ASIAN PACIFIC JOURNAL OF ALLERGY AND IMMUNOLOGY (2000) 18: 209-214

Nitric Oxide Production by Murine Spleen Cells Stimulated with *Porphyromonas gingivalis*-Derived Lipopolysaccharide

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SUMMARY **The aim of the present study was to determine whether *Porphyromonas gingivalis*-lipopolysaccharide (Pg-LPS) may stimulate nitric oxide (NO) production by murine spleen cells. Spleen cells derived from Balb/c mice were cultured in the presence of Pg-LPS or LPS from *Salmonella* Typhosa. The cell were also cultured in the presence of Pg-LPS with or without L-arginine, L-arginine plus N^G-monomethyl-L-arginine (NMMA), or IFN-gamma. Furthermore, the plastic non-adherent spleen cells were stimulated with Pg-LPS and L-arginine. The results showed that Pg-LPS failed to stimulate splenic NO production by themselves. Exogenous L-arginine or IFN-gamma up-regulated the NO production of Pg-LPS-stimulated spleen cells, but the stimulatory effects of L-arginine were completely blocked by NMMA. It was also demonstrated that in the presence of Pg-LPS and L-arginine, splenic macrophages were the cellular source of NO. These results suggest, therefore, that *P. gingivalis*-LPS may induce murine splenic macrophages to produce NO in a L-arginine and an IFN-gamma-dependent mechanism.**

Nitric oxide (NO) is a gaseous free radical and known to help regulate the immune response since NO is produced by the immunocompetent cells such as macrophages. Indeed, this molecule has been shown to play an important role in the development of autoimmune diseases as well as bacterial, fungal, parasitic and tumor induced immune responses.[1] The biosynthesis of NO is dependent on the presence of L-arginine which is catalyzed by nitric oxide synthetase (NOS) to generate citrulline and NO. Cytokines such as IFN-gamma and bacterial antigens such as lipopolysaccharide (LPS), known as a macrophage activator, are all capable of inducing NO production by macrophages. Previous evidences have indicated that murine spleen cells also produced NO when stimulated with antigens such as pertussis toxin and *Escherichia coli*-derived fimbria.[2,3]

Porphyromonas gingivalis, a black pigmented gram negative bacterium, has been implicated in the development of chronic inflammatory periodontal disease (CIPD). *P. gingivalis*-LPS (Pg-LPS) is well known to be one of the major virulence factors capable of stimulating host immune responses and periodontal tissue destruction.[4-6] The ability of this bacterial factor to induce NO production by murine spleen cells is, however, not known. Recent evidences have demonstrated that NO could be produced by murine peritoneal macrophages stimulated with Pg-LPS, although the levels of these gaseous molecules were 100 fold less than those produced by cells stimulated with LPS from *E. coli*.[7-9] In contrast, we have recently found that Pg-LPS may stimulate NO production of a murine macrophage-like cell line (RAW264.7 cells) in the presence of exogenous L-arginine, suggesting that NO production of murine macrophages stimulated with Pg-LPS is an exogenous L-arginine-dependent mechanism (Sosroseno

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et al., unpublished results). The aim of this study therefore was to determine whether Pg-LPS may stimulate NO production by murine spleen cells

MATERIALS AND METHODS

Female 6-8 weeks old Balb/c mice were sacrificed by CO_2 asphyxiation. Spleen were aseptically excised and spleen cells obtained through mincing on a sterile steel grid. After washing and counting the viable cells, 2 x 10^5 cells in 200 µl of RPMI-1640 medium, supplemented with 10% fetal calf serum, 1% glutamine and 1% antibiotics were cultured in a 96 round bottom well plate (Nunc, Denmark). All reagents for cell cultures were purchased from Sigma Chemical Co., St. Louis, MO. *P. gingivalis* ATCC33277-derived lipopolysaccharide (Pg-LPS), a generous gift from Professor Greg Seymour (Univeristy of Queensland, Brisbane, Australia), at various concentrations was used. As a positive control, cells were cultured in the presence of *Salmonella* Typhosa-LPS (ST-LPS) (Sigma). In other experiments, cell cultures were added with or without L-arginine (Sigma), L-arginine plus N^G-monomethyl-L-arginine (NMMA) (Sigma), or recombinant mouse IFN-gamma (R & D System, MN). In order to determine whether splenic macrophages were responsible for NO production, spleen cells were fractionated into adherent and non-adherent cells by adding 1 x 10^8 cells in 10 ml of RPMI-1640 medium with 5% fetal calf serum into a sterile polystyrene petri dish for 2 hours at 37°C. The non-adherent cells were harvested by washing the plates two times with warm medium containing fetal calf serum. These cells microscopically contained less than 1% macrophages as determined by Giemsa staining and 2 x 10^5 cells in 200 µl of the culture medium were cultured in the presence of 10 µg of Pg-LPS and 500 µM of L-arginine. All cultures were done in triplicates and incubated in a humidified atmosphere of 5% CO_2 in air at 37°C for 4 days.

