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Effect of L-N^6-(1-iminoethyl)-lysine, an inducible nitric oxide synthase inhibitor, on murine immune response induced by *Actinobacillus actinomycetemcomitans* lipopolysaccharide

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Background and Objectives: Inducible nitric oxide synthase (iNOS) activity is known to regulate the immune response. The present study was carried out to determine the effect of L-N^6-(1-iminoethyl)-lysine (L-NIL), an iNOS inhibitor, on the induction of immune response to *Actinobacillus actinomycetemcomitans* lipopolysaccharide in mice.

Material and Methods: BALB/c mice were sham-immunized (group I), immunized with *A. actinomycetemcomitans* lipopolysaccharide (group II) or treated with L-NIL AND immunized with *A. actinomycetemcomitans* lipopolysaccharide (group III). All animals were then challenged with viable *A. actinomycetemcomitans*. The levels of serum nitric oxide (NO), specific immunoglobulin G (IgG) isotypes and both interferon-γ and interleukin-4, as well as spleen cell-derived iNOS activity, before and after bacterial challenge, were assessed. The diameter of skin lesions was also determined. Serum and spleen cells from the above groups were adoptively transferred to the recipients that were then subsequently challenged with live bacteria.

Results: Treatment with L-NIL suppressed serum NO and splenic iNOS activity, but enhanced serum-specific IgG2a antibody and interferon-γ levels. The lesions in L-NIL-treated mice healed much more rapidly. Transfer with serum and cells from L-NIL-treated and *A. actinomycetemcomitans* lipopolysaccharide-immunized donors resulted in rapid healing of the lesions in the recipients.

Conclusion: It is suggested that treatment with L-NIL in mice immunized with *A. actinomycetemcomitans* lipopolysaccharide may shift the immune response towards a protective T helper 1-like immunity against *A. actinomycetemcomitans*-induced infection.

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Nitric oxide (NO) is a reactive free radical involved in the regulation of physiological and pathophysiological mechanisms in cardiovascular, nervous and immunological systems. This gaseous molecule is a product of L-arginine metabolism catalyzed by three nitric oxide synthase (NOS) isoforms, namely the neural form (nNOS), the endothelial form (eNOS) and the inducible form (iNOS) (1). Whilst nNOS and eNOS participate in the homeostatic control of biological processes, iNOS is induced by pro-inflammatory stimuli to produce large amounts of NO (1,2). Pharmacological inhibition of *in vivo* iNOS activity by using L-N^6-(1-iminoethyl)-lysine (L-NIL) has been shown to ablate NO production and exacerbate inflammatory diseases, such as leishmaniasis (3) and *Mycobacterium*-induced granulomatous lesion, in mice (4), but to reduce renal disease in mice (5). These studies demonstrated that NO production generated by iNOS activity may up- or down-regulate the inflammatory response.

In chronic periodontitis, the precise mechanism(s) of the host's immune response remains to be determined (6,7). However, the number of $iNOS^+$ gingival cells in patients with periodontal diseases has been shown to be higher than that in healthy subjects, but to decrease following periodontal therapy (8–13). This seems to suggest that increased expression of iNOS in the inflamed periodontal tissues may be induced by interferon-γ and lipopolysaccharide from periodontopathic bacteria (8,14). However, the exact mechanism(s) by which the expression of iNOS may regulate the course of periodontal diseases in humans remains unclear. Recent studies, using animal models, may provide some insights into the possible role of iNOS in periodontal diseases. When iNOS-deficient and wild-type mice were challenged intra-orally with *Porphyromonas gingivalis* serum antibody, specific immunoglobulin G (IgG) to *P. gingivalis and* cytokine levels were comparable between both groups of animals (15). However, neutrophil functions were altered and alveolar bone loss failed to occur in *P. gingivalis*-inoculated iNOS-deficient mice (15,16). Similar results have also been observed upon the administration of mercaptoethylguanidine, an inducible nitric oxide synthase inhibitor, which suppressed alveolar bone loss in ligature-induced periodontitis in rats (17). These previous reports appear to indicate that NO production, as a result of iNOS activity, may play a crucial role in alveolar bone loss and bacteriocidal activity stimulated by neutrophils, but not the adaptive immunocompetent cell activation that occurs during the course of periodontal disease.

