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Effect of exogenous nitric oxide on murine immune response induced by *Aggregatibacter actinomycetemcomitans* lipopolysaccharide

W. Sosroseno[1], P. S. Bird[2], G. J. Seymour[3]

[1]School of Dentistry, AIMST University, Semeling, Bedong, Kedah, Darul Aman, Malaysia, [2]Oral Biology and Pathology, School of Dentistry, University of Queensland, Brisbane, Queensland, Australia and [3]Faculty of Dentistry, The University of Otago, Dunedin, New Zealand

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Background and Objective: Elevated nitric oxide (NO) has been associated with destructive periodontal disease. The aim of the present study was to test the hypothesis that exogenous NO may inhibit a protective immune response to *Aggregatibacter actinomycetemcomitans* lipopolysaccharide (LPS) in a murine model.

Material and Methods: Mice of the BALB/c strain were sham immunized, immunized with *A. actinomycetemcomitans* LPS, treated with *S*-nitroso-*N*-acetyl penicillamine (SNAP; a NO donor) and immunized with *A. actinomycetemcomitans* LPS or treated with SNAP plus 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) and immunized with *A. actinomycetemcomitans* LPS. All animals were then challenged subcutaneously with viable *A. actinomycetemcomitans*. The serum-specific immunoglobulin G (IgG) subclasses and both interferon-γ (IFN-γ) and interleukin-4 (IL-4) as well as splenic inducible nitric oxide synthase (iNOS) activity before and after bacterial challenge were assessed. The diameter of skin lesions was determined. Groups of mice were treated with L-N^6-(1-iminoethyl)-lysine (L-NIL), an iNOS inhibitor, or 1*H*-(1,2,4)oxadiazolo(4,3-a) quinoxalin-1-one (ODQ), a guanylyl cyclase inhibitor, prior to injections with SNAP and/or *A. actinomycetemcomitans* LPS, and the skin lesions were assessed.

Results: Treatment with SNAP increased the iNOS activity, suppressed both serum-specific IgG2a and IFN-γ levels, and delayed the healing of the lesions. These SNAP-induced immune alterations were restored by treatment with carboxy-PTIO. Pretreatment with L-NIL resulted in partial healing, whereas pretreatment with ODQ induced a delayed healing of the lesions.

Conclusion: The present study suggests that exogenous NO may suppress a protective T helper 1-like murine immune response to *A. actinomycetemcomitans* LPS by an endogenous NO-independent but a cyclic GMP-dependent mechanism.

Wihaskoro Sosroseno, School of Dentistry, AIMST University, Semeling, Bedong 08100, Kedah, Darul Aman, Malaysia
Tel: + 601 2965 2034
Fax: + 604 442 2887
e-mail: wsosroseno@yahoo.com

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Nitric oxide (NO) is a product of L-arginine metabolism catalysed by a family of nitric oxide synthases (NOS). This gaseous molecule plays a crucial role in cardiovascular, neural, endocrine and immunological systems (1). Three NOS isoforms [neural NOS (nNOS or NOS-1), inducible NOS (iNOS or NOS-2) and endothelial NOS (eNOS or NOS-3)] are recognized (2). Nitric oxide has been shown to be involved in the induction of the T helper type 1 (Th1)-like immune response, since it may up- or downregulate the iNOS expression by murine macrophages (3–6), and both interferon (IFN)-γ production and interleukin (IL)-12 receptor expression by murine T cells (6–8). Nitric oxide also regulates murine splenic antibody production (9), natural killer cell function (10), neutrophil migration (11), dendritic cell function (12), macrophage phagocytic activity (13) and dendritic cell-derived chemokine production (14). It appears that the regulatory role of NO in antigen presentation (14), cytokine production (14–16) and immune cell migration (11,17) is dependent on the cyclic GMP pathway. Together, these studies indicate that the NO/cGMP pathway plays a crucial role in murine innate and adaptive immune responses.

The expression of iNOS in inflamed gingival tissue has been shown to be significantly increased compared with that in healthy tissues, and this decreases after periodontal therapy (18–22), suggesting that NO may play a crucial role in the progression of periodontal disease. However, the exact mechanism(s) by which NO regulates the immunopathogenesis of periodontal disease in humans remains unclear. In rodent models, alveolar bone loss failed to occur in in *Porphyromonas gingivalis*-infected iNOS-deficient mice and in ligature-induced periodontitis in iNOS-inhibitor-treated rats (23–25). It has also been demonstrated that reduced bacterial clearance but not the induction of antigen-specific serum immunoglobulin G (IgG) antibody occurs in *Porphyromonas gingivalis*-infected iNOS-deficient mice (26). Therefore, these previous reports seem to suggest that NO may regulate bone resorption and innate but not adaptive immunity during the course of periodontal disease.

