Yonago Acta medica 1998;41:7–15

Effect of a Nitric Oxide Donor on Intracellular Cytokine Production in Normal Human Peripheral Lymphocytes

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It has been recently suggested that nitric oxide (NO) plays an important role in modulating immune responses including helper T (Th) cell differentiation. To investigate the effect of NO on cytokine production in T cells, we examined in vitro the percentage of interferon (IFN)-γ and interleukin (IL)-4 producing cells by the intracellular cytokine staining method with flow cytometry. The percentage of IFN-γ and IL-4 producing cells reached maximal value 8 h after stimulation by phorbol 12-myristate 13-acetate (PMA) and ionomycin. When an NO donor, sodium nitroprusside (SNP), was co-incubated with PMA and ionomycin for 8 h, nitrite levels increased in a dose dependent manner for SNP (*P* = 0.007; Friedman test). The percentage of IFN-γ producing cells was diminished in consequence of the increasing doses of SNP (*P* = 0.002; Friedman test). While the percentage of IL-4 producing cells tended to be diminished by SNP, this difference, however, was statistically not significant (*P* = 0.062; Friedman test). Therefore, we have suggested that NO might affect Th cell differentiation through inhibition of Th1-cytokine production and might cause a Th2 cell predominant state.

Key words: IFN-γ; IL-4; intracellular cytokines; NO; sodium nitroprusside

Nitric oxide (NO) has many biological functions, for example, vasodilation (Palmer et al., 1987), platelet aggregation (Radomski et al., 1987), microbicidal activity (Liew, 1993), neurotransmitter (Bredt and Snyder, 1994) and inflammatory response (Moilanen and Vapaatalo, 1995). NO also plays an important role in the pathophysiology of airway diseases as suggested from measurement of NO in the exhaled air of patients with airway inflammation. It has been reported that there was a marked increase in the concentration of exhaled NO in patients with bronchiectasis (Kharitonov et al., 1995) and bronchial asthma (Alving et al., 1993). For asthmatic patients, NO may have a beneficial effect, so far as it is a bronchodilator (Högman et al., 1993). However, a high concentration of NO may have cytotoxic effects on airway epithelial cells and this could contribute to epithelial shedding as described in asthmatic airways (Kanazawa et al., 1997).

The effect of NO on the function of lymphocytes involving T helper (Th) cell differentiation into Th1 cells, which produce mainly interferon (IFN)-γ, and/or into Th2 cells, which produce mainly interleukin (IL)-4, has been of great interest, because the imbalance between Th1- and Th2-cytokine was one of the pathogenesis of atopic diseases including asthma (Umetsu and DeKruyff, 1997). Taylor-Robinson and colleagues (1994) reported that NO inhibited the proliferation of Th1 cells and the production of Th1-cytokines but did not affect Th2 cells. On the other hand, Bauer and colleagues (1997) reported that NO inhibited the production of both Th1- and Th2-cytokines from activated human

Abbreviations: c-AMP, cyclic AMP; FACS, flow cytometric activated cell scan; FITC, fluorescein isothiocyanate; IFN-γ, interferon–γ; IL, interleukin; NO, nitric oxide; NOS, NO synthase; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; PKA, protein kinase A; PMA, phorbol 12-myristate 13-acetate; SIN-1, 3-morpholinosydnonimine N-ethylcarbamide; SNP, sodium nitroprusside; Th cell, helper T cell

T cells. Thus, the relationship between NO and Th cell differentiation has not yet been clearly understood.

Recently, the assessment of intracellular cytokines has become available. This method is suitable for identifying heterogeneous T cells with respect to cytokine-producing patterns (Th1 or Th2). Furthermore, analysis at the single cell level is enough to estimate cytokine production for short time cultures (Jung et al, 1993). On the other hand, the analysis of the secretion of cytokines in supernatants requires culturing cells for a longer time (Jung et al, 1993; Gonzalez et al, 1994). Thus, we considered that this method was more physiologically relevant to study the relationship between NO and Th cell differentiation. NO is generated from L-arginine by NO synthases (NOS) (Nathan and Xie, 1994) in vivo, while in vitro chemical compounds such as sodium nitroprusside (SNP) and 3-morpholinosydnonimine N-ethylcarbamide (SIN-1) spontaneously release NO in aqueous solution and have been used as NO donors.

