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Increased exhaled nitric oxide in active pulmonary tuberculosis due to inducible NO synthase upregulation in alveolar macrophages

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Increased exhaled nitric oxide in active pulmonary tuberculosis due to inducible NO synthase upregulation in alveolar macrophages. C-H. Wang, C-Y. Liu, H-C. Lin, C-T. Yu, K.F. Chung, H-P. Kuo. ©ERS Journals Ltd 1998.
ABSTRACT: Nitric oxide (NO) plays an important role in resistance to *Mycobacterium tuberculosis* infection. Our aim was to determine whether inducible NO synthase (iNOS) expression and generation of reactive nitrogen intermediates (RNI) by alveolar macrophages (AM) are increased in patients infected with *M. tuberculosis*.

NO levels in the exhaled air of 19 active pulmonary tuberculosis (TB) and 14 control subjects were measured using a chemiluminescence NO analyser. The expression of iNOS on AM was studied by labelling AM with anti-mac iNOS polyclonal antibody analysed with a flow cytometer. The spontaneous generation of RNI by cultured AM was also measured. Data are presented as mean±SEM.

The level of NO in exhaled air was higher in patients with active TB (16.2±1.2 parts per billion (ppb)) compared to control subjects (6.5±0.9 ppb), p<0.0001. Exhaled NO decreased with anti-TB treatment. Compared to control subjects (29.0±4.5 fluorescence intensity (FI)), iNOS expression on AM was upregulated in TB patients (86.3±12.5 FI) p<0.001 and the capacity for spontaneous generation of nitrite was enhanced. Nitrite production was inhibited by N^G-monomethyl-L-arginine (L-NMMA), a competitive inhibitor of iNOS. The expression of iNOS on AM was related to the concentration of exhaled NO (r=0.66, p<0.001) and the nitrite generation capacity of AM (r_s=0.77, p<0.001).

We conclude that the increase in exhaled nitric oxide observed in patients with active pulmonary tuberculosis is due to an upregulation of inhaled NO synthase expression in alveolar macrophages which have an enhanced capacity for nitric oxide production.
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Nitric oxide (NO) plays a major role in the pulmonary host-defence mechanism in response to infections and is implicated in bacteriostatic as well as bactericidal processes [1, 2]. NO is generated through the L-arginine pathway and is converted to the related reactive nitrogen intermediates (RNI) (NO_3^- and NO_2^-). In inflammatory responses, NO is produced by the inducible form of NO synthase (iNOS), which is mostly from inflammatory cells, such as macrophages [3–5]. NO production and iNOS expression by alveolar macrophages (AM) is upregulated in response to heat-killed *Mycobacterium tuberculosis* instilled into the lungs of rats [6] and plays a role in limiting the growth of *Mycobacterium avium complex* [7, 8]. Murine macrophages activated by interferon (IFN)-γ and tumour necrosis factor (TNF)-α are capable of killing *M. tuberculosis in vitro via* the generation of RNI by iNOS [9]. In addition, pretreatment with NO synthase (NOS) inhibitors profoundly increases bacterial burden, pathological tissue damage and mortality in mice infected with *M. tuberculosis* [10], thus indicating that NO plays an important role in resistance to *M. tuberculosis* infection in the mouse. A recent report has shown that iNOS messenger ribonucleic acid (mRNA) is upregulated on AM in patients with active pulmonary tuberculosis (TB) [11], however, it is not known whether the iNOS enzyme activity is also enhanced and leads to increased generation of RNI by AM against *M. tuberculosis.*

NO can be detected in the expired air of animals and humans [12]. Recent reports have shown that the level of exhaled NO is elevated in patients with bronchial asthma and bronchiectasis [13, 14], and have suggested that this is due to iNOS upregulation induced by cytokines such as IFN-γ, TNF-α and interleukin (IL)-1β [15] released in chronic airway inflammation. Thus, the measure of exhaled NO concentration may gauge the extent of NO-mediated inflammatory responses in the airways. In this study, we measured exhaled NO levels in patients with active pulmonary TB and determined the cellular source of NO by examining the expression of iNOS and the release of RNI from AM.