Aggregatibacter (*Actinobacillus*) *actinomycetemcomitans*, a gram-negative oral bacterium, is thought to play a major role in the development of aggressive periodontal disease, involving rapid gingival and alveolar bone destruction (27,28). Previously, we and others have shown that lipopolysaccharide (LPS) isolated from this periodontopathogen stimulated murine macrophage cell lines and spleen cells to produce NO (29–31). Indeed, our recent studies revealed that inhibition of iNOS activity by treatment with L-N^6-(1-iminoethyl)-lysine (L-NIL), an iNOS inhibitor, increases a protective Th1-like murine immune response to *A. actinomycetemcomitans* LPS *in vivo* and *in vitro* (32,33). However, the effect of exogenous NO in the immune response to this periodontopathic bacterium is unknown. Since NO has been shown to downregulate the induction of murine Th1-like immunity (8), the aim of the present study was to determine the effect of exogenous NO on *A. actinomycetemcomitans* LPS-induced immune responses in mice.

Material and methods

Preparation of *A. actinomycetemcomitans* lipopolysaccharide

Aggregatibacter actinomycetemcomitans Y4 (serotype b) was grown in Todd–Hewitt broth supplemented with 1% (w/v) yeast extract (Difco Laboratories, Detroit, MI, USA) in an atmosphere of,10% O_2, 10% CO_2 and 80% N_2 at 37°C. Lipopolysaccharide from this periodontopathic bacterium was isolated using a hot phenol–water extract, treated with nuclease (Sigma, St Louis, MO, USA), and then lyophilized as previously described (31).

Immunization procedure

Female BALB/c mice (6–8 weeks old) were divided into four groups (I–IV), each consisting of three to five mice. Group I mice were injected intraperitoneally with 100 μL of PBS alone (sham immunized) weekly for 2 weeks and group II mice received an intraperitoneal injection of 100 μg of *A. actinomycetemcomitans* lipopolysaccharide in phosphate-buffered saline (PBS) weekly for 2 weeks. Group III mice were injected subcutaneously with 100 μL of PBS containing *S*-nitroso-*N*-acetyl penicillamine (SNAP; Sigma), a NO donor, at 3 μM/kg body weight (34) 15 min before immunization with *A. actinomycetemcomitans* LPS and every 3 days throughout the 2 week experimental period. Group IV mice were injected subcutaneously with 100 μL of PBS containing SNAP and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO; Sigma), a NO scavenger, at 3 and 30 μM/kg body weight, respectively, 15 min before immunization with *A. actinomycetemcomitans* LPS and every 3 days throughout the 2 week experimental period. A preliminary study showed that the dose of carboxy-PTIO, which was 10 times higher than that of SNAP, was sufficient to neutralize nitric oxide release from SNAP *in vitro* (data not shown). Treatment with SNAP and/or carboxy-PTIO did not affect the weight gain of any mice (data not shown). The experimental procedures were approved by the Ethical Committee of Universiti Sains Malaysia, Kota Bharu, Malaysia, and Gadjah Mada University, Yogyakarta, Indonesia.

Inducible nitric oxide synthase activity assay

In separate experiments, mice were divided into four groups (groups I–IV) treated as above. Spleens were aseptically removed from mice in groups I–IV 1 day before (day –1) and at days 5, 10 and 20 after bacterial challenge, and single cell suspensions were prepared by teasing through sterile stainless-steel grids. Three to five mice in every group were killed by CO_2 asphyxiation at each time point of examination. The cells were washed three times in RPMI 1640 medium (Sigma), lysed by incubating with 100 μL of 0.1% Triton X-100 and shaken for 30 min. The protein concentration of the cell lysates was measured using the Bradford protein

assay (Bio-Rad, Hercules, CA, USA). The iNOS activity was assessed as previously described (35). Briefly, 50 μg of cell lysate were incubated for 120 min at 37°C in 100 μL of 20 mM Tris-HCl (pH 7.9) containing 4 μM tetrahydobiopterin, 4 μM flavin adenine dinucleotide, 3 mM dithiothreitol, 2 mM NADPH and 2 mM L-arginine. The reaction was then stopped by adding lactate dehydrogenase (20 U/mL) and the levels of nitrite were measured using the Griess reagent as described previously (31). The non-specific absorbance was subtracted from the absorbance reading in wells containing all reagents except for NADPH and L-arginine. Unless stated otherwise, all materials were purchased from Sigma.