In this study, we investigated the effect of the NO donor on the production of intracellular IFN-γ and IL-4 in lymphocytes.

Materials and Methods

Preparation of peripheral blood mononuclear cells

Peripheral venous blood was collected into heparinized tubes from 8 healthy subjects, 4 males and 4 females. The average age of the 8 subjects was 34.7 years. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (specific gravity 1.077 g/mL) (Pharmacia Uppsala, Sweden) density gradient centrifugation for 30 min at room temperature. PBMCs-rich fraction was collected from the interface and washed twice with Mg^{2+}- and Ca^{2+}- free phosphate buffered saline (PBS) (Research Institute for Medical Disease, Osaka, Japan). PBMCs were finally resuspended in Roswell Park Memorial Institute Media 1640 with 25 mmol/L HEPES and 0.3 mmol/L glutamine (BioWhitker, Walkersville, MD) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Cansera International Inc., Rexdale, ON, Canada), 100 unit/mL penicillin, 100 mg/mL streptomycin (BioWhitker), and 2×10^{-5} mol/L 2-mercaptoethanol (Wako Life Science Co., Ltd., Tokyo, Japan).

Stimulation of PBMCs

PBMCs (1.0×10^6 cells/mL) were stimulated with phorbol 12-myristate 13-acetate (PMA) (10 ng/mL) (Wako) and ionomycin (1 μmol/L) (Sigma, St Louis, MO) in the presence of brefeldin A (10 μg/mL) (Sigma), in order to store cytokines at the Golgi complex level, with or without SNP (0–800 μmol/L) (Wako) at 37˚C under 5% CO_2 humidified atmosphere in 24-well microtiter plates (Nunc, Roskilde, Denmark) for 4, 8 and 12 h.

Concentration of nitrites in supernatants

After 4, 8, and 12 h of stimulation, the supernatants of the cultured PBMCs were collected and centrifuged for 5 min at 1500 rpm. Nitrite concentration in the supernatants was analyzed by the Griess method using a commercially available nitrite/nitrate assay kit-C (Wako). In brief, 100 μL of unknown samples and serial dilution of sodium nitrite standards were added to the 96-well microtiter plate (Nunc) and then 50 μL of solution A, which consisted of 58 mmol/L of sulfanilamide in 5% phospholic acid, was added to each well. After incubating for 5 min at room temperature, 50 μL of solution B, which consisted of 3.86 mmol/L of 1-naphthylenediamine, was added to each well followed by incubating for 10 min at room temperature. The optic density of each well was read at 540 nm in a Micro plate reader (Tosoh Ltd., Tokyo). The detection range was 10.0–100 μmol/L of nitrite.

Analysis of cellular viability

Cellular viability was determined by the dye exclusion test using trypan blue (GIBCO, Grand Island, NY). After 8 h of stimulation, cultured

PBMCs were collected by centrifugation for 5 min at 1500 rpm. The supernatants were removed and the cells were resuspended in 1 mL of PBS. Then, 10 μL of the cell suspension and 10 μL of 0.4% trypan blue were mixed. The number of cells in 10 μL of the mixture was counted using a hemocytometer. Cellular viability was expressed as the percentage of cells excluding the dye to total inspected cells by the hemocytometer.

