macrophages may be independent of Pg-LPS.

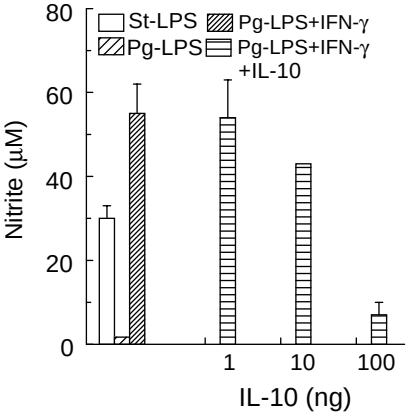

Fig. 6. Mean and standard deviation of NO production of RAW 264.7 cells stimulated with *P. gingivalis* LPS and IFN-γ with or without IL-10. The cells were stimulated with 100 ng of *S. typhosa* LPS (St-LPS) or 100 ng of *P. gingivalis* LPS (Pg-LPS) with or without 200 units of rIFN-γ or with 100 ng of *P. gingivalis* LPS (Pg-LPS) plus 200 units of rIFN-γ and various concentrations of IL-10 for 24 h. NO levels in the culture supernatants were determined and the levels of unstimulated cell cultures subtracted. Where the bar is not shown, the error was so small as not to be graphically distinct.

Discussion

The results of the present study show that following stimulation with Pg-LPS, the macrophage cell line, RAW 264.7 cells, did not produce NO, whereas following St-LPS stimulation, these cells induced NO production. This may be due to the structural differences between the biologically active component, lipid A of St-LPS and Pg-LPS which may, in turn, lead to difference degrees of bacterial virulence. For example, lipid A of Pg-LPS-induced mitogenic cell proliferation in both LPS-non-responder (C3H/HeJ) and –responder (C3H/HeN) mice, but was less capable of stimulating IL-1 α mRNA expression than lipid A from enteric bacterial LPS (27, 28), indicating that lipid A of Pg-LPS is less virulent than that of enteric bacteria. Tanamoto et al. showed that lipid A of Pg-LPS induced NO production by murine macrophage-like cells (J774-1 cells), but it was 100-fold less than that produced by the cells stimulated with lipid A of enterobacterial LPS (36). *Francisella tularensis* LPS, which is structurally different from that of enteric bacteria, also failed to induce NO production by murine peritoneal macrophages (3). This is further supported by the observation that detectable NO levels were produced by murine macrophage cell lines stimulated with *Actinobacillus actinomycetemcomitans* LPS, which is structurally similar to that of enteric bacteria (5, 35). Furthermore, unlike the results of the present study, Frolov et al. have found that peritoneal macrophages harvested after an intraperitoneal injection with thioglycolate or heat-killed *P. gingivalis* and then restimulated with Pg-LPS *in vitro* produced detectable levels of NO but not TNF-α (9). The reason for this discrepancy is not clear. Pre-exposure of mice to antigens such as heat-killed *P. gingivalis in vivo* might preferentially preactivate macrophages to produce NO production *in vitro*. Alternatively, the differences between the present study and previous reported results on Pg-LPS-induced NO production by murine macrophages may be related to different bacterial strains of *P. gingivalis* used in the studies. Shapira and colleagues reported that the LPS of *P. gingivalis* strain A7436 induced low levels of NO by murine peritoneal macrophages, while that *P. gingivalis* W50 enhanced both TNF-α and NO secretion (32). Thus, it is possible that the Pg-LPS from ATCC 33277 used in the present study may be a non-inducer of NO similar to that of A7436.

The requirement of L-arginine to generate NO, a reaction catalyzed by NOS, is well known (17, 20, 22). Bacterial LPS alone is able to induce NO production by RAW 264.7 cells (12, 35), indicating that endogenous L-arginine in these cells is adequate for optimal NO production. NO production could be detected if exogenous L-arginine was added in the cultures of Pg-LPS-stimulated RAW 264.7 cells, indicating an exogenous L-arginine-dependent mechanism. The exogenous L-arginine would be converted by NOS to generate NO, since the arginine analog, NMMA, suppressed NO production of RAW 264.7 cells in the presence of Pg-LPS and L-arginine. The exact mechanism by which Pg-LPS-stimulated murine macrophages require exogenous L-arginine to produce NO is unknown. Presumably, the addition of exogenous L-arginine to Pg-LPS-stimulated murine macrophages may act as a signal to elevate the production of intracellular cationic amino acid transporter 2, an essential protein for L-arginine uptake, thereby increasing L-arginine metabolism (18).