Actinobacillus actinomycetemcomitans is a gram-negative oral bacterium that is strongly associated with periodontal disease, especially the aggressive forms of disease. Infection with this oral bacterium induced soft tissue lesion and alveolar bone loss in rodent models (18–21), suggesting that tissue damage caused by *A. actinomycetemcomitans* infection in these animals may mimic human periodontal disease. Lipopolysaccharide isolated from this periodontopathogen is able to induce the host's immune response, as well as both soft tissue damage and alveolar bone loss (22). We and others have shown that lipopolysaccharide from *A. actinomycetemcomitans* stimulates murine macrophage cell lines and spleen cell-derived macrophages to produce NO (23–25). Therefore, the aim of the present study was to determine the effect of L-NIL, a selective iNOS inhibitor, on the immune response induced by *A. actinomycetemcomitans* lipopolysaccharide in mice.

Material and methods

Preparation of *A. actinomycetemcomitans* lipopolysaccharide

A. 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 37°C, 10% O_2, 10% CO_2 and 80% N_2. Lipopolysaccharide from this periodontopathic bacterium was isolated using hot phenol-water, treated with nuclease (Sigma, St Louis, MO, USA) and then lyophilized, as previously described (25).

Immunization procedure

Female BALB/c mice (6–8 wk old) were divided into three groups, each consisting of three to five mice. Group I mice were injected intraperitoneally with 100 μl of phosphate-buffered saline (PBS) alone (sham-immunized). Group II mice received an intraperitoneal injection of 100 μg of *A. actinomycetemcomitans* lipopolysaccharide in PBS, weekly for 2 wk. Group III mice were injected intravenously, via the lateral tail vein, with 100 μl of PBS containing L-NIL (Sigma) at 5 mg/kg body weight, 1 d before immunization with *A. actinomycetemcomitans* lipopolysaccharide and every 3 d throughout the experiment. The doses and frequency of L-NIL treatment were shown to inhibit iNOS activity in the spleen cells (see below). The weight gain of L-NIL-treated mice was also unaltered (data not shown). The experimental procedures were approved by the Ethical Committee of Universiti Sains Malaysia, Malaysia, and Gadjah Mada University, Yogyakarta, Indonesia.

Serum nitrite assay

Sera were obtained from the blood collected from the tail vein before (day −1), and on days 5, 10 and 20 after, bacterial challenge. The levels of serum nitrite were assessed as described previously (26). Briefly, 100 μl of serum was mixed with 449 μl of solution containing 0.25 U nitrate reductase (25 μl), 2.5 μM flavin adenine dinucleotide (FAD) (25 μl), 28 mM potassium phosphate (pH 7.5, 200 μl), 25 μM NADPH (4 μl) and 145 μl of water, and incubated for 2 h at 37°C. After the addition of 5 μg of lactate dehydrogenase (1 μl), 0.1 mM pyruvate (60 μl) and 44.5 μl of water, the solution was incubated for 30 min at 37°C. Nitrite levels were then assessed by the Griess reagent, as described previously (25). Briefly, 100 μl of sample was mixed with an equal volume of the Griess reagent (1% sulfanilamide, 0.1% naphthlethylenediamine dihydrochloride in 2.5% phosphoric acid) and read

in an automated reader (μQuant spectrophotometer; Biotek-Instrument Inc., Winooski, VT, USA) at 540 nm. A standard curve was prepared with sodium nitrite. All materials were purchased from Sigma.