Fluorescent staining of cells

The cells were washed twice with PBS and then resuspended in 50 μL of PBS containing 0.1% sodium azide (Wako) and 1% FBS (staining buffer). Next, they were incubated with 10 μL of Cy-Chrome conjugated anti-human-CD3 monoclonal antibody (PharMingen, San Diego, CA) for 30 min in the dark at 4˚C. Then they were washed twice in staining buffer, and fixed throughly with 100 μL of 4% paraformaldehyde for 20 min in the dark at 4˚C. After washing twice with staining buffer, they were resuspended in 50 μL of PBS containing 0.1% sodium azide, 1% FBS and 0.1% saponin (Sigma) (permeabilization buffer) and incubated with 0.5 μL of fluorescein isothiocyanate (FITC) conjugated mouse anti-human IFN-γ IgG1 antibody (PharMingen) and 1 μL of phycoerythrin (PE) conjugated mouse anti-human IL-4 IgG2a antibody (PharMingen). For negative control staining, 0.5 μL of FITC conjugated mouse IgG1 antibody (PharMingen) and 1 μL of PE conjugated mouse IgG2a antibody (PharMingen) were used. Each tube was incubated for 30 min in the dark at 4˚C. Then the cells were washed twice with permeabilization buffer and resuspended in staining buffer.

Flow cytometry

Flow cytometry was performed with 1.0×10^6 cells per sample by FACSCalibur (Becton Dickinson, Mountain View, CA). It was equipped with a 15-mW argon ion laser and appropriate filters for FITC (530 nm), PE (585 nm) and Cy-Chrome (> 650 nm) were used. Lymphocytes were analyzed by selective gating based on forward and side scatter parameters with CD3 positive cells using CELLQuest software (Becton Dickinson). Cytokine-producing cells were analyzed by detection of FITC and PE staining, and the results were expressed as the percentage of cytokine-producing cells in a proportion of CD3 positive lymphocytes. Statistical markers were set using an isotype matched negative control.

Morphology of intracellular IFN-γ producing cells

For immunofluorescent staining of intracellular IFN-γ, PBMCs were stimulated with PMA and ionomycin in the presence of brefeldin A for 8 h and were collected. After washing with PBS, the cells were fixed with 100 μL of 4% paraformaldehyde for 20 min, and then were centrifuged for 5 min at 1500 rpm. Then they were resuspended in 50 μL of permeabilization buffer and 10 μL of mouse anti-human IFN-γ IgG1 antibody (PharMingen) was added to the cell suspension and incubated for 30 min in the dark at 4˚C. After washing with staining buffer, the cells were resuspended in 50 μL of permeabilization buffer and incubated with 10 μL of gout anti-mouse IgG1 FITC antibody (Southern Biotechnology Associates, Inc., Birmingham, AL) for 30 min in the dark at 4˚C. The cells were washed with permeabilization buffer and resuspended in staining buffer. The cells were transferred to slides and mounted with glycerol (Wako) and were examined by fluorescence microscopy (Olympus, Tokyo).

Statistical analysis

Data were presented as mean ± SD. The results of the percentage of CD3 antigen expressed cells, the percentage of intracellular cytokine-producing cells and nitrite level were shown by skewed distribution. Nonparametric analysis was carried out with the Friedman test for comparison among multiple groups, and the Wilcoxon test for differences between the 2 groups. P values less than 0.05 were considered to be statistically significant. These statistical analyses were performed with the Stat View 4.11 statistics package (Abacus Concepts, Berkeley, CA).

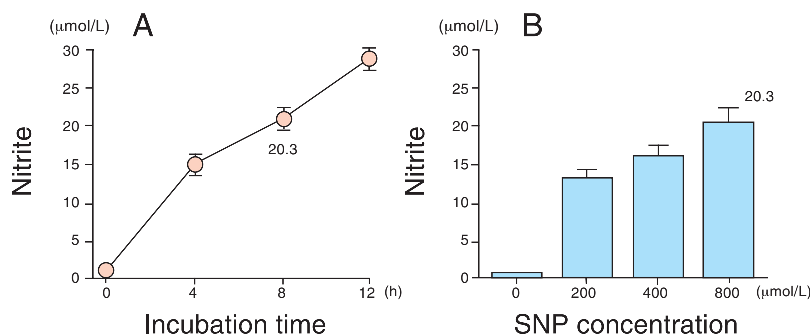

Fig. 1. Concentration of nitrite in the culture medium measured by Griess method. PBMCs were stimulated with PMA (10 ng/mL) and ionomycin (1 μmol/L), and SNP was added in the culture medium. Nitrite level was measured after 4, 8 and 12 h incubation with 800 μmol/L of SNP (**A**), and after 8 h incubation with 200, 400 and 800 μmol/L of SNP (**B**). Results are expressed as mean ± SD of 4 individual experiments. Nitrite level in the culture medium increased significantly in a time and a dose dependent manner for SNP ($P = 0.007$ and $P = 0.007$, respectively; Friedman test).