Nitric oxide (NO) production was determined by the Griess reaction.[2] Briefly, 100 µl of culture supernatants were mixed with an equal volume of the Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid) and read in an automated plate reader at 540 nm. Nitrite levels were calculated from a standard curve prepared with sodium nitrite. All reagents for NO assay were purchased from Sigma. NO levels of samples were deduced by those of unstimulated cell cultures.

The data were analyzed by one-way analysis of variance using the Minitab package (Minitab Inc., State College, PA).

RESULTS

Spleen cells were cultured in the presence of ST-LPS or Pg-LPS for 4 days and subsequently, NO production was assessed from the culture supernatants (Fig. 1). NO was detected when spleen cells were stimulated with 10 ng of ST-LPS. The levels of this gaseous molecule in the cultures stimulated with 100 ng of ST-LPS were not significantly different as compared with those from the cultures stimulated with 10 ng of the same antigen ($p > 0.05$). In contrast, NO

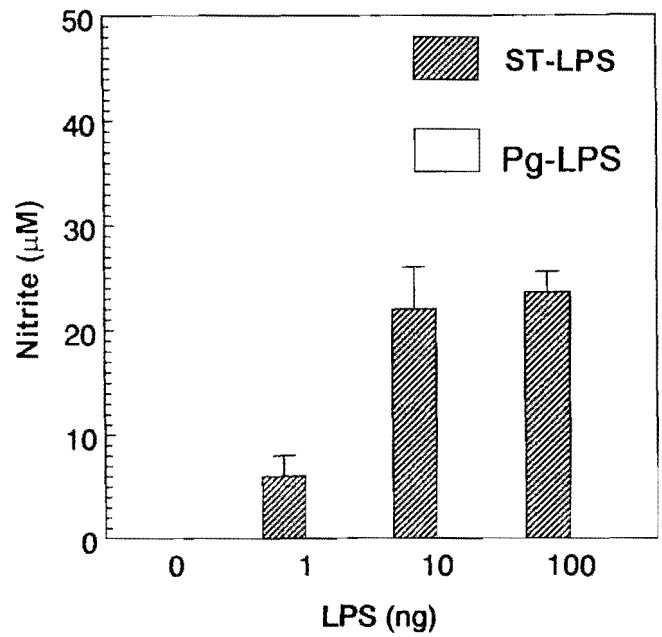

Fig. 1 Mean and standard deviation of NO produced by lipopolysaccharide-stimulated murine spleen cells. Balb/c-derived spleen cells were stimulated with *P. gingivalis*-LPS (Pg-LPS) or *Salmonella* Typhosa-LPS (ST-LPS). After 4 days, NO was assessed from the culture supernatants.

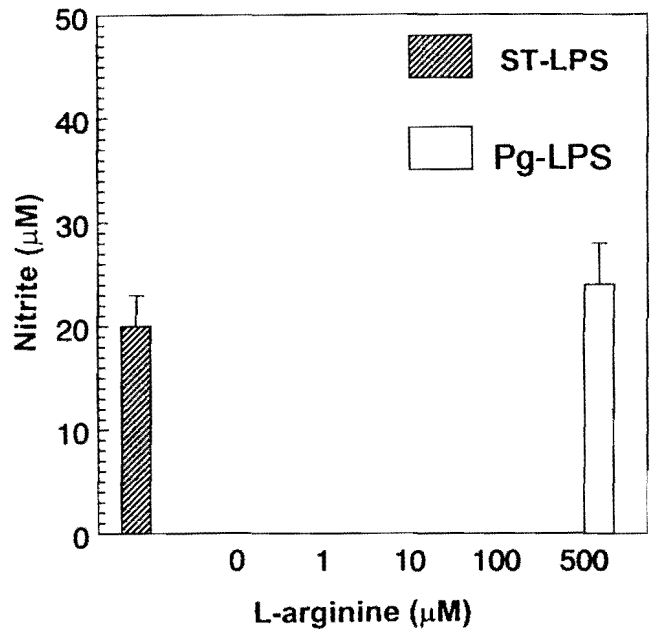

Fig. 2 The effects of exogenous L-arginine on the mean and standard deviation of NO produced by murine spleen cells stimulated with *P. gingivalis*-LPS (Pg-LPS). As a control, the cell cultures were stimulated with *Salmonella* Typhosa-LPS (ST-LPS).