Enzyme-linked immunosorbent assay (ELISA)

Sera were obtained from the blood collected from the tail vein before (day –1) and at days 5, 10 and 20 after bacterial challenge. The levels of specific antibody were assessed by ELISA as previously described (32). Unless stated otherwise, all materials were purchased from Sigma. Briefly, 96-well plates (Nunc, Roskilde, Denmark) were coated with 50 μL of PBS containing 0.2 μg/mL of *A. actinomycetemcomitans* LPS and incubated overnight at 4°C. Non-specific binding sites were blocked by 1% dry skimmed milk powder in PBS containining 0.05% Tween. After washing, 100 μL of diluted samples were added and incubated for 1 h at room temperature. After further washing, 100 μL of biotin-conjugated goat anti-mouse IgG1, IgG2a, IgG2b and IgG3 antibodies diluted in dry milk-containing PBS-tween 1:5000 were added, incubated for 1 h and washed. One hundred microlitres of horseradish peroxidase diluted in dry milk-containing PBS-tween 1:10,000 were added and incubated for 30 min. Following washing, colour was developed by adding 3, 3′5,5′-tetramethylbenzidrine substrate and incubated for 10 min. The colour reaction was stopped by adding HCl and read at an absorbance of 450 nm on an automated reader (Biotek-Instruments, Winooski, VT, USA). Phosphate-buffered saline alone and serum from *A. actinomycetemcomitans* LPS-hyperimmunized mice were used as the negative and positive controls, respectively, on every plate.

Cytokine assay

The levels of serum IFN-γ and IL-4 were assessed using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Primary lesion induced by *A. actinomycetemcomitans*

Abscess formation was induced as described previously (32). Briefly, 1 week after the last immunization, mice were inoculated with viable *A. actinomycetemcomitans* organisms (1×10^{10} in 100 μL of sterile PBS each site) at two sites on the shaved dorsal surface of the abdomen approximately 1 cm on either side of the mid-line. Mice were examined for lesions at the injection sites, and the diameter of the lesions was measured using a digital vernier caliper at days 1, 5, 10, 15 and 20 after bacterial inoculation.

The role of endogenous nitric oxide

In a separate experiment, mice were divided into five groups (groups A–E), each consisting of three to five mice. Group A mice were injected intraperitoneally with 100 μL of PBS weekly for 2 weeks and group B mice were injected intraperitoneally with 100 μg of *A. actinomycetemcomitans* LPS in 100 μL of PBS weekly for 2 weeks. Group C mice were injected subcutaneously with 100 μL of PBS containing SNAP (Sigma) before immunization with *A. actinomycetemcomitans* LPS as described for group B. Group D mice were injected intravenously with 100 μL of PBS containing L-N^6-(1-Iminoethyl)-lysine (L-NIL), an iNOS inhibitor (Sigma), at 5 mg/kg body weight, 1 day before immunization with *A. actinomycetemcomitans* LPS as described previously (32). Group E mice were injected with L-NIL 1 day before subcutaneous injection with SNAP and intraperitoneal injection with LPS as above. Treatment with L-NIL and/or SNAP was repeated every 3 days throughout the experiments. One week after the last immunization, primary lesion was induced by viable *A. actinomycetemcomitans* as described in the preceeding subsection.

The role of cyclic GMP

In a further experiment, mice were divided into five groups (groups a–e), each consisting of three to five mice. Group a mice were injected intraperitoneally with 100 μL of phosphate-buffered saline (PBS) alone weekly for 2 weeks and group b mice with 100 μg of *A. actinomycetemcomitans* LPS in 100 μL of PBS weekly for 2 weeks. Group c mice were injected subcutaneously with 100 μL of PBS containing SNAP (Sigma), before immunization with *A. actinomycetemcomitans* LPS as for group b mice. Group d mice were injected intraperitoneally with 100 μL of PBS containing 1*H*-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ; Sigma), a guanylyl cyclase inhibitor, at 5 μM/kg body weight, 30 min before immunization with *A. actinomycetemcomitans* LPS as described previously (11). Group e mice were injected with ODQ; 15 min later the animals were injected subcutaneously with SNAP and, after a further 15 min, they were immunized with *A. actinomycetemcomitans* LPS. Treatment with ODQ and/or SNAP was repeated every 3 days throughout the experiments. One week after the last immunization, primary lesion was induced by viable *A. actinomycetemcomitans* as described above.