Results

Nitrite assay

The nitrite level increased in a time (Fig. 1A) and a dose dependent manner (Fig. 1B) for SNP ($P = 0.007$, $P = 0.007$, respectively; Friedman test). The nitrite level was below any detectable limit in the supernatants when PBMCs were cultured without SNP. After 8 h incubation, 800 μmol/L of SNP released approximately 20.3 μmol/L of nitrite in the culture medium.

Viability assay

The viability of PBMCs was not affected by any concentrations of SNP without stimulation from PMA and ionomycin for 8 h ($P > 0.05$; Friedman test). With stimulation from PMA and ionomycin for 8 h, it was shown to have diminished the viability of PBMCs ($P = 0.001$; Friedman test). The viability in the presence of 800 μmol/L SNP was significantly diminished compared with that in the absence of SNP ($P = 0.012$; Wilcoxon test) (Table 1).

CD3 positive lymphocyte percentage of PBMC

The mean percentage of CD3 positive lymphocytes was 52.1% (range from 44.6% to 64.3%) (Table 2). The percentage of CD3 positive lymphocytes was not affected by stimulation with PMA and ionomycin, or any concentrations of SNP for 8 h of incubation ($P > 0.05$; Friedman test) (Table 2).

Table 1. Viability of PBMCs estimated by dye exclusion test using trypan blue

	SNP (μmol/L)			
	0	200	400	800
Without PMA and ionomycin	98.5 ± 1.5%	98.9 ± 1.4%	98.5 ± 1.0%	97.0 ± 3.1%
With PMA and ionomycin	97.1 ± 3.1%	96.8 ± 3.1%	96.1 ± 1.8%	83.2 ± 8.7%*

Results are expressed as mean ± SD of 8 individual experiments.
* $P = 0.012$, between viability of PBMCs in the presence of 800 μmol/L of SNP and in the absence of SNP when stimulated with PMA and ionomycin (Wilcoxon test).

Table 2. The percentage of CD3 positive lymphocytes in 1.0 × 10⁴ of PBMCs

	SNP (μmol/L)			
	0	200	400	800
Without PMA and ionomycin	49.9 ± 3.9%	52.1 ± 6.6%	51.5 ± 6.2%	51.1 ± 5.3%
With PMA and ionomycin	54.8 ± 5.2%	52.7 ± 5.0%	53.2 ± 4.9%	51.5 ± 5.7%

Results are expressed as mean ± SD of 8 individual experiments.

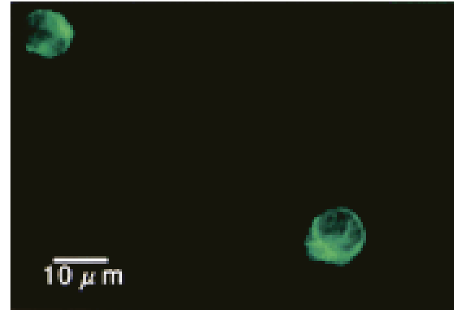

Fig. 2. Intracellular IFN-γ staining morphology visualized by fluorescent microscopy. IFN-γ producing cells exhibited maximal fluorescence in the cytoplasma close to the nuclei.

IFN-γ-staining morphology in individual cells

Figure 2 shows IFN-γ positive cells which exhibited maximal fluorescence in the cytoplasma close to the nuclei, reflecting the accumulation of IFN-γ in the Golgi apparatus.