Material and methods

Study population

Nineteen patients with active pulmonary TB (13 males and 6 females, aged 59.1±4.5 yrs) were studied prior to anti-TB treatment. None of these patients were current smokers. For all patients, at least one recent sputum specimen

was positive for acid-fast bacilli on microscopic examination and all sputum cultures grew *M. tuberculosis*. The nutritional status was assessed by the measurement of body mass, height, triceps skin-fold thickness, mid-arm circumference and serum albumin level. Patients with poor nutritional status (body mass <90th percentile or mid-arm circumference and triceps skin-fold thickness <25th percentile) were prospectively excluded from this study to avoid the confounding effect of poor nutritional status on immunity. Patients with asthma, chronic obstructive pulmonary disease, bronchiectasis, systemic or local airway inflammatory diseases (lupus erythromatosus, sepsis, and pneumonia), diabetes mellitus, and malignancy were also excluded from this study. None took corticosteroids or other immunosuppressants.

The control group consisted of 14 healthy, nonsmoking subjects including eight males and six females, with a mean age of 47.3±4.3 yrs. Among them, six presented with little haemoptysis. In all of them, fibreoptic bronchoscopy and chest radiographs were within normal limits. The other eight subjects were volunteers. None of these subjects had a history or evidence of lung disease based on physical, chest radiographic, chest computed tomography and bronchoscopic examinations. None of them had suffered any upper respiratory tract infection within the last 6 weeks or were on antibiotics or other medications at the time of evaluation. The research protocol was approved by Chang Gung Memorial Hospital Research Committee. Informed consent was obtained from all subjects.

Protocol

In all patients and normal control subjects, exhaled NO was measured at entry. In TB patients, the levels were also measured after 3 months of treatment. Patients and normal subjects underwent bronchoscopy with bronchoalveolar lavage (BAL). Patients also had transbronchial lung biopsy on admission.

Disease extent on chest radiograph and bacterial loads in sputum

In all TB patients, morning sputum collected on three different days was stained for acid-fast bacilli by the auramine fluorescent stain. Bacterial counts were determined as previously described [16]. Briefly, the grading was differentiated into four categories: 0: absence of bacilli; 1: 1–9 bacilli; 2: 10–29 bacilli; and 3: >30 bacilli·30 oil-immersion fields^{-1}. The grading of bacterial counts from the specimens on three different days were summed up as an index of bacterial load of sputum. In all patients, a posterior-anterior chest radiograph was taken at the time of hospital admission. The extent of disease on the chest radiograph was determined according to the grading proposed by the World Health Organization (1960) [17]: 0 = no involvement; 1= trivial; 2 = slight; 3 = limited; 4 = moderate; 5 = extensive; 6 = gross. The grading of the patient's radiograph was the consensus of two pulmonary physicians.

Measurement of exhaled NO

The level of exhaled NO was measured using a chemiluminescence analyser (NOA Model 280; Sievers, Boulder, CO, USA), which was adapted for on-line recording of NO concentration. To minimize the influence of environmental air on the level of exhaled NO, all subjects breathed NO free gas (21% O_2 and 79% N_2) for 3 min. To decrease the possibility of a time-dependent change in sensitivity in the NO analyser, the baseline NO concentration in ambient air was kept to a similar level (±3 parts per billion (ppb)). Subjects wore noseclips and were positioned either sitting or standing. They were asked to perform a slow vital capacity manoeuvre over 30–45 s into a wide-bore Teflon tube. NO was sampled from a side arm and continuously into the analyzer at a flow rate of 200 mL·min^{-1}. The optimal flow rate into the analyser is 200–500 mL·min^{-1}. Over this flow range, the response of the chemiluminescence analyzer is independent of the flow into the analyser and small changes in flow rate will not cause changes in the response of the NO machine. The factory calibration was performed at this flow rate, thus, we used this flow rate to monitor the level of exhaled NO. Three successive and the highest values of plateau levels were recorded to obtain the mean values.

