The Role of Nitric Oxide in the Immune Response of Tuberculosis

Nitric oxide (NO) formed by the action of inducible form of nitric oxide synthase (iNOS), reacts with oxygen radical forming reactive nitrogen intermediate (RNI). NO and related RNI have been reported to posses antimycobacterial activity. Macrophages can inhibit the proliferation of *Mycobacterium tuberculosis* by producing NO. In murine models, the ability of macrophages to produce NO can determine the susceptibility of the host to *M. tuberculosis* and the virulence of *M. tuberculosis*. However, it is still not clear whether NO is involved in the defense mechanism against *M. tuberculosis* in humans. We have demonstrated that human peripheral blood mononuclear cells (PBMC) and airway epithelial cells can express iNOS mRNA expression and produce NO production in response to tubercle bacilli stimulation. Furthermore, H37Ra, avirulent strain of *M. tuberculosis*, induces a larger amount of NO in cultured PBMC than H37Rv, virulent strain, does. There was no difference in NO production between healthy volunteers and patients with tuberculosis. NO production in airway epithelial cells is closely related with IFNγ concentration. The balance of stimulatory cytokines and inhibitory cytokines for NO production may play a critical role in the defense mechanism against *M. tuberculosis* considering that NO production is upregulated by IFNγ, TNFα, and IL-1β and downregulated by IL-10 and TGFβ. The study of immune response to *M. tuberculosis* including NO production may give us a better understanding of the pathogenesis of tuberculosis.

Key Words : *Nitric oxide, tuberculosis, cytokines, nitric oxide synthase*

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INTRODUCTION

Tuberculosis (Tbc) still remains an urgent global health problem, with approximately 1 billion people presently infected with *M. tuberculosis* and about 3 million deaths per year (1). Although the prevalence of pulmonary Tbc in Korea has markedly decreased during recent decades, the prevalence of pulmonary Tbc in Korea was as much as 1.0% of general population in 1995 (2). Therefore, it is urgent to spend more effort and money in controlling Tbc.

Following exposure to *M. tuberculosis*, some individuals (about 10%) develop active Tbc, while the majority do not. It is not clear what factors determine the development of active disease among individuals infected by *M. tuberculosis*. It would be interesting to disclose the factors why some develop active disease after exposure and why the others do not.

Macrophages have been reported to play a pivotal role in the immune response against mycobacteria by producing cytokines, such as TNFα, IL-1β (3). TNFα and IL-1β, along with IFNγ produced by T-lymphocytes, can induce nitric oxide (NO) in macrophages via the action of inducible form of the enzyme nitric oxide synthase (iNOS). NO and related reactive nitrogen intermediate (RNI) can kill and/or inhibit intracellular pathogens like mycobacteria (4, 5). IFNγ 'knockout' mice that are not capable of producing NO and RNI in response to tubercle bacilli, experience a fulminant course of tuberculosis suggesting the role of NO and RNI in the defense mechanism against *M. tuberculosis* (6). There is evidence that the production of NO and related RNI is well correlated with antimycobacterial effect of murine macrophages. There is, however, few report on the antimycobacterial effect of human macrophages and its correlation with the production of cytokines and NO.

Airway epithelial cells as well as macrophages can produce NO in response to the stimulation of TNFα, IL-1β, and IFNγ (7). We have demonstrated that airway epithelial cells can produce NO in response to the stimulation of tubercle bacilli. Tubercle bacilli which enter the airway stimulate alveolar macrophages and induce proinflammatory cytokines, such as TNFα and IL-1β. These cytokines stimulate adjacent airway epithelial cells in a paracrine fashion, resulting in production of NO. By producing NO, airway epithelial cells are able

to actively participate in airway inflammatory process and the defense mechanism against *M. tuberculosis*.

The purpose of the present review is to describe the role of NO in the defense mechanism against *M. tuberculosis* and regulatory mechanism for the production of NO in macrophages and airway epithelial cells.

NITRIC OXIDE

There has been an explosion of information about NO which appears to be involved in an extraordinary range of functions including vascular regulation, neurotransmission, cytotoxicity, and host defense (8). NO is formed when the guanido group of the essential amino acid L-arginine is cleaved forming NO and L-citrulline. The reaction is catalyzed by nitric oxide synthase (NOS).