Statistical analysis

All data were analysed by a repeated measures analysis using a statistical package (SPSS Inc., Chicago, IL, USA).

Results

Splenic iNOS activity

The splenic iNOS activity in all groups of animals was assessed before and after bacterial challenge (Fig. 1).

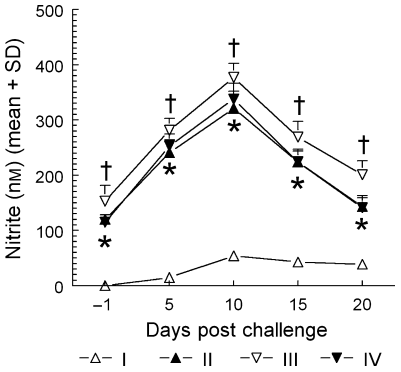

Fig. 1. Effect of exogenous NO treatment on the levels of iNOS activity in mice induced by *A. actinomycetemcomitans* LPS. Group I mice were sham immunized; group II, *A. actinomycetemcomitans* LPS immunized; group III, SNAP treated and *A. actinomycetemcomitans* LPS immunized; and group IV, SNAP plus carboxy-PTIO treated and *A. actinomycetemcomitans* LPS immunized. *Significant difference between group II or III and I at $p < 0.05$; †significant difference between group III and I at $p < 0.05$.

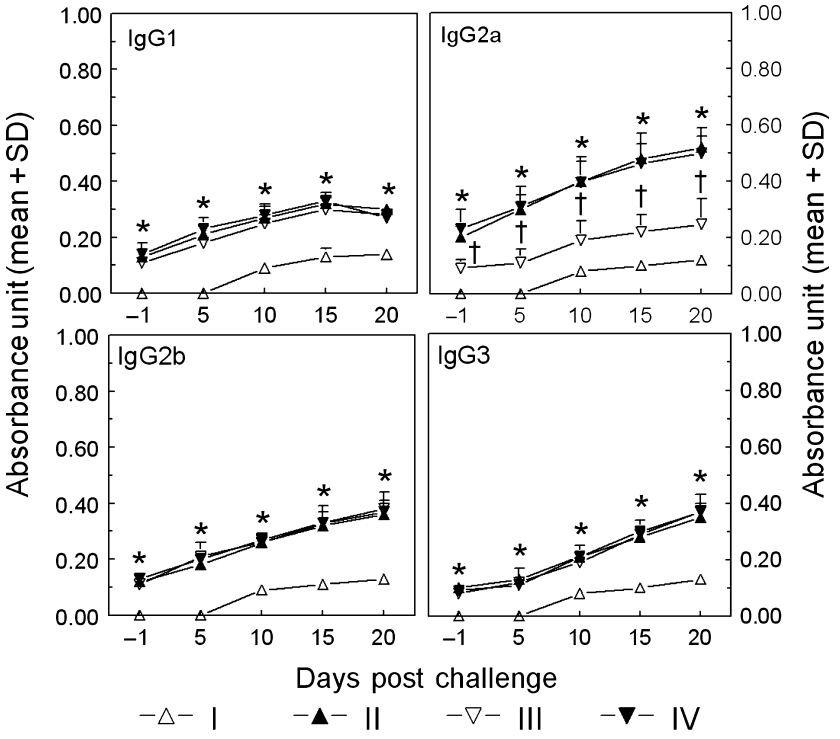

Fig. 2. Effect of exogenous NO treatment on the levels of serum IgG isotypes specific to *A. actinomycetemcomitans* LPS in mice. The groups of animals are the same as those described in the legend to Fig. 1. *Significant difference between group II or III and I at $p < 0.05$; †significant difference between group III and I at $p < 0.05$.

Splenic iNOS activity in groups II, III and IV could be detected at day –1, peaked at day 10 and returned to baseline levels by day 20 ($p < 0.05$). The levels of splenic iNOS activity in groups II, III and IV were significantly higher than those in group I ($p < 0.05$). No significant differences were observed between the levels of iNOS activity in groups II and IV throughout the experiment ($p > 0.05$). Of interest, the levels of iNOS activity in group III were significantly higher than those in groups II and IV on all days of assessment ($p < 0.05$).