To ascertain that the level of exhaled NO was indicative of the inflammatory state of the lower airways, the concentrations of NO in 13 patients with active TB was sampled directly at the level of vocal cords, main carina, left main bronchus, right main bronchus and the lesion site before the lavage procedure and compared with the concentration of exhaled NO. Using fibreoptic bronchoscopic guidance, airway gas samples were collected continuously through the sampling port of bronchoscope and serially analysed for NO concentration using a chemiluminescence analyser (NOA Model 280; Sievers).

Preparation of lower respiratory tract cells

BAL was performed in all study subjects using five aliquots (50 mL each) of 0.9% saline solution as described previously [16, 18]. Briefly, sterile saline solution was introduced into the right fourth or fifth subsegmental bronchus in normal subjects. In TB patients, BAL was performed in the involved segment. The lavaged fluid was retrieved by gentle aspiration, pooled and was filtered through two layers of sterile gauze. The BAL fluid was centrifuged at 600 × g for 20 min at 4°C. The cell pellet was washed sequentially and resuspended in RPMI-1640 (GIBCO, Grand Island, NY, USA) supplemented with 5% heat-inactivated foetal calf serum (FCS; Flow Laboratories, Paisley, Scotland, UK) at 10^6 cells·mL^{-1}. The cell viability was determined by trypan blue exclusion. Differential cell counts were determined by counting 500 cells on cytocentrifuge preparations using a modified Wright-Giemsa stain.

Culture of AM

AMs were placed in plastic culture dishes in RPMI-1640, allowed to adhere for 90 min and washed three times with warm RPMI-1640 to remove nonadherent cells. Adherent cells were scraped off with a sterile rubber policeman. The cells were resuspended (10^6 cells·mL^{-1}) in RPMI-1640 medium containing 5% FCS, 100 U·mL^{-1} penicillin, and 100 µg·mL^{-1} streptomycin. Then, the purified AM were placed in 12 well petri dishes at 10^6 cells·mL^{-1} for 24 h at 37°C, 5% CO_2. The culture supernatant was collected and frozen at -70°C before measuring

RNI production. To determine the generation of RNI was specific for NO production, AM from nine patients with active TB or eight control subjects were cultured at 10^6 cells·mL^{-1} in the presence or absence of an NOS inhibitor, N^G-monomethyl-L-arginine (L-NMMA) at a final concentration of 1 mM (Calbiochem, La Jolla, CA, USA) for 24 h at 37°C, 5% CO_2. The supernatants were stored at -70°C for measurement of RNI.

Expression of iNOS in AM

BAL cells, at a concentration of 1×10^6 cells·mL^{-1}, were fixed with 4% paraformaldehyde at room temperature for 10 min and then washed twice with phosphate-buffered saline (PBS) pH 7.0. The cell pellet was resuspended in 20 µL of 3.7% n-octyl-β-D-glucopyranoside (Sigma, St Louis, MO, USA), and then incubated for 5 min at room temperature before two extensive washes with PBS. Thereafter, the cells were incubated with anti-iNOS rabbit polyclonal antibody (Transduction Laboratories, Lexington, KY, USA; diluted 1:5 in PBS (20 µL)) in the dark for 1 h at 4°C. Rabbit immunoglobulin (Ig)G was used as control. The cells were washed twice with PBS, the cell pellet was labelled with 5 µL fluorescein isothiocyanate (FITC) conjugated swine anti-rabbit IgG (Dakopatts, Glostrup, Denmark) and placed in the dark for 30 min at 4°C. Cells were analysed by flow cytometry after two extensive washings with cold PBS containing 5% FCS. The main fluorescence intensity (FI) of AM labelled with the IgG control antibody was always less than 10.

Flow cytometric analysis

Analysis was performed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) and LYSYS II software (Becton Dickinson). All fluorescence was measured using logarithm amplification. Ten thousand events were collected for each sample. Cells were gated on the basis of forward scatter and side scatter into lymphocytes, macrophages and granulocytes. The ability of the gates to segregate cells was checked using CD14 and CD3 antibodies to determine the purity of the macrophages and lymphocytes gates, respectively. The remaining cell group was indicated as granulocytes. Less than 5% of cells in the macrophage gate expressed CD3 and less than 0.4% of cells in the lymphocyte gate expressed CD14. Control of rabbit IgG was used to give a measure of nonspecific binding using FITC-conjugated swine anti-rabbit immunoglobulin (Dakopatts) against which results were expressed as a mean FI in arbitrary units transformed to a linear scale from the $\log_{10}$ channel number of mean fluorescence, for a particular cell marker.