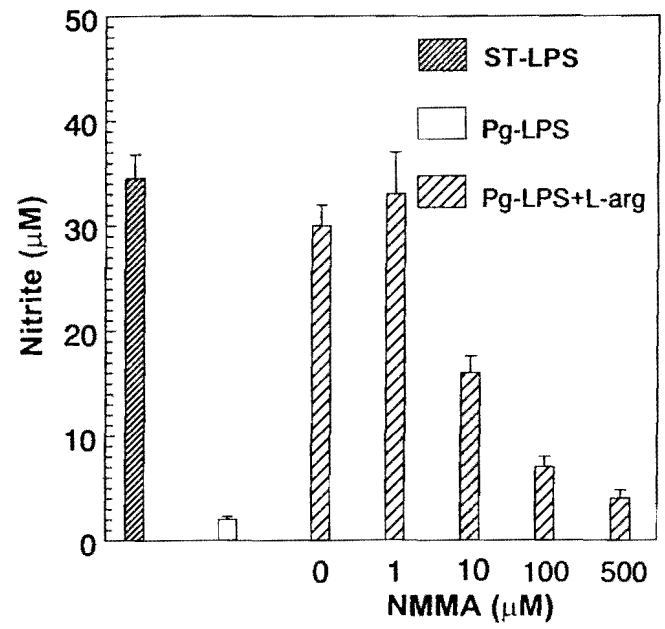

Fig. 3 The effects of NMMA, a L-arginine analog, on the mean and standard deviation of NO produced by murine spleen cells stimulated with *P. gingivalis*-LPS (Pg-LPS) in the presence of 500 μM of L-arginine (Pg-LPS+L-arg). The cells stimulated with *Salmonella* (ST-LPS) or Pg-LPS alone served as a positive or negative control, respectively.

production could not be detected in the spleen cell cultures stimulated with Pg-LPS at concentration up to 100 ng ($p > 0.05$).

In order to determine whether NO production from Pg-LPS-stimulated murine spleen cells required exogenous L-arginine, spleen cell cultures were stimulated with 100 ng of Pg-LPS together with increased concentration of L-arginine (Fig. 2). NO could only be detected when cells were stimulated with Pg-LPS and 500 μM of L-arginine ($p < 0.01$), but its levels were still lower than those from cultures stimulated with 1 ng of ST-LPS ($p < 0.05$). The requirement of exogenous L-arginine could also be demonstrated by the way that 10 μ M of NMMA, a L-arginine analogue, could partially block NO production of spleen cell cultures stimulated with 100 ng of Pg-LPS and 500 μM of L-arginine ($p < 0.05$) (Fig. 3). NO production from these cell cultures was completely inhibited by 500 μM of NMMA ($p < 0.01$).

IFN-gamma is well known for its ability to induce NO production from murine macrophages. Detectable NO production in spleen cell cultures stimulated with 100 ng of Pg-LPS was seen in the presence of IFN-gamma at concentration of 200 units ($p < 0.01$) (Fig. 4). The levels of NO in these cell cultures were significantly increased when 500 units of this cytokine was added ($p > 0.05$).

In order to assess the cellular source of NO in the spleen cell cultures, the plastic non-adherent cells were stimulated with 100 ng of Pg-LPS and 500 μM of L-arginine (Fig. 5). NO production in the non-adherent cell cultures was completely suppressed as compared

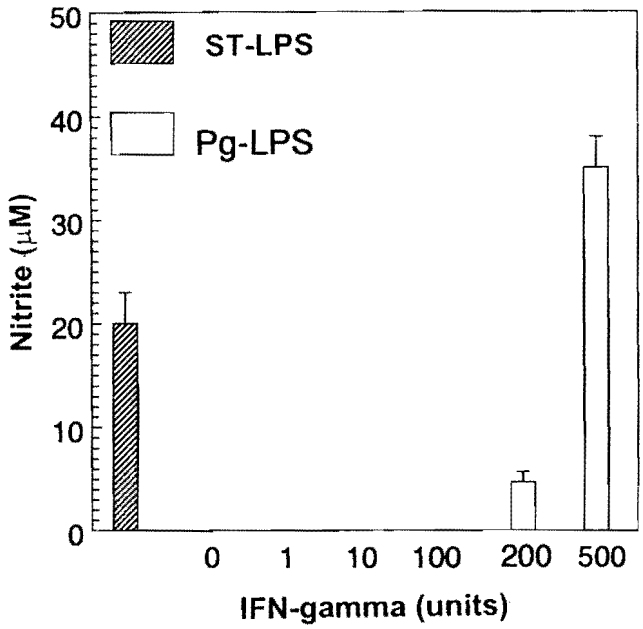

Fig. 4 The effects of IFN-gamma on the mean and standard deviation of NO produced by murine spleen cells stimulated with *P. gingivalis*-LPS (Pg-LPS). As a control, the cell cultures were stimulated with *Salmonella* Typhosa-LPS (ST-LPS).