The levels of serum-specific antibodies

The levels of serum-specific antibodies in all groups of animals are depicted in Fig. 2. The levels of serum IgG isotypes to *A. actinomycetemcomitans* LPS in groups II, III and IV were significantly higher than those in group I throughout the experiments ($p < 0.05$). The levels of serum IgG1, IgG2b and IgG3 between group II, III and IV were not significantly different ($p > 0.05$). In sharp contrast, the levels of serum IgG2a in group III were significantly lower than those in groups II and IV, but higher than those in group I throughout the period of the experiment ($p < 0.05$).

The levels of serum cytokines

The levels of serum IFN-γ and IL-4 in groups II, III and IV were significantly higher than those in group I ($p < 0.05$; Fig. 3A and 3B, respectively). However, the levels of serum IFN-γ in group III were significantly lower than those in groups II and IV throughout the period of the experiment ($p < 0.05$; Fig. 3A). The levels of serum IL-4 in groups II, III and IV were comparable ($p > 0.05$).

Primary lesion

Skin lesions after injection with live *A. actinomycetemcomitans* in all groups of mice could be observed within 24 h after bacterial challenge (Fig. 4). The lesion sizes were not significantly different ($p > 0.05$) between groups II and IV from day 1 to 20 and were much smaller than those in group I from day 1 to 20 ($p < 0.05$). Interestingly, the diameters of the lesions in group III were significantly lower than those in group I, but higher than those in group II and IV from day 1 to 20 ($p < 0.05$).

The role of endogenous nitric oxide

Our previous study showed that the induction of a murine immune response to *A. actinomycetemcomitans* is dependent upon iNOS activity (32,33). Therefore, the next experiments were to delineate whether the effect of exogenous NO on the skin abscess formation induced by viable *A. actinomycetemcomitans* was independent of the action of endogenous NO (Fig. 5). The results showed that, the lesions in group B healed rapidly compared with those in group A ($p < 0.05$). The lesions in group C healed slightly more rapidly than those in group A ($p < 0.05$). Treatment with L-NIL in group D resulted in rapid healing of the lesions compared with

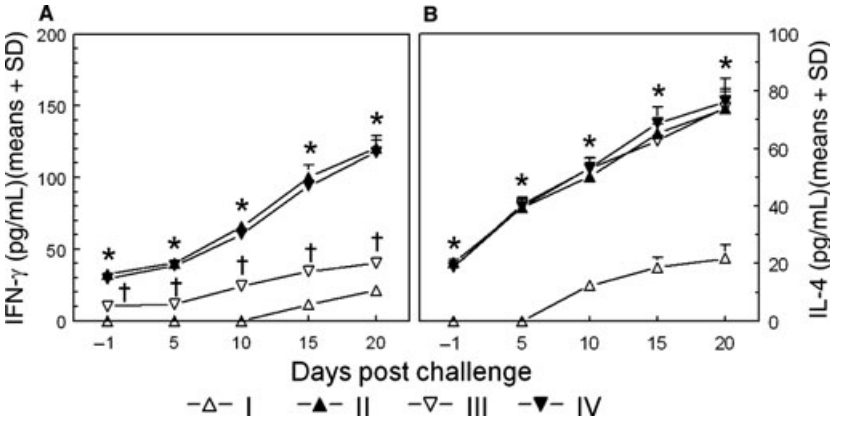

Fig. 3. Effect of exogenous NO treatment on the levels of serum IFN-γ (A) and IL-4 (B) in mice induced by *A. actinomycetemcomitans* LPS. The groups of animals are the same as those described in the legend to Fig. 1. *Significant difference between group II or III and I at $p < 0.05$; †significant difference between group III and I at $p < 0.05$.