Quantification of nitrite production by AM

To measure the concentration of nitrite, 50 µL of lavage fluid or culture supernatant was added to the purge vessel containing 5 mL of a reducing solution (1% potassium iodide (KI) in acetic acid) to convert nitrite into NO. NO was then quantitated using a specific chemiluminescence analyser (NOA Model 280 Sievers). Conversion of standard mixtures of nitrite and nitrate solutions to NO was 94% when compared with calibrated standards of NO gas.

Immunohistochemical staining of lung tissue

Transbronchial lung biopsies were obtained from radiographically-affected areas as part of our work-up of patients with active pulmonary TB. Lung tissue specimens were fixed in 10% formalin and embedded in paraffin, cut at 4 mm and picked up on aminopropyltriethoxysilane coated slides. As controls, we studied surgically resected lung tissue specimens from two heart transplant donors. Two antibodies were used for NOS detection: 1) a polyclonal rabbit antibody to the murine macrophages iNOS which immunoreacted with human iNOS (Anti-macNOS; Transduction Laboratories, Lexington, KY, USA); and 2) a monoclonal mouse antibody to constitutive NOS (cNOS) from human endothelium (Anti-ECNOS, Transduction Laboratories). At the dilution used, the antiserum has proven reactivity with endothelial and neuronal cNOS isoenzymes from several species including humans [19, 20]. Controls included a nonspecific purified rabbit IgG. After being dewaxed thoroughly in xylene and rinsed in absolute alcohol, sections were incubated in 3% hydrogen peroxide (H_2O_2) in methanol for 30 min to quench endogenous peroxidase. Then the sections were microwaved in citric acid buffer with 0.1% Triton for 5 min to enhance antigen exposure and incubated in 0.2% normal swine serum (Dakopatts) for 30 min to block the positive and negative charges of tissues. Afterwards, the sections were subjected to 1 h incubation with the specific anti-NOS antibody (diluted 1: 200) or the nonspecific purified rabbit IgG (diluted 1:200) as a control. Antibody labelling was visualized using an avidin-biotin complex method (labelled streptavidin biotin (LSAB) 2 kit; Dakopatts, and diaminobenzidene (DAB) peroxidase substrate kit; Vector Laboratories, Burlingame, CA, USA). The slides were then counter-stained with haematoxylin, dehydrated through graded alcohols and xylene and mounted.

Statistical analysis

Data are presented as mean±SEM. One-way analysis of variance (ANOVA) for mixed design was used to compare values of more than two different experimental groups. If variance among groups was noted, a Bonferroni test was used to determine significant differences between specific points within groups. The data was analysed by Student's t-test for paired or unpaired data. For data with uneven variation, a Mann-Whitney U-test or Wilcoxon signed rank test was used for unpaired or paired data, respectively. The relationship between the mean FI of iNOS expression, the level of exhaled NO, the disease extent on chest film and bacterial load on sputum was sought by linear regression test or Spearman's rank correlation test. A p-value of less than 0.05 was considered statistically significant.

Results

Cell profiles in BAL

There was a significant increase in the total cell count in patients with active pulmonary TB (43.6±11.6 $\times10^6$ cells) compared to control subjects, (11.9±1.8 $\times10^6$ cells) $p<0.05$. Cell concentration was 4.4±1.1 $\times10^4$ cells·mL^{-1}

in patients with active TB compared to 1.4±0.3 $\times 10^4$ cells·mL^{-1} in control subject ($p<0.05$). The proportion of lymphocytes and neutrophils was significantly higher in patients with TB (12.9±1.3 and 12.3±2.7%, respectively) compared to control subjects (8.8±1.3 and 1.4±0.3%, respectively), $p<0.05$ and $p<0.001$, respectively. Reciprocally, the percentage of macrophages was significantly lower in patients with TB (74.6±2.4%, n=19) compared to control subjects (89.7±1.2%, n=14), $p<0.01$. The recovery rate of BAL was significantly lower in patients with active pulmonary TB (46.5±3.9%) compared to control subjects (65.4±3.3%), $p<0.001$.