Several species of NOS have been characterized and several distinct NOS genes have been identified (9). NOS can exist as constitutive forms (cNOS) which are basally expressed in endothelial, neuronal and other cells and are Ca^{2+}-calmodulin dependent. There are also inducible forms (iNOS) which may be expressed after exposure to certain cytokines and endotoxin. iNOS does not require calmodulin.

iNOS is induced by several cytokines including IFNγ, IL-1β, and TNFα as well as by endotoxin. Induction of iNOS results in the production of larger amount of NO and may contribute to the host defense against the infection of bacteria and parasites (8). NO reacts with oxygen radicals forming reactive nitrogen intermediates. NO and subsequent RNI have been reported to be involved in the stasis and killing bacteria including mycobacteria, and parasites suggesting the role of NO in the host defense (10).

NITRIC OXIDE AND TUBERCULOSIS

When tubercle bacilli enter the alveoli, they are engulfed by alveolar macrophages, which perform three important functions (11). First, alveolar macrophages produce proteolytic enzymes and other metabolites that exhibit mycobatericidal effects. Second, alveolar macrophages process and present mycobacterial antigens to T-lymphocytes. Expression of antigen through this pathway induces expansion of specific $CD4^+$ lymphocytes, the cell population that is central to acquired resistance to *M. tuberculosis*. Third, alveolar macrophages produce a characteristic pattern of soluble mediators (cytokines) in response to *M. tuberculosis*, including IL-1, IL-6, IL-10, TNFα, and transforming growth factor beta (TGFβ). These cytokines, along with IFNγ produced by T-lymphocytes, have the potential to exert potent immunoregulatory effects and to mediate the clinical manifestation of tuberculosis. These cytokines can also regulate the production of NO in macrophages and adjacent airway epithelial cells.

Lipoarabinomannan (LAM) is a major cell wall-associated glycolipid produced by *M. tuberculosis* (12). LAM has been designated as a possible virulence factor of mycobacteria by virtue of its ability to scavenge potentially cytotoxic oxygen free radicals and its capacity to block the transcriptional activation of IFNγ inducible genes in human macrophage-like cell lines (13). Chatterjee and colleagues have isolated LAM from different strains of *M. tuberculosis*, the avirulent H37Ra (a highly attenuated laboratory strain) and the virulent Erdman strain (14). Thereafter, LAM has been used for in vitro stimulation instead of live tubercle bacilli.

Stimulation with H37Ra LAM could induce TNFα secretion at 1000 ng/ml and NO production in cultured murine bone marrow-derived macrophages, but stimulation with Erdman LAM could not. Addition of IFNγ enhanced TNFα secretion and NO production in response to H37Ra LAM. In contrast, Erdman LAM could not induce macrophage TNFα secretion and NO production even in the presence of IFNγ (15). Virulent strain of *M. tuberculosis*, Erdman, can survive and proliferate in host since they did not induce a immune response in macrophages and are able to escape from the defense mechanism of host against *M. tuberculosis*. These findings suggest that virulence of *M. tuberculosis* may be determined by presence or absence of an appropriate immune response of the host including NO generation.

Barrera and colleagues (16) reported that nitrite production was significantly different between bacillus Calmette-Guerin(BCG)-susceptible and BCG-resistant mice. When stimulated with IFNγ, nitrite production was significantly higher in macrophages derived from BCG-resistant mice than in those cells from BCG-sensitive mice. Nitrite production was well correlated with mycobacteriostatic effect and this effect was blocked by NOS inhibitor, N-MMLA. These findings suggest that NO has an important role in the defense mechanism against *M. tuberculosis*, and the development of disease after exposure to *M. tuberculosis* may be determined by an appropriate immune response including NO production.

In contrast to the findings described earlier, Brown and colleagues (17) reported that NO production did not affect the antimycobacterial activity of macrophages from BCG-sensitive and BCG-resistant mice. They suggested that *Nramp* (natural resistance associated-macrophage protein) gene may be involved in the susceptibility and resistance of mice to mycobacteria (17,18). However,

they studied the effect of corticosteroid rather than natural resistance to mycobacteria, and NMMA, a NOS inhibitor, did affect the antimycobacterial effect of macrophages stimulated with IFNγ suggesting the role of NO in the defense mechanism against mycobacteria.

It seems evident that NO and related RNI produced by macrophages are involved in the defense mechanism against *M. tuberculosis* by inhibiting the proliferation of *M. tuberculosis* in mice. However, there is little data on the role of NO in human defense mechanism against tuberculosis.

NO PRODUCTION IN HUMAN MACROPHAGES

It is curious that while human macrophages has been reported to produce RNI in amounts sufficient for killing of *M. avium* in vitro (19), the results of efforts to demonstrate NO production in human macrophages have been inconsistent. There is, however, evidence to suggest that human macrophages have the potential to produce NO. Individuals experiencing sepsis (20) or undergoing cytokine therapy for tumor (21) generate a large amount NO suggesting the presence of iNOS in human macrophages.