INOS activity assay

Spleens were aseptically removed from 1 d before (day −1), and on days 5, 10 and 20 after, bacterial challenge, and single-cell suspensions were prepared by teasing through sterile stainless steel grids. The cells were washed three times in RPMI-1640 and then lysed by incubating with 100 μl of 0.1% Triton X-100 and shaking 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 (4). Briefly, 50 μg of cell lysate was incubated for 120 min at 37°C in 100 μl of 20 mM Tris-HCl (pH 7.9) containing 4 μM BH_4, 4 μM FAD, 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 by the Griess reagent, as described above. The nonspecific 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

Serum was obtained, as described above, and the levels of specific antibody were assessed by enzyme-linked immunosorbent assay (ELISA), as previously described (27). 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* lipopolysaccharide and incubated overnight at 4°C. Non-specific binding sites were blocked by 1% dry milk powder in PBS containing 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 antimouse IgG1, IgG2a, IgG2b or IgG3 (diluted in 1 : 5000) was added, incubated for 1 h and washed. Then 100 μl of horseradish peroxidase, diluted 1 : 10.000 in dry milk PBS, was added and incubated for 30 min. Following washing, color was developed by adding 3,3′5,5′-tetramethyl benzidrine substrate and incubated for 10 min. The color reaction was stopped by adding HCl and the plates were read at an absorbance of 450 nm on automated reader (MQUANT). PBS only, and serum from *A. actinomycetemcomitans* lipopolysaccharide-hyperimmunized mice, were used as the negative and positive controls, respectively, on every plate.

Cytokine assay

The levels of serum interferon-γ and interleukin-4 were assessed by ELISA kits (R & D Systems, Minneapolis, MN, USA), as described by the manufacturer.

Primary lesion induced by *A. actinomycetemcomitans*

Abscess formation was induced, as described previously (18,19). Briefly, 1 wk after the last immunization, mice were inoculated with viable *A. actinomycetemcomitans* organisms (1×10^{10} in 100 μl of sterile PBS at each site) at two sites on the shaved dorsal surface of the abdomen, ≈1 cm either side of the midline. Mice were examined for the lesions at the injection sites, and the diameter of the lesions was measured using a digital venier caliper on days 1, 5, 10, 15 and 20.

Serum transfer

One week after the last immunization, mice from all groups were killed by CO_2 asphyxiation and blood was obtained by cardiac puncture. Serum was obtained by centrifugation and 50 μl of serum samples were injected intravenously to the recipients via the lateral vein of the tail base (27). The recipients were divided into three groups, each consisting of three to five mice. Groups A, B and C were transferred with the sera from sham-, *A. actinomycetemcomitans* lipopolysaccharide- and L-NIL + *A. actinomycetemcomitans* lipopolysaccharide-immunized mice, respectively. Abscess formation was induced 24 h after serum transfer, and the diameter of the lesions was assessed as described above, under the heading 'Primary lesion induced by *A. actinomycetemcomitans*'.

Cell transfer

One week after the last immunization, mice from all groups were killed by CO_2 asphyxiation and their spleens were obtained. Single-cell suspension was prepared by teasing through sterile stainless steel grids. Forty million viable cells in sterile saline were transferred to the recipients, via the lateral tail vein, using an insulin syringe (27). The recipients were divided into three groups, each consisting of three to five mice. Groups 1, 2 and 3 were transferred with cells from sham-, *A. actinomycetemcomitans* lipopolysaccharide- and L-NIL + *A. actinomycetemcomitans* lipopolysaccharide-immunized mice, respectively. The abscess formation was induced 24 h after serum transfer, and the diameter of the lesions was assessed as described above, under the heading 'Primary lesion induced by *A. actinomycetemcomitans*'.

Statistical analysis

All data were analyzed by a repeat-measurement analysis using a statistical package (SPSS; SPSS Inc., Chicago, IL, USA).