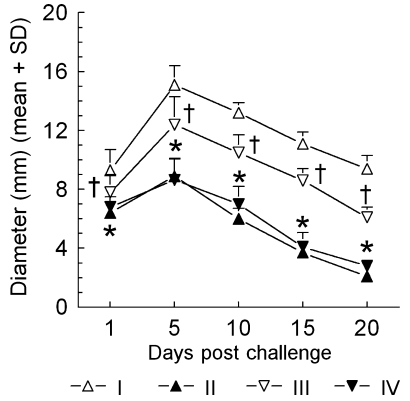

Fig. 4. Effect of exogenous NO treatment on diameter of abscess induced by *A. actinomycetemcomitans* in mice. The groups of animals are the same as those described in the legend to Fig. 1. *Significant difference between group II or III and I at $p < 0.05$; †significant difference between group III and I at $p < 0.05$.

those in group B ($p < 0.05$). Interestingly, the diameters of the lesions in group E, treated with L-NIL prior to injection with SNAP, were higher than those in group B but lower than those in group C from day 5 to 20 ($p < 0.05$).

The role of cyclic GMP

The involvement of cyclic GMP in the effect of exogenous NO on the skin abscess formation induced by live *A. actinomycetemcomitans* was assessed by treatment with ODQ, a guanylyl

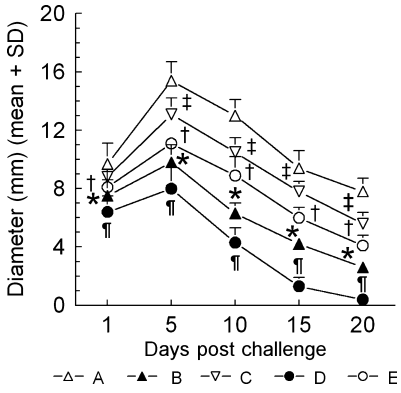

Fig. 5. Effect of L-NIL treatment on diameter of abscess in SNAP-treated and *A. actinomycetemcomitans* LPS-immunized mice. Group A mice were sham immunized; group B, *A. actinomycetemcomitans* LPS immunized; group C, SNAP treated and *A. actinomycetemcomitans* LPS immunized; group D, L-NIL treated and *A. actinomycetemcomitans* LPS immunized; and group E, L-NIL and SNAP treated and *A. actinomycetemcomitans* LPS immunized. *Significant difference between group B and A at $p < 0.05$; ‡significant difference between group C and A at $p < 0.05$; ¶significant difference between group D and A or B at $p < 0.05$; †significant difference between group E and A, B, C or D at $p < 0.05$.

cyclase inhibitor (Fig. 6). As observed in the preceeding subsection and Fig. 5, the lesions in group c, treated with SNAP prior to immunization with *A. actinomycetemcomitans* LPS, healed much more slowly than those in

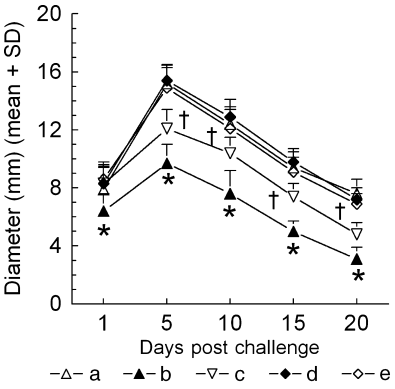

Fig. 6. Effect of ODQ treatment on diameter of abscess in SNAP-treated and *A. actinomycetemcomitans* LPS-immunized mice. Group a mice were sham immunized; group b, *A. actinomycetemcomitans* LPS immunized; group c, SNAP treated and *A. actinomycetemcomitans* LPS immunized; group d, ODQ treated and *A. actinomycetemcomitans* LPS immunized; and group e, ODQ and SNAP treated and *A. actinomycetemcomitans* LPS immunized. *Significant difference between group b and a, c, d, or e at $p < 0.05$; †significant difference between group c and a, b, d or e at $p < 0.05$.

group b ($p < 0.05$). In sharp contrast, no significant difference between the diameters of the lesions in groups a, d and e could be found from days 1 to 20 ($p > 0.05$).

Discussion

The present study showed that exogenous NO increased the levels of iNOS activity in the *A. actinomycetemcomitans* LPS-immunized mice and that carboxy-PTIO, a NO scavenger, ablated this stimulatory effect of exogenous NO. This has been confirmed in previous reports, where exogenous NO increased iNOS expression in stimulated murine macrophages (5,6). Multiple injections of exogenous NO at a physiological dose in an animal model also enhanced the iNOS activity *in vivo* (34). However, conflicting reports have shown that exogenous NO may inhibit the activity of iNOS in stimulated murine macrophages (3,4). The exact explanation for this apparent discrepancy is not clear. One possibility may be simply due to different doses of NO, such that high

doses of exogenous NO may suppress the iNOS activity in stimulated murine macrophages (3,4).