When we stimulated cultured peripheral blood mononuclear cells (PBMC) with relatively low concentration of tubercle bacilli (5×10^5 bacilli/ml), nitrite concentration was minimal in supernatant of cultured PBMC. When cultured PBMC were stimulated with a higher concentration of tubercle bacilli (5×10^7 bacilli/ml), nitrite concentration in supernatant of cultured PBMC was significantly increased (22, Fig. 1). Interestingly, nitrite concentration in supernatants of cultured PBMC stimulated with H37Ra, avirulent strain of *M. tuberculosis*, was significantly higher than in those stimulated H37Rv, virulent strain of *M. tuberculosis* (Fig.1). This result supports the theory that the virulence of *M. tuberculosis* may be determined by whether or not appropriate immune response including NO production is present in the host. However, we could not find any difference in NO production in cultured PBMC from healthy volunteer and from patients with tuberculosis.

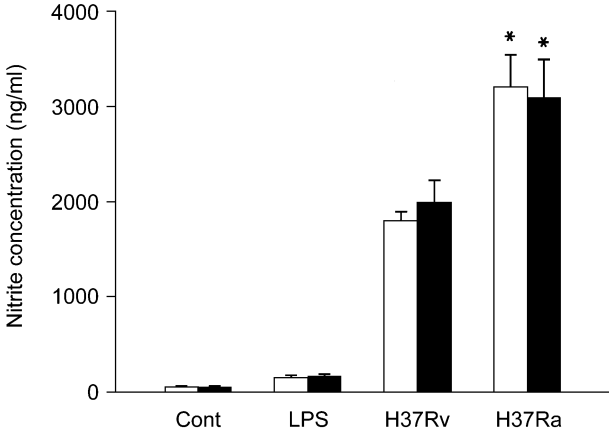

Fig. 1. NO production in cultured PBMC. Nitrite concentration was higher in PBMC stimulated H37Ra than in PBMC stimulated with H37Rv. Open bars represent data from healthy volunteer and solid bars represent data from patients with tuberculosis. Data are expressed as mean±SEM.
n=7, *p<0.05 compared with H37Rv.

Recently, Wang and colleagues (23) reported that the magnitude of iNOS expression was significantly increased in both alveolar macrophages and peripheral blood monocytes from Tbc patients compared to those cells from normal subjects. They also found that both alveolar macrophages and peripheral blood monocytes from active Tbc patients produced more nitrite than those cells from normal subjects.

In patients with active Tbc, two hypotheses may be speculated. First, NO production may be increased to overcome the infection of *M. tuberculosis*. Large amount of NO and related RNI produced by the patients can inhibit the proliferation of *M. tuberculosis*. The report by Wang and colleagues (23) supports this hypothesis. Second, NO production may be decreased in patients with tuberculosis, since they cannot appropriately produce NO in response to the stimulation of tubercle bacilli (16). This hypothesis means that tuberculosis may develop in individuals who cannot elicit appropriate immune response after exposure to *M. tuberculosis*. PBMC from patients with multi-drug resistant Tbc have been reported to produce a large amount of IL-10 (24), which is known to inhibit the function of macrophages and to reduce NO production (25, 26), supporting the second hypothesis. It is still not clear whether NO production is related with the pathogenesis of pulmonary tuberculosis in human and further studies should be followed to clarify the role of NO in tuberculosis.

NO PRODUCTION IN AIRWAY EPITHELIAL CELLS

Airway epithelial cells have been reported to be able to actively participate in the pathogenesis of pulmonary inflammatory diseases by producing several cytokines and chemokines (27-29). Airway epithelial cells can produce chemokines such as IL-8 and RANTES in response to tubercle bacilli stimulation indicating that airway epithelial cells may play a certain role in the pathogenesis of tuberculosis (30, 31). However, there has been no report which shows NO production in airway epithelial cells in response to the stimulation of tubercle bacilli.

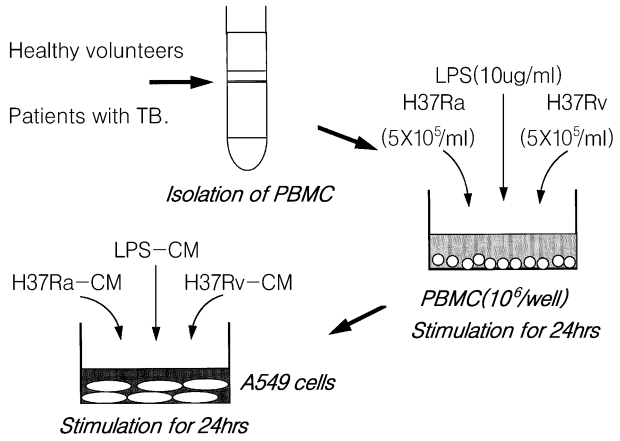

Fig. 2. Study design to study the NO production in A549 cells. A549 cells were stimulated with supernatants of cultured PBMC.