Exhaled NO and iNOS expression in AM

The level of exhaled NO was significantly higher in patients with active TB (16.2±1.2 ppb) compared to control subjects (6.5±0.9 ppb) $p<0.0001$ (fig. 1). In patients with active TB (n=13), the NO concentrations sampled in lower airways (vocal cords, 16.7±1.1; main carina, 16.9±1.1; left main bronchus, 15.8±0.8; right main bronchus, 16.8±1.1; the lesion site, 16.7±1.1 ppb) were not significantly different from the level of NO in exhaled breath (16.8±1.1 ppb) and were highly correlated with the corresponding exhaled NO levels (vocal cords, r=0.89; main carina, r=0.87; left main bronchus r=0.78; right main bronchus, r=0.77; the lesion site, r=0.82, $p<0.001$, for each). The concentration of exhaled NO from TB patients was significantly decreased from a pretreatment value of 16.5±1.4 ppb to 8.5±0.8 ppb after standard anti-TB treatment for 3 months ($p<0.0001$) (fig. 1). The exhaled NO levels at 3 months showed no difference when compared with the control. The magnitude of iNOS expression on AM was significantly greater in TB patients (86.3±12.5 FI) compared to control subjects (29.0±4.5 FI, n=14, $p<0.001$) (fig. 2). The magnitude of iNOS expression on AM was highly correlated with the level of exhaled NO either in all study subjects (r=0.66, $p<0.0001$, fig. 3) or in TB patients (r=0.74, $p<0.0005$), but not with the disease extent on chest radiographs or with the bacterial load on sputum. The expression of iNOS in lymphocytes or neutrophils was

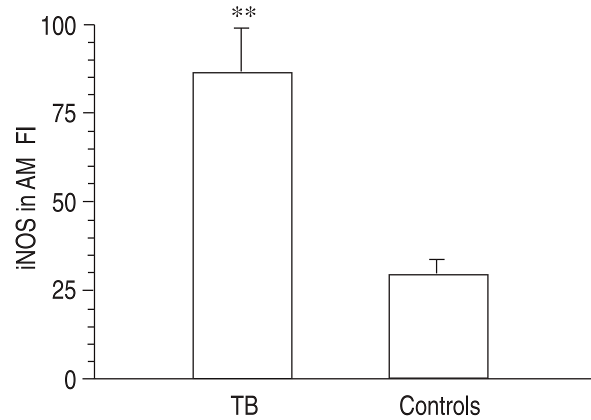

Fig. 2. – The fluorescence intensity (FI) of inducible nitric oxide synthase (iNOS) in alveolar macrophages (AM) from control subjects (n=14) and patients with active pulmonary tuberculosis (TB) (n=19). Values are presented as mean±SEM. **: $p<0.001$ *versus* control subjects.

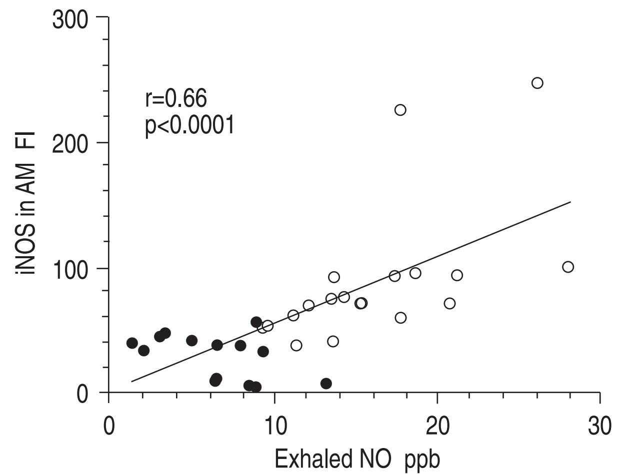

Fig. 3. – The correlation between the magnitude of inducible nitric oxide synthase (iNOS) expression on alveolar macrophages (AM) and the concentration of exhaled nitric oxide (NO). ○ : patients with active pulmonary tuberculosis (TB) (n=19); ●: control subjects (n=14). ppb: parts per billion.