Results

Serum NO levels and splenic iNOS activity

Both serum NO levels and the splenic iNOS activity in all groups of animals were assessed before and after bacterial challenge (Fig. 1A,B). The levels of serum NO and splenic iNOS activity in group II were detectable on day −1 and increased dramatically up to day 10, but thereafter steadily decreased to day 20 ($p < 0.05$). The results of the

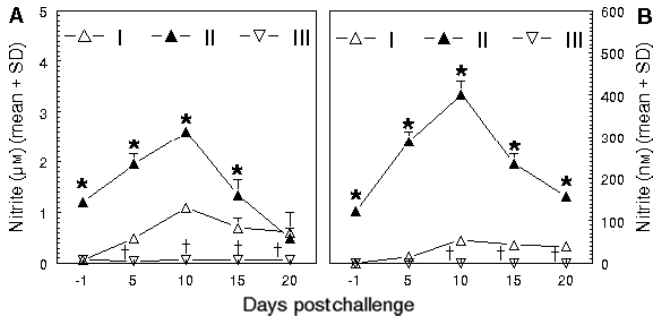

Fig. 1. Effect of L-N^6-(1-iminoethyl)-lysine (L-NIL) treatment on the levels of serum nitric oxide (NO) (A) and inducible nitric oxide synthase (iNOS) (B) induced *by Actinobacillus actinomycetemcomitans* lipopolysaccharide in mice. Groups I and II were sham-immunized and *A. actinomycetemcomitans* lipopolysaccharide-immunized mice, respectively. Group III was L-NIL-treated and *A. actinomycetemcomitans* lipopolysaccharide-immunized mice. *Significant difference between groups II and I at $p < 0.05$; †significant difference between groups III and I at $p < 0.05$. SD, standard deviation.

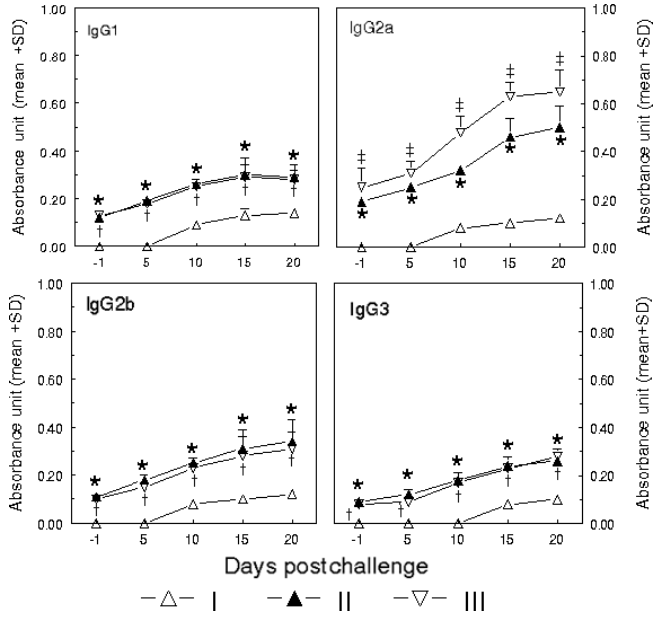

Fig. 2. Effect of L-N^6-(1-iminoethyl)-lysine (L-NIL) treatment on the levels of serum immunoglobulin G (IgG) isotypes specific to *Actinobacillus actinomycetemcomitans* lipopolysaccharide in mice. The groups of animals are the same as those described in Fig. 1. *Significant difference between groups II and I at $p < 0.05$; †significant difference between groups III and I at $p < 0.05$; ‡significant difference between groups III and II at $p < 0.05$. IgG, immunoglobulin G; SD, standard deviation.

present study also showed that the levels of serum NO and splenic iNOS activity in group II were higher than those in group I on day −1 up to day 20 ($p < 0.05$). On the other hand, serum NO levels and splenic iNOS activity in group III remained low before and after the bacterial challenge and remained lower than those in groups I and II from day 10 to day 20 ($p > 0.05$).