Therefore, it would be interesting to see whether airway epithelial cells can produce NO in response to the stimulation of tubercle bacilli. We stimulated cultured PBMC with tubercle bacilli first and then stimulated cultured A549 cells with conditioned media of cultured PBMC (Fig. 2). Since direct stimulation of A549 cells with either LPS or tubercle bacilli did not induce iNOS mRNA expression and NO production, we hypothesized that tubercle bacilli may stimulate alveolar macrophages resulting in production of proinflammatory cytokine such as IL-1β, TNFα, and IFNγ, and these cytokines may stimulate adjacent structural cells such as alveolar epithelial cells in a paracrine fashion resulting in the production of NO in vivo (29, 30).

iNOS mRNA expression and NO production in cultured A549 cells were induced by the stimulation of tubercle bacilli-conditioned media (Tbc-CM) of cultured PBMC (Fig. 3, Fig. 4) indicating that airway epithelial cells can participate in the immune response against *M. tuberculosis* in humans. However, there was no difference in iNOS mRNA expression and NO production in cultured A549 cells stimulated with either H37Rv-CM or H37Ra-CM. In this experiment, we used 5×10^5 tubercle bacilli/ml for stimulation. If we had used higher number of tubercle bacilli for stimulation, we might have seen a difference between H37Rv and H37Ra stimulated groups.

Concentrations of proinflammatory cytokines, such as IL-1β, TNFα, and IFNγ, were also increased in supernatants of cultured PBMC stimulated with tubercle bacilli (Fig. 5). These findings suggest that the study design we have used can correctly represent the pathophysiological events in vivo, which occur during *M. tuberculosis* infection.

NO production in A549 cells was well correlated with the concentration of IFNγ in supernatant of cultured PBMC. IFNγ may be the most important cytokine for inducing NO production in airway epithelial cells. This finding is consistent with in vitro study which shows that iNOS mRNA expression was induced only when IFNγ was included in the stimulation (Fig. 6).

REGULATION OF NO PRODUCTION

As previously described, NO production is enhanced by IFNγ and TNFα. In contrast, NO production by murine macrophages can be downregulated by various

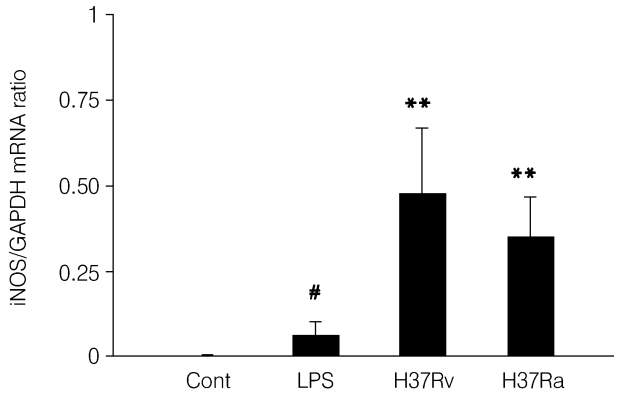

Fig. 3. iNOS mRNA expression in A549 cells. iNOS mRNA expression was increased in A549 cell stimulated with tubercle bacilli-CM or LPS-CM. Data are expressed as mean± SEM. n=9, # $p<0.05$ compared with control, ** $p<0.01$ compared with LPS.

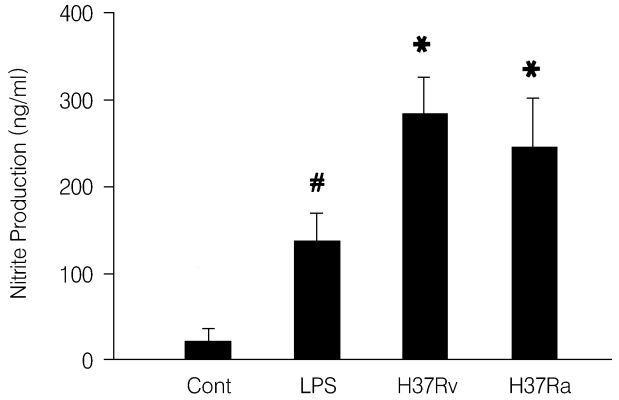

Fig. 4. NO production in A549 cells. NO production was increased in A549 cells stimulated with tubercle bacilli-CM or LPS-CM. Data are expressed as mean±SEM. n=9. * $p<0.05$ compared with LPS, # $p<0.05$ compared with control.