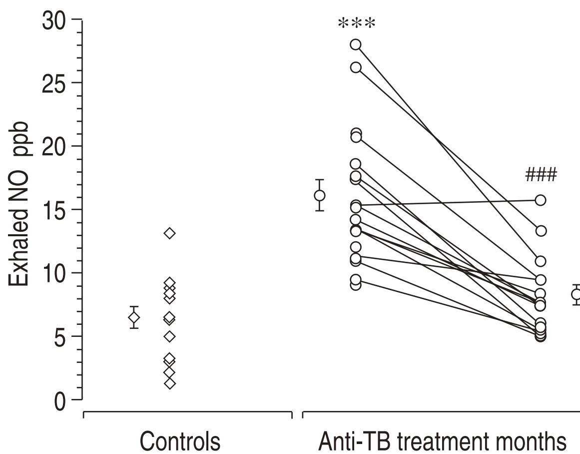

Fig. 1. – The level of exhaled nitric oxide (NO) in control subjects (◇) (n=14) and patients with active pulmonary tuberculosis (TB) (○) prior to (n=19) and after (n=15) receiving anti-TB treatment for 3 months. Values are presented as mean±SEM. ***: $p<0.0001$ *versus* controls. ###: $p<0.0001$ *versus* TB patients prior to treatment. ppb: parts per billion.

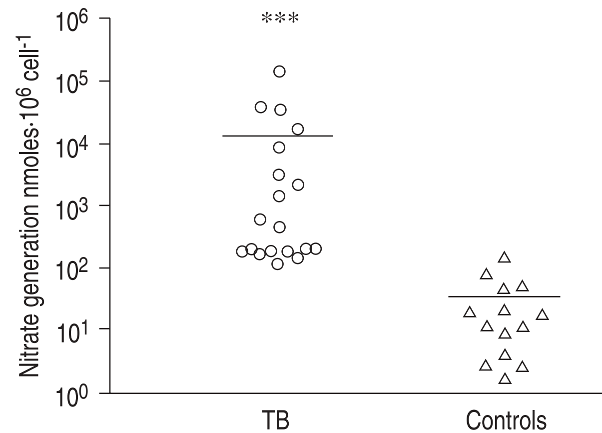

Fig. 4. – The generation of nitrite produced by alveolar macrophages (AM) in control subjects (n=14) and patients with active pulmonary tuberculosis (TB) (n=19) after 24 h culture. Values are presented as mean±SEM. The horizontal bars represent mean values for each group. ***: $p<0.0001$ *versus* control subjects.

very low both in patients with TB (4.7±0.5 and 11.4±1.1 FI, respectively, n=19) and in controls (3.4±0.3 and 10.3±1.7 FI, respectively, n=14). There was no significant difference between these two groups.

Nitrite generation from cultured AM

There was a significantly higher level of nitrite in the supernatant of BAL fluid retrieved from patients with active TB (51.2±7.8 nmoles·mL^{-1}, n=19) compared to that retreived from control subjects (30.2±3.7 nmoles·mL^{-1}, n=14, $p<0.05$). The concentration of nitrite in unstimulated medium alone (15.5 nmoles·mL^{-1}) was substracted from AM cultures. Cultured AM from patients with active TB spontaneously released nitrite in culture medium (13,250.0±7,655.0 nmoles·10^{6} cells^{-1}) to a much greater extent than that released from control subjects (35.7±12.7 nmoles·10^{6} cells, $p<0.0001$) (fig. 4). The nitrite generation by cultured AM was significantly related to the expression of iNOS on AM (r_s=0.77, n=33, $p<0.0001$). The spontaneous generation of nitrite by cultured AM from patients with TB (2,058.6±1,792.9 nmoles·10^{6} cells^{-1}, n=9) was significantly inhibited in the presence of L-NMMA (950.7±848.8 nmoles·10^{6} cells, n=9, $p<0.01$). However, there was no significant change in the nitrite production from AM of control subjects in the presence (27.1±3.8 nmoles·10^{6} cells^{-1}) or absence (29.3±4.3 nmoles·10^{6} cells^{-1}) of L-NMMA.