Flow cytometric analysis of IFN-γ and IL-4 producing cells

When 800 μmol/L of SNP was added in the culture medium with stimulation for 8 h, the percentage of IFN-γ producing cells was markedly diminished (from 15.2% to 7.7%), while the percentage of IL-4 producing cells was diminished slightly (from 2.9% to 2.1%) (Fig. 3A and B). Negative staining with isotype control antibodies is shown in Fig. 3C.

Time course of intracellular IFN-γ and IL-4 staining gated on CD3 positive lymphocytes

The percentage of IFN-γ and IL-4 producing cells in CD3 positive lymphocytes reached maximal value at 8 h after stimulation of PMA and ionomycin without SNP ($P < 0.05$; Friedman test). The percentage of IFN-γ producing cells in CD3 positive lymphocytes was markedly diminished by 800 μmol/L of SNP each time. IL-4 producing cells in CD3 positive lymphocytes were also diminished by 800 μmol/L of SNP each time. When stimulated for 8 h, the mean percentage of IFN-γ producing cells in CD3 positive lymphocytes was 21.9% without SNP and 12.1% with 800 μmol/L of SNP.

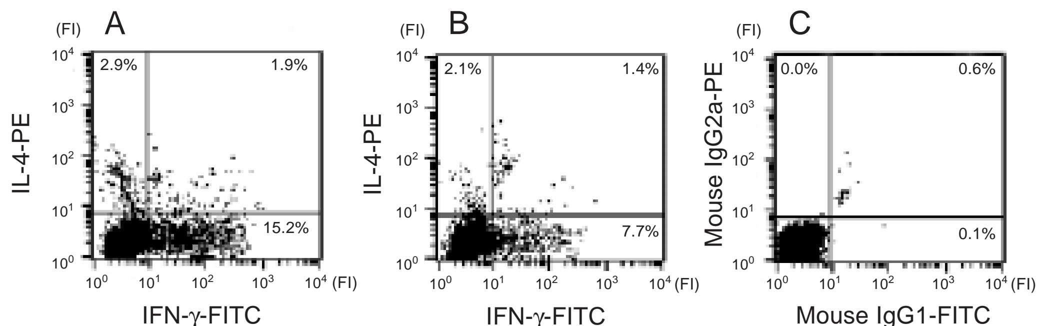

Fig. 3. Intracellular detection of IFN-γ and IL-4 producing cells gated on CD3 positive lymphocytes from a healthy subject by flow cytometry. PBMCs were stimulated with PMA (10 ng/mL) and ionomycin (1 μmol/L) (**A**) or together with 800 μmol/L of SNP (**B**). Negative staining with isotype control antibodies is shown in **C**. FI, fluorescence intensity.

When stimulated for 8 h, the mean percentage of IL-4 producing cells in CD3 positive lymphocytes was 3.4% without SNP and 2.2% with 800 μmol/L of SNP. Before stimulation, the mean percentage of IFN-γ or IL-4 producing cells in CD3 positive lymphocytes was below 1% (Fig. 4).

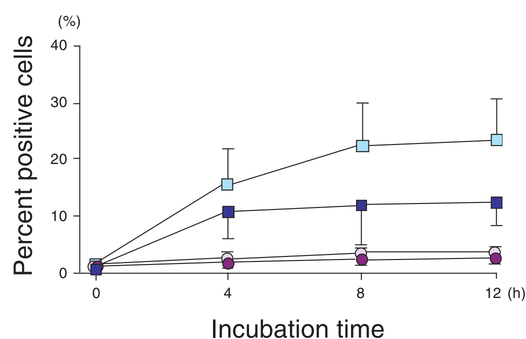

Fig. 4. Time course of percentage of intracellular staining for IFN-γ (□, ■) and IL-4 (○, ●) with CD3 positive lymphocytes from 4 healthy subjects. PBMCs were stimulated with PMA (10 ng/mL) and ionomycin (1 μmol/L) without SNP (□, ○) or with 800 μmol/L of SNP (■, ●). The percentage of positive cells was shown to be the rate of staining cells by FITC or PE in CD3 positive lymphocytes using flow cytometry. Results are expressed as mean ± SD of 4 individual experiments. The percentage of IFN-γ producing cells (□) and IL-4 producing cells (○) reached maximal value after 8 h stimulation without SNP ($P < 0.05$; Friedman test). The percentage of IFN-γ producing cells (■) and IL-4 producing cells (●) diminished by SNP at each of the time point.