Noda & Amano showed that, in the presence of IFN-γ, bacterial LPS-stimulated RAW 264.7 cells produced 5–6 times more NO than cells stimulated with LPS alone, indicating that this cytokine potentiates NO production of LPS-stimulated macrophages (26). However, in the present study, increased NO production of RAW264.7 cells incubated with Pg-LPS plus IFN-γ was not significantly different from that of cells stimulated with IFN-γ alone, suggesting that this cytokine may fail to act as a cofactor to restore the effects of Pg-LPS on macrophage-induced NO synthesis. Indeed, the addition of anti-MuIFN-γ antibodies to RAW264.7 cells that were incubated with Pg-LPS plus IFN-γ led to a complete suppression of the effect of this cytokine on NO production, confirming an independent mechanism of IFN-γ and Pg-LPS on NO synthesis. Both LPS and IFN-γ regulated mouse NOS transcription at different sites of the NOS promoters. LPS involves binding of necrosis factor (NF)-κB heterodimers, p50/c-Rel and p50/RelA at the NOS promoter, whereas IFN-γ involves binding of IFN-1 in the NOS promoter (23, 37). The failure of IFN-γ to upregulate the effect of Pg-LPS on NO production seen in the present study shows that Pg-LPS may be unable to bind the NOS promoter complex and, subsequently, be unable to inducte of NOS transcription, whereas IFN-γ remains independent of the induction of binding of IRF-1 to the NOS promoter, leading to stimulation of RAW 264.7 cell-induced NO production.

Following the addition of IL-10, NO production by RAW 264.7 cells stimulated with Pg-LPS and IFN-γ was suppressed, confirming that increased NO production was due solely to the stimulatory effect of the latter cytokine, but not Pg-LPS. It seems plausible that the mechanism by which IL-10 inhibits NO production of IFN-γ-activated macrophages is through suppression of TNF-α, a cytokine which potentiates the effect of IFN-γ on NO production (6, 29).

The extrapolation of this study in the course of CIPD remains speculative. Increased levels of L-citruline, a metabolism of L-arginine catalyzed by iNOS, observed in homogenized gingival tissues of patients with periodontal disease have been reported, suggesting that high levels of NO production may be synthesized by inflamed gingiva (24). Indeed, iNOS at both gene and protein levels was increased in the inflamed gingiva as compared with healthy gingiva (14, 19, 21). In these studies, this enzyme appeared to be produced by gingival macrophages, fibroblasts and endothelial cells. Furthermore, Kendal et al. demonstrated that higher levels of iNOS expression of gingival fibroblast cultures were only observed when the cultures were stimulated with a combination of IFN-γ and *P. gingivalis* LPS and not with only one of these stimulators (19). However, that study did not examine whether gingival macrophages/monocytes might produce NO after stimulation with IFN-γ and *P. gingivalis* LPS. Nevertheless, the results of the presence study seem to support this previous study in sense that only in the presence of IFN-γ can *P. gingivalis* LPS stimulate NO production. Previous reports have shown that oral bacterial LPS is absorbed in the dental root surfaces and gingival tissues of patients with periodontal disease (2, 8). Increased IFN-γ levels in gingival tissues of these patients have been documented (11, 37, 38). It is possible, therefore, that both oral bacterial LPS and IFN-γ may be partly responsible for the elevated NO production observed in inflamed gingiva (19, 21, 24). As yet, the present study also indicated that IL-10 may downregulate RAW264.7 cell-derived NO production induced by both *P. gingivalis* LPS and IFN-γ. A role for high levels of IL-10 has been suggested in the development of destructive periodontal disease (11, 34). The levels of NO in the inflamed gingiva may be dependent on the cytokine profiles in the gingival milieu and, thus, on the stages of periodontal disease.

Acknowledgments

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