Immunohistochemical localization of iNOS expression

The anti-iNOS polyclonal antibody strongly reacted with AM from patients with active pulmonary TB, while there was very faint labelling of AM from controls. The

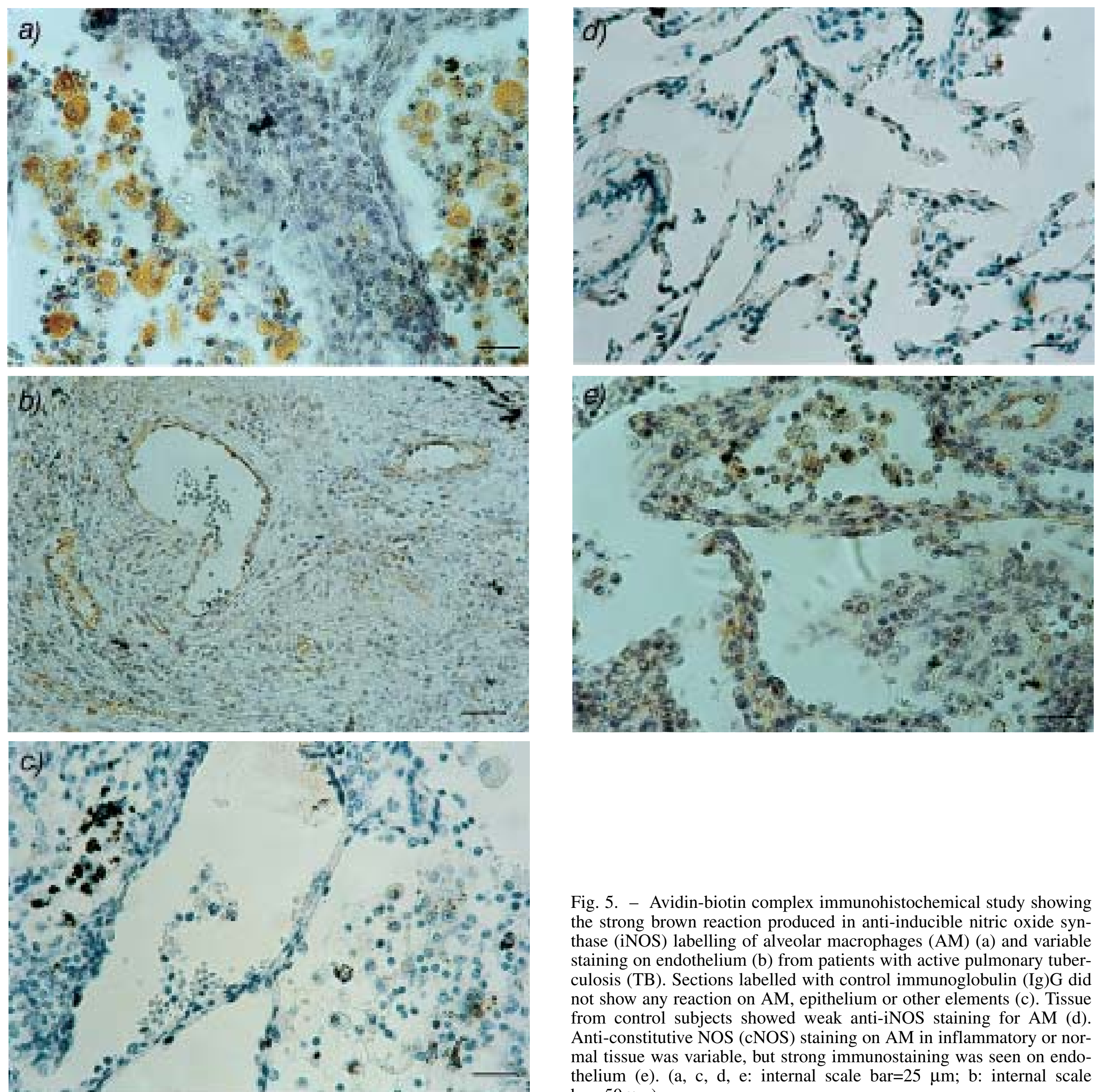

Fig. 5. – Avidin-biotin complex immunohistochemical study showing the strong brown reaction produced in anti-inducible nitric oxide synthase (iNOS) labelling of alveolar macrophages (AM) (a) and variable staining on endothelium (b) from patients with active pulmonary tuberculosis (TB). Sections labelled with control immunoglobulin (Ig)G did not show any reaction on AM, epithelium or other elements (c). Tissue from control subjects showed weak anti-iNOS staining for AM (d). Anti-constitutive NOS (cNOS) staining on AM in inflammatory or normal tissue was variable, but strong immunostaining was seen on endothelium (e). (a, c, d, e: internal scale bar=25 µm; b: internal scale bar=50 µm).

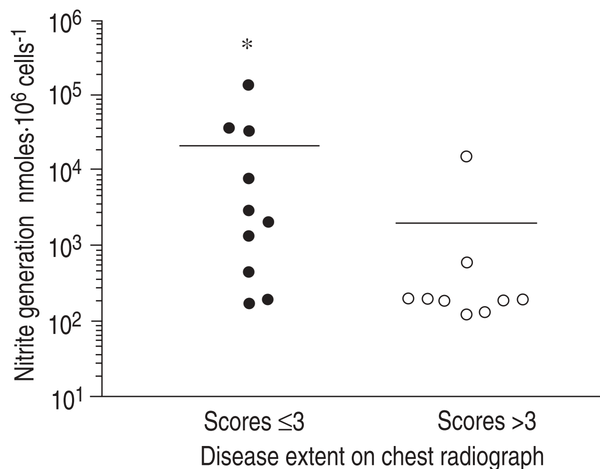

Fig. 6. – The spontaneous generation of nitrite by cultured alveolar macrophages (AM) from patients with active pulmonary tuberculosis (TB) with a chest radiographic score >3 (n=9) or ð3 (n=10). Values are presented as mean±SEM. The horizontal bars represent mean values for each group. *: $p<0.05$ *versus* TB patients with a chest radiographic score >3.

frequency of positive immunostaining on AM from TB patients was higher than that of controls (74.5±4.4%, n= 19 compared to 15.4±6.1%, n=14, $p<0.0001$). Anti-iNOS antibody strongly reacted with AM, but not with the airway epithelium or the alveolar epithelium in patients with active pulmonary TB (fig. 5a and b). Anti-cNOS antibody strongly labelled endothelial cells but weakly AM or interstitial cells (fig. 5d).