The effect of different amounts of SNP on intracellular IFN-γ and IL-4 producing lymphocytes

When PBMCs were stimulated with PMA and ionomycin for 8 h, the percentage of IFN-γ producing cells gated on CD3 positive lymphocytes was diminished by SNP in a dose-dependent manner ($P = 0.002$; Friedman test). The percentage of IFN-γ producing cells in the presence of 800 μmol/L of SNP was significantly diminished compared with that of cells in the absence of SNP ($P = 0.012$; Wilcoxon test) (Fig. 5A). The percentage of IL-4 producing cells gated on CD3 positive lymphocytes tended to be diminished by SNP; however, this difference was statistically not significant ($P = 0.062$; Friedman test) (Fig. 5B).

Discussion

We investigated the effects of an NO donor on the production of IFN-γ and IL-4 at the single cell level. When PBMCs were stimulated with PMA and ionomycin for 8 h, the percentage of IFN-γ and IL-4 producing cells in CD3 positive lymphocytes were 21.9% and 3.4%, respectively. These results were consistent with the observation of Jung and colleagues (1995). In the experiment of co-incubation with SNP, the percentage of IFN-γ producing cells diminished significantly. While the percentage of IL-4 producing cells tended to be diminished, this difference, however, was statistically not significant.

The viability of PBMCs estimated by the dye exclusion test using trypan blue was not affected, when 800 μmol/L of SNP was added without PMA and ionomycin into the culture medium, while the viability of PBMCs was diminished when 800 μmol/L of SNP was added with PMA and ionomycin into the culture medium. So we suggested that SNP had no cytotoxic effect on PBMCs per se. SNP was reported to induce apoptosis of murine neuroblastoma (Yamada et al., 1996) and a human cancer cell line established from squamous cell carcinoma of the tongue (Sumitani et al., 1997). The reduction of the viability of PBMCs stimulated with PMA and ionomycin in the presence of SNP might be partially due to apoptosis. However, we could have excluded the apoptotic cells, which were shown to be a lower size than the living cells (Swat et al., 1991), to be gated on lymphocytes by forward and side scatter properties using flow cytometry. Furthermore, the percentage of CD3 positive lymphocytes was not affected by SNP, or PMA and ionomycin. So in all the data obtained from CD3 positive gating, the reduction of the percentage of IFN-γ producing cells might not result from cell death or apoptosis.

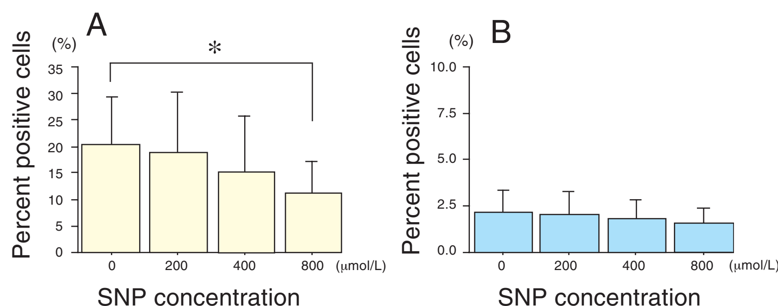

Fig. 5. The effect of different amounts of SNP on intracellular IFN-γ (**A**) and IL-4 staining (**B**). PBMCs were stimulated with PMA (10 ng/mL) and ionomycin (1 μmol/L) with 200, 400, 800 μmol/L of SNP. Results were expressed as mean ± SD of 8 individual experiments. The percentage of IFN-γ producing cells was significantly diminished by 800 μmol/L of SNP (*P = 0.012; Wilcoxon test). The percentage of IL-4 producing cells was slightly diminished by SNP, however, statistically not significant (P = 0.062; Friedman test).