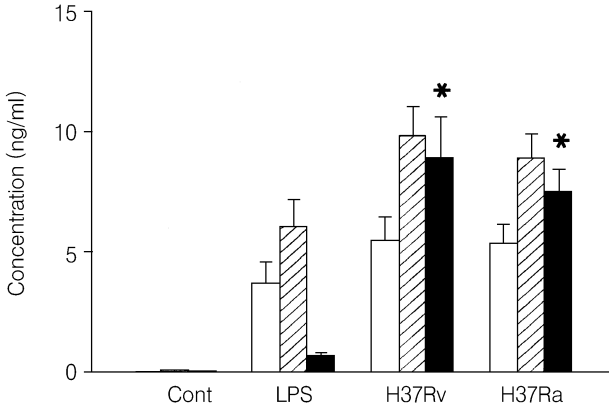

Fig. 5. Concentration of cytokines in supernatants of cultured PBMC. IFNγ concentration was higher in cultured PBMC stimulated with tubercle bacilli than in cells stimulated with LPS. Note that IFNγ concentrations were well correlated with NO production in A549 cells. Open bars represent IL-1β, hatched bars represent TNFα, and solid bars represent IFNγ concentrations. Data are expressed as mean±SEM. n=9. *p<0.01 compared with LPS.

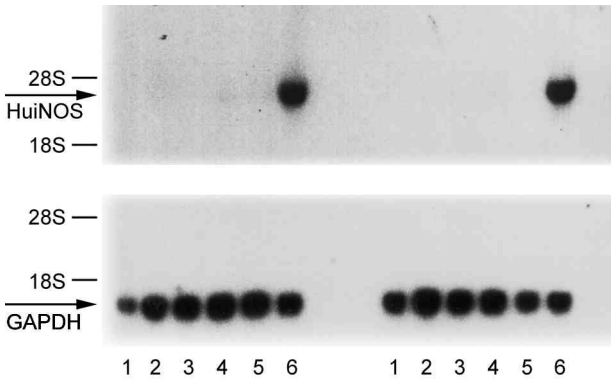

Fig. 6. Northern blot analysis for iNOS mRNA in A549 cells stimulated with various combinations of cytokines. Lane 1 represents control, lane 2 represents IL-1β, lane 3 represents TNFα, lane 4 represents IFNγ, lane 5 represents IL-1β and TNFα, and lane 6 represents IL-1β, TNFα, and IFNγ stimulation, respectively. Concentration of cytokines was 10 ng/ml. Note that iNOS mRNA expression was induced in A549 cells only stimulated with combination of IL-1β, TNFα, and IFNγ.

Th2 cytokines, including IL-4, IL-10, and TGFβ (26, 32-34). The mechanism that these inhibitory cytokines inhibit NO production is still unclear.

Among cytokines produced by T-cells, IL-10 is an important negative regulator of immune response and IL-10 negatively regulate Th1 cells as well as macrophages (35). IL-10 has been shown to inhibit the antimycobacterial activity of macrophages in vitro and could account for the ability of mycobacteria to survive intracellularly. Transgenic mice that secret IL-10 from the T cell compartment could not clear the mycobacterial infection and developed large bacterial burdens (36). In contrast, IL-10-deficient mice were highly resistant to intracellular pathogen during the course of infection (37). Infusion of a large dosage of the monoclonal anti-IL-10 resulted in a very significantly diminished mycobacterial growth in mice (38). These findings suggest that IL-10 inhibit the resistance to *M. tuberculosis* infection.

NO production may be regulated by balance between stimulatory cytokines, such as TNFα and IFNγ, and inhibitory cytokines, such as IL-10 and TGFβ. The immune response to tuberculosis is a double-edged sword that may contribute to both clearance of infection and tissue damage. If the balance between stimulatory cytokines and inhibitory cytokines for NO production is lost, NO production in response to mycobacterial infection may be inadequately high or low resulting in disseminated tissue damage or the inability to clear infection.

We do not know the reason why some individuals develop tuberculosis after exposure to *M. tuberculosis* while the others do not, and why tuberculosis is progressive despite appropriate anti-tuberculosis medication in some individuals. The study of immune response to *M. tuberculosis* including NO production may give us a better understanding of the pathogenesis of tuberculosis and an answer to those questions.

Acknowledgments

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