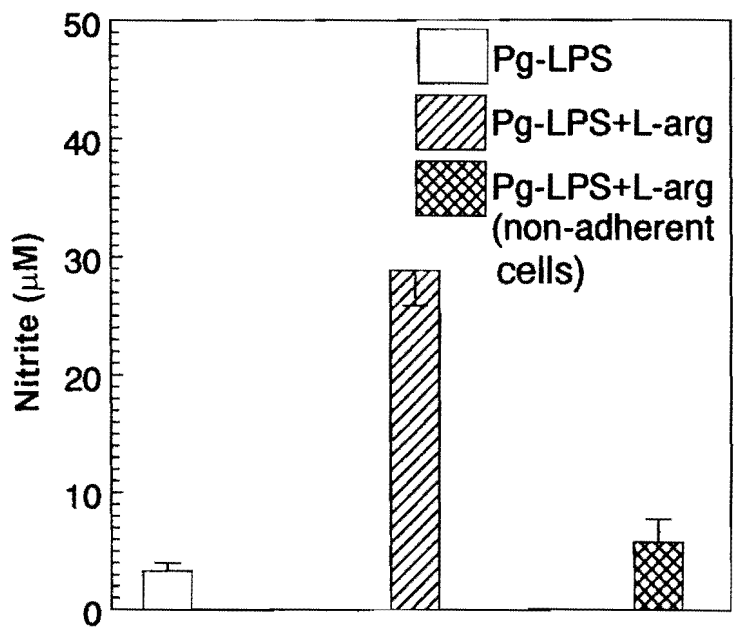

Fig. 5 Mean and standard deviation of NO produced by plastic non-adherent spleen cells stimulated with *P. gingivalis*-LPS (Pg-LPS) in the presence of L-arginine. The non-adherent spleen cells were cultured in the presence of 10 μg of Pg-LPS and 500 μM of L-arginine. As a positive control, bulk spleen cells were cultured in the presence of Pg-LPS and L-arginine.

with that in the bulk spleen cell cultures ($p < 0.01$). Indeed, the levels of NO from the non-adherent cells were not statistically different with those from the bulk cultures stimulated only with 100 ng of LPS ($p > 0.05$), indicating that NO may only be produced by the plastic-adherent spleen cells.

DISCUSSION

NO is now recognized as an important intracellular and intercellular regulatory molecule for immune responses and is produced by oxidation of the terminal guanidine nitrogen atom of L-arginine, a reaction catalyzed by the NADPH-dependent enzyme NOS.[1] Bacterial LPS known as a potent macrophage activator induces NO production via its ability to increase NOS expression. Murine spleen cells have been demonstrated to produce NO following stimulation with pertussis toxin and *E. coli* fimbriae.[2,3] Of interest, stimulation with LPS derived from *P. gingivalis* (Pg-LPS) at concentration up to 100 ng failed to induce NO production by murine spleen cells. The precise reason to explain the failure of Pg-LPS to induce NO production, however, remains unclear. One possibility is that the structural differences between biologically active lipid A of enteric bacterial LPS and that of *P. gingivalis* may account for the results of the present study. Evidences have shown that the lipid A isolated from *P. gingivalis*-LPS failed to stimulate IL-1 production and was 100-fold less potent to induce murine macrophage-derived NO production,[7,10] suggesting that LPS from *P. gingivalis* is less virulent as compared with that from enteric bacteria. Alternatively, LPS derived from an oral bacterial strain used in the present study may be considered as a NO non-inducer. Shapira

and colleagues[9] have shown, in this respect, that LPS from *P. gingivalis* strain A7436 but not W50 produced detectable NO levels by murine peritoneal macrophages. Thus, it would seem possible that the ability of LPS derived from *P. gingivalis* ATCC33277 to induce NO production may be similar to that from strain W50.