The levels of serum-specific antibodies

After challenge with *A. actinomycetemcomitans*, the levels of serum antibody-specific IgG isotypes in all groups of mice increased steadily from day 5 to day 20 (Fig. 2). The levels of serum IgG isotypes to *A. actinomycetemcomitans* lipopolysaccharide in groups II and III were significantly higher than those in group I on day 0, before bacterial challenge and throughout the experimental period ($p < 0.05$). No significant difference between the levels of serum antigen-specific IgG1, IgG2b and IgG3 in group II and group III were observed ($p > 0.05$). Interestingly, the levels of serum antigen-specific IgG2a in group III were significantly higher than those in group II at every time point of antibody assessment ($p < 0.05$).

Serum cytokine levels

The levels of serum interferon-γ and interleukin-4 in groups II and III increased from day −1 to day 20 ($p < 0.05$) (Fig. 3A,B). However, serum interferon-γ and interleukin-4 in group I were detected only on days 10 and 5, respectively ($p < 0.05$). The levels of these cytokines in groups II and III were significantly higher than the levels in group I ($p < 0.05$). Interestingly, the levels of serum interferon-γ in group III were higher than those in group II before challenge (−1) and throughout the experimental period ($p < 0.05$). In sharp contrast, the levels of serum interleukin-4 in groups II and III were comparable ($p > 0.05$).

Primary lesion

The skin abscess formation after injection with live *A. actinomycetemcomitans* was observed in all groups of animals within 24 h after bacterial challenge (Fig. 4). The peak diameter of the lesion in all groups of mice was reached on day 5. However, the lesions in group II decreased rapidly when compared with those in group I ($p < 0.05$). Interestingly, the lesions in group III healed more rapidly than those in group II and were undetectable on day 20 ($p < 0.05$).

Serum and cell transfer

When serum and spleen cells from *A. actinomycetemcomitans* lipopolysaccharide-immunized mice were transferred to the recipients, which were then challenged with *A. actinomycetemcomitans*, the lesions in groups B and b were reduced rapidly

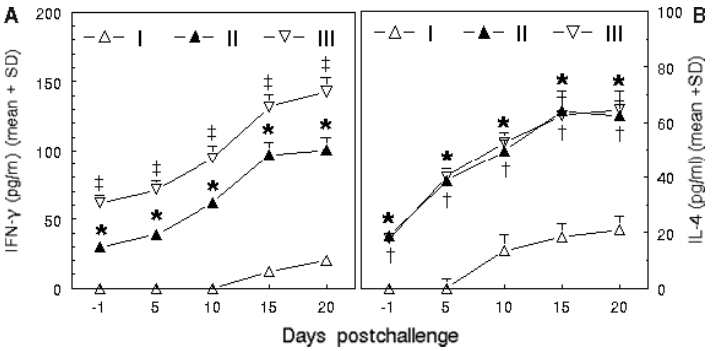

Fig. 3. Effect of L-N^6-(1-iminoethyl)-lysine (L-NIL) treatment on the levels of serum interferon-γ (IFN-γ) (A) and interleukin-4 (IL-4) (B) induced by *Actinobacillus actinomycetemcomitans* lipopolysaccharide in mice. The groups of animals are the same as those described in Fig. 1. *Significant difference between groups II and I at $p < 0.05$; †significant difference between groups III and I at $p < 0.05$; ‡significant difference between groups III and II at $p < 0.05$. SD, standard deviation.