There have been previous reports that exogenous NO donors could down-regulate the production of IFN-γ and IL-2 (Benbernou et al., 1997; Taylor-Robinson et al., 1994) as well as up-regulate the production of IL-4 (Chang et al., 1997) from T lymphocytes in murine. Therefore, it has been considered that NO was one of the modulators of Th cell differentiation in the murine system as well as in human lymphocytes as demonstrated by our results.

A controversial observation to our results was reported by Bauer and colleagues (1997), in that the NO donor inhibited both IFN-γ and IL-4 production from anti-CD3 activated human T cells. As to this discrepancy, two reasons could be considered. First, NO is produced and it functions on a micro environmental level and the half-life of NO is less than 10 s in vivo (Ignarro, 1990). And in Bauer's report, IL-4 level was estimated by enzyme-linked immunosorbent assay in the culture supernatant after one day of stimulation. Therefore, we reasoned that our culture system involving a shorter period of time might be physiologically relevant to study the effect of NO on T cell function. Secondly, Bauer and others used SIN-1, which releases both NO and superoxide anion; but NO and superoxide rapidly react to form peroxynitrite anion. Peroxynitrite anion was reported to induce endothelial cell injury (Beckman et al., 1990) and neuronal cell death (Lipton et al., 1993).

The mechanism of the regulating balance between Th1- and Th2-cytokines has been of great interest because it may determine the outcome of allergic and infectious diseases. It seemed that NO could play a role in regulating the development of Th cell differentiation and could polarize activated T cells to the Th2 profile from ours and other previous reports (Taylor-Robinson et al., 1994; Chang et al., 1997). Asthmatic patients showed elevated levels of NO in exhaled air (Alving et al., 1993) and Th2 predominance in the airway or peripheral blood was reported by several groups (Rousst et al., 1991; Walker et al., 1992). Barnes and Liew (1995) speculated that elevated NO in the asthmatic airway might play an important role in amplifying and perpetuating Th2 mediated inflammatory response through inhibition of Th1 mediated immune response. Our results may imply that NO in a local inflammatory site might be attributed to Th2-dominant inflammation.

The mechanism of the single inhibition of Th1 cytokine has not yet been understood clearly. Taylor-Robinson (1997) reported that IL-2 production was inhibited by NO produced from Th1 cells, and exogenous IL-2 reserved the NO-mediated down-regulation of Th1 proliferation. In addition, the inhibition of Th1 cytokines by NO may represent an important feedback mechanism preventing overexpression of Th1 cells or excessive production of NO. Recent

reports showed intracellular cyclic AMP (c-AMP) elevating agents, such as PGE_2, pentoxifylline and cholera toxin inhibited selectively Th1 cell proliferation or cytokine secretion, while these agents did not inhibit Th2 proliferation and production of cytokines (Muñoz et al., 1990; Betz and Fox, 1991; Benbernou et al., 1995, 1997). Therefore, it is suggested that c-AMP and protein kinase A (PKA) pathway, which is considered to be activated by c-AMP, may play an important role in determining the balance between Th1 and Th2 cytokine profiles in T cell line. Soff and workers (1997) reported that SNP activated PKA in rat smooth muscle cells. Therefore, our results that SNP inhibited Th1 cytokine, but not Th2 cytokine production might be caused by activation of PKA. However, we have no evidence that PKA activation actually occurred by SNP in lymphocytes. Further study is required to elucidate this hypothesis.

In conclusion, we demonstrated that NO might affect the diminishing of IFN-γ production by lymphocytes, but not significantly enough to diminish IL-4 production. Our findings suggest that NO modulates the immune system by the imbalance of Th1/Th2.

Acknowledgments: The authors wish to thank Prof. Takao Sasaki, Third Dept. of Internal Medicine, Prof. Kazuo Shiraki, Dept. of Pediatrics and Prof. Keisuke Satoh, Dept. of Pharmacology, Faculty of Medicine, Tottori University, for their helpful suggestions in this study.

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(Received December 17, 1997, Accepted January 8, 1998)