NO was detected from Pg-LPS-stimulated cell cultures in the presence of 500 μM of L-arginine, indicating that NO production from murine spleen cells stimulated with Pg-LPS is a L-arginine-dependent mechanism. L-arginine is known as a substrate catalyzed by NOS to generate NO and citrulline.[1] The results of the present study would seem to indicate that Pg-LPS might be unable to induce potent signals necessary for the metabolisms of endogenous L-arginine as seen by the fact that exogenous L-arginine restored the ability of this LPS to induce splenic NO production. It has been shown, in this respect, that exogenous L-arginine could act as a signal to elevate the production of intracellular cationic amino acid transporter 2 (CAT2), an essential protein for L-arginine uptake, thereby increasing L-arginine metabolisms.[11] Furthermore, elevated intracellular L-arginine concentration may also suppress the activation of arginase, an enzyme acting to catalyze the metabolism of L-arginine to generate urea.[12] Thus, the possibility that exogenous L-arginine may elevate the ability of *P. gingivalis* to induce signal(s) for splenic NO production by down-regulating arginase but up-regulating NOS activation can not be ruled out and remains to be further investigated.

It has been suggested that NMMA is an arginine analog metabolized by and subsequently bound to NOS, thereby preventing NOS-catalyzing L-arginine metabolism.[13] This agent is competitive if simultaneously added with L-arginine. Therefore, that NMMA inhibited NO production by spleen cells in the presence of the exogenous L-arginine and Pg-LPS supports the notion that stimulatory effects of Pg-LPS on NO production by murine spleen cells required exogenous L-arginine.

In the presence of IFN-gamma, NO could be detected in Pg-LPS-stimulated murine spleen cells, indicating that IFN-gamma may restore the ability of Pg-LPS to induce splenic NO production. The results of the present study are in accordance with previous findings showing that IFN-gamma induces high levels of NO production by macrophages both *in vivo* and *in vitro* and anti-IFN-gamma antibodies block the stimulatory effects of this cytokine.[14-17] It should, however, be kept in mind that both IFN-gamma and bacterial LPS independently stimulate NO production. This cytokine involves binding of interferon regulatory factor-1 (IRF-1) in the NOS promoter, whereas the bacterial LPS involves binding of NF-κB heterodimers, p50/c-RelA at the NOS promoter (see ref. 1 for review). Our recent findings also indicated that the ability of IFN-gamma to up-regulate NO production of Pg-LPS-stimulated murine macrophages was completely blocked by anti-IFN-gamma antibodies regardless of the presence of Pg-LPS (Sosroseno *et al.* unpublished results). Thus, a possibility that this cytokine may independently elevate NO production of *P. gingivalis*-LPS-stimulated spleen cells regardless the presence of this bacterial LPS can not be ruled out and needs to be elucidated.

Antigens such as pertussis toxin and *E. coli*-derived fimbriae were all capable of stimulating NO production;[2,3] however, these studies did not show the cellular source of splenic NO. The ability of splenic macrophages to produce NO when stimulated with LPS from oral bacterium has not yet been reported. Of interest, failure of the non-adherent spleen cells to produce NO in the presence of Pg-LPS and L-arginine suggests that NO in the spleen cell cultures was produced by splenic macrophages. These results are not surprising, since the abilities of activated murine macrophages to produce NO are well known.[1] Similarly, previous studies have also demonstrated that NO could be produced by Pg-LPS-stimulated murine peritoneal macrophages.[7-9] As yet, whether macrophages in the lymphoid organs such as the spleen may be the only cellular source of NO remains to be further investigated.

The extrapolation of the present study to the immunopathogenesis of CIPD is speculative. It has been hypothesized that the early periodontal lesion is regulated by IFN-gamma-producing Th1 cells whereas the destructive lesion is modulated by IL-4-producing Th2 cells.[18,19] In a murine model, the course of abscess formation induced by live *P. gingivalis* organisms seemed to be modulated by the activation of Th2 cells in mice immunized with antigens derived from this periodontopathogen.[20] Since NO levels have been associated with the development of Th1 cells due to the fact that IFN-gamma up-regulates but IL-4 down-regulates NO production,[1] failure of Pg-LPS to stimulate murine slenic NO production as seen in the present study may support the hypothesis that immune response to *P. gin-*

givalis is associatedwith the development of Th2 cells.[18,19]

ACKNOWLEDGEMENTS

I would like to thank to Professor Greg. Seymour (Brisbane) for providing Pg-LPS, and to both Betty and Eka for their assistance. While employed by GMU, WS was supported by RUT III, from the Ministry of Research and Technology, the Indonesian Government.

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