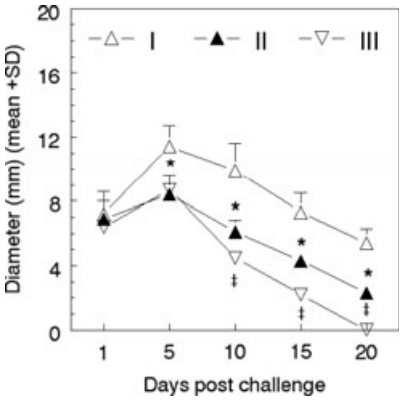

Fig. 4. Effect of L-N^6-(1-iminoethyl)-lysine (L-NIL) treatment on the diameter of abscess induced by *Actinobacillus actinomycetemcomitans* lipopolysaccharide in mice. The groups of animals are the same as those described in Fig. 1. *Significant difference between groups II and I at $p < 0.05$; ‡significant difference between groups III and II at $p < 0.05$. SD, standard deviation.

compared with those in groups A and a, which were transferred with serum and spleen cells from sham-immunized mice, respectively ($p < 0.05$) (Fig. 5A,B). Interestingly, lesion diameter in groups C and c was lower than that in groups B and b by day 10 and was undetectable on day 20, respectively ($p < 0.05$).

Discussion

The present study was conducted to determine the effect of L-NIL, a selective iNOS inhibitor, on the immune response induced by *A. actinomycetemcomitans* lipopolysaccharide. L-NIL is an L-arginine analogue which binds onto iNOS 30-fold more strongly than eNOS and blocks NO production from iNOS activity (28). The results of the present study showed that the levels of serum NO and iNOS activity in L-NIL-treated mice remained low throughout the experiments. This was not unexpected, as significant reduction of NO levels in various body fluids, such as serum, peritoneal fluid and urine, following treatment with L-NIL in animal models, have been reported (5,29,30). Increases in both serum NO and splenic iNOS activity in mice injected with *A. actinomycetemcomitans* lipopolysaccharide before bacterial challenge are in accordance with previous studies showing that injection with bacterial lipopolysaccharide increased both serum NO and iNOS activity (29,31).

Increased serum-specific IgG isotypes in mice injected with *A. actinomycetemcomitans* lipopolysaccharide before bacterial challenge, as seen in present study, were supported by a previous report showing that injection with *A. actinomycetemcomitans* lipopolysaccharide induced increased the level of serum-specific IgG in mice (32). Interestingly, treatment of mice with L-NIL before immunization with *A. actinomycetemcomitans* lipopolysaccharide, followed by bacterial challenge, resulted in preferentially increased levels of serum-specific IgG2a, suggesting that reduced iNOS activity may up-regulate the production of serum *A. actinomycetemcomitans* lipopolysaccharide IgG2a. An increased serum IgG2a response in autoimmune interstitial nephritis rats pretreated with L-NIL has also been reported (33). However, the exact mechanism by which serum *A. actinomycetemcomitans* lipopolysaccharide-specific IgG2a was increased in L-NIL-treated mice, as seen in the present study, is unknown. Interferon-γ appears to stimulate IgG2a production selectively (34), while there is no evidence to suggest that NO may directly or indirectly involve in immunoglobulin class switching. Indeed, the present study

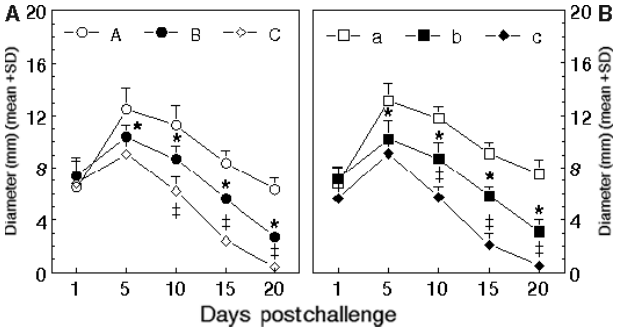

Fig. 5. Effect of serum (A) and spleen cell (B) transfer on the diameter of abscess induced by *Actinobacillus actinomycetemcomitans* lipopolysaccharide in the recipient mice. Groups A and a were transferred with serum or cells from the sham-immunized donor, respectively. Groups B and b were transferred with serum or cells from an *A. actinomycetemcomitans*-immunized lipopolysaccharide donor, respectively. Groups C and c were transferred with serum or cells from an L-NIL-treated + *A. actinomycetemcomitans* lipopolysaccharide-immunized donor, respectively. *Significant difference between groups II and I at $p < 0.05$; ‡significant difference between groups III and II at $p < 0.05$. SD, standard deviation.