The present study showed that exogenous NO preferentially inhibited the production of serum specific IgG2a antibody to *A. actinomycetemcomitans* LPS. Interferon-γ is known to preferentially regulate the production of IgG2a in the murine immune system (36,37). Indeed, the present study demonstrated that exogenous NO inhibited the levels of serum interferon-γ in mice treated with carboxy-PTIO and SNAP and immunized with *A. actinomycetemcomitans* LPS, which reversed this suppressive effect of exogenous NO. These results are in accordance with previous reports that exogenous NO inhibited the production of interferon-γ by murine T cells, perhaps via the inhibition of interleukin-12 (4,6,38). Therefore, the suppressed production of interferon-γ in exogenous NO-treated animals seen in the present study may preferentially downregulate serum-specific IgG2a antibodies, thereby inhibiting the *A. actinomycetemcomitans* LPS-induced murine Th1-like immune response.

The treatment with exogenous NO in the present study delayed healing of the lesions induced by live *A. actinomycetemcomitans*, but healing was restored by carboxy-PTIO. Indeed, the results of the present study also indicate that treatment with L-NIL only partly restored healing of the lesions in exogenous NO-treated animals, suggesting that exogenous NO alone, independent of the action of endogenous NO, may still be able to suppress induction of the immune response to *A. actinomycetemcomitans* LPS. Furthermore, protection against skin lesions induced by periodontopathic bacteria in a murine model has been shown to depend on a Th1-like immune response (32,39,40). Therefore, it seems plausible that exogenous NO may directly suppress the protective Th1-like immune response, which may lead to delayed healing of the lesions induced by *A. actinomycetemcomitans*.

Previous reports have indicated that induction of the immune response is dependent on the NO/cGMP pathway, which plays a crucial role in the regulation of antigen presentation (14), cytokine production (14–16) and immune cell migration (11,17). Thus, the results showing that inhibition of guanylyl cyclase activity led to suppression of the *A. actinomycetemcomitans*-induced murine immune response in the presence of exogenous NO suggest that induction of the murine immune response to *A. actinomycetemcomitans* LPS is also dependent on the NO/cGMP pathway. However, whether or not the NO/cGMP pathway might regulate antigen presentation, cytokine production and/or cell migration during the course of the *A. actinomycetemcomitans*-induced murine immune response remains to be further investigated.

Extrapolation of the results of the present study to the immunopathogenesis of periodontal disease in humans remains speculative. Nitric oxide has been shown to play a crucial role in alveolar bone loss and the activation of innate immunity in experimental periodontitis in animal models (23–26). The fact that the murine immune response to *A. actinomycetemcomitans* LPS is regulated by both endogenous and exogenous NO, as seen in the present and previous studies (32,33), suggests that the induction of an immune response to periodontopathogens in the periodontal tissues may be determined by the sum of both endogenous and exogenous NO levels at the inflamed site. Indeed, iNOS protein in periodontal diseased patients is expressed by gingival inflammatory cells, endothelial cells and keratinocytes (18–21), all of which may contribute to the total amount of NO at the periodontal diseased site. In addition, the levels of NO in the gingival crevicular fluid obtained from periodontal diseased patients were significantly higher than those in healthy subjects (41,42), indicating that high levels of NO synthesized by different types of activated gingival cells may downregulate the local protective immune response to periodontopathogens, thereby accelerating the course of periodontal disease. If so, the local administration of iNOS inhibitor as a supplemental treatment might enhance the local protective immunity and, hence, have a beneficial therapeutic effect on periodontal disease. This is speculative, however, and needs to be confirmed by further work.

In conclusion, the present study showed that treatment with SNAP in *A. actinomycetemcomitans* LPS-immunized mice increased the iNOS activity, preferentially suppressed both serum interferon-γ and specific IgG2a antibody levels, and delayed the healing of the *A. actinomycetemcomitans*-induced lesions, and these parameters could be restored by carboxy-PTIO. Pretreatment with L-NIL in the SNAP-treated and *A. actinomycetemcomitans* LPS-immunized mice induced a partial healing of the lesions, whereas pretreatment with ODQ in these mice delayed the healing of the lesions. Therefore, the results of the present study suggest that exogenous NO may suppress a protective T lymphocyte helper 1-like murine immune response to *A. actinomycetemcomitans* LPS by an endogenous NO-independent but a cGMP-dependent mechanism.

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