also indicated that treatment with L-NIL before immunization and throughout the experiments augment the levels of serum interferon-γ but does not affect the levels of serum interleukin-4, as also seen in previous studies (33,35). Therefore, it suggests that L-NIL-reduced iNOS activity in mice immunized with *A. actinomycetemcomitans* lipopolysaccharide and challenged with *A. actinomycetemcomitans* might lead to increased serum interferon-γ, which in turn may stimulate preferentially specific IgG2a production. Previous studies have reported that interferon-γ levels increased *P. gingivalis*-specific IgG2a in interleukin-10-depleted mice, as well as in interleukin-12-treated mice (36,37), supporting the results of the present study. Most recently, Tanaka and colleagues also demonstrated that interferon-γ is prerequisite for the production of IgG2 by human mononuclear cells stimulated with *A. actinomycetemcomitans* (38). Furthermore, increased serum interferon-γ and specific IgG2a levels in L-NIL-treated mice, seen in the present study, seem to indicate that treatment with this iNOS inhibitor may skew the immune response towards a T helper 1-like response, a similar phenomenon observed in a previous report (33). As seen in the present study, treatment with L-NIL in mice resulted in more rapid healing of *A. actinomycetemcomitans*-induced abscesses than in non-L-NIL-treated mice. Leukocyte migration to the diseased sites, leucocyte adhesion, the expression of adhesion molecules and the production of specific antibody in the L-NIL-treated mice were unaltered (30,39), suggesting that both innate and adaptive immunity in the diseased sites in L-NIL-treated mice may be intact. Our previous studies indicated that protection against a *P. gingivalis* infection in mice may be dependent on the T helper 1 response and, subsequently, an IgG2a response (40). A T helper 1-like immune response in interleukin-10-depleted mice stimulates rapid healing of *P. gingivalis*-induced abscesses (36). Therefore, one may assume that the T helper 1 immune response specific to *A. actinomycetemcomitans* lipopolysaccharide, caused by L-NIL-reduced iNOS activity, may be protective to *A. actinomycetemcomitans*-induced soft tissue lesions in mice. This assumption is further strengthened by the results of the present study, showing that adoptive transfer of serum and spleen cells derived from L-NIL-treated mice resulted in more rapid healing of *A. actinomycetemcomitans*-induced lesions in the recipients than those from non-L-NIL-treated animals. Thus, serum containing high levels of both serum interferon-γ and specific IgG2a, or spleen cells containing preferentially activated T helper 1 cells, might stimulate a more rapid healing process in *A. actinomycetemcomitans*-induced soft tissue lesions in the recipients.

The extrapolation of the present study to periodontal diseases in humans remains speculative. The number of iNOS-positive gingival cells was increased in patients with periodontal diseases (8–12). Interestingly, periodontal treatment in these patients reduced the expression of gingival iNOS (13). These previous observations suggest that increased iNOS activity in inflamed gingival tissue may be associated with periodontal disease severity, perhaps by suppression of periodontopathogen-stimulated protective T-cell activation. If so, a supplemental treatment, by inhibition of iNOS activity, may have a beneficial therapeutic effect on periodontal diseases, because it may up-regulate the T helper 1-type immune response which may, in turn, reduce the progresion of periodontal diseases. This requires further investigation.

In conclusion, the present study showed that treatment with L-NIL resulted in augmentation of both serum *A. actinomycetemcomitans* lipopolysaccharide IgG2a and interferon-γ levels, as well as rapid healing of *A. actinomycetemcomitans*-induced lesions, suggesting that reduced iNOS activity may stimulate a protective T helper 1-like immunity in *A. actinomycetemcomitans*-induced infections in mice.

Acknowledgements

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