Relationship between the capacity of nitrite production by AM and disease extent

Cultured AM from TB patients with more limited disease (grading of chest radiographs ð3 (n=10)) exhibited a greater capacity for spontaneous generation of nitrite (23,340.0±14,010.0 nmoles·10^6 cells^{-1}) than those retrieved from patients with more extensive disease (grading of chest radiographs >3 (2,033.0±1795.0 nmoles·10^6 cells^{-1}, $p<0.05$)) (fig. 6).

Discussion

We have demonstrated that exhaled NO level is increased in patients with active pulmonary TB and that it returns to normal levels after anti-TB treatment. This increased level of NO in exhaled breath was also observed in the trachea and main bronchi. iNOS expression in alveolar macrophages was also found to be upregulated and there was an enhanced capacity for spontaneous generation of RNI. The magnitude of iNOS expression on AM was closely related to the level of exhaled NO and to the capacity for spontaneous production of nitrite from cultured AM. Immunohistochemistry revealed that iNOS was upregulated mainly in macrophages in lung biopies, with little or no expression in epithelial cells. cNOS expression was also weakly positive on AM in the areas of inflammation, but it was present on endothelial cells. Therefore, our study strongly indicates that the increase in exhaled NO is likely the result of enhanced iNOS expression and increased activity of lung macrophages. This study confirms a recent report showing upregulation of iNOS mRNA in AM of patients with active pulmonary TB [11].

The mechanisms responsible for the upregulation of iNOS and enhanced NO production from AM in patients with TB is not known. Although cytokines released in the immune response against *M. tuberculosis*, such as TNF-α, IL-1β and IFN-γ [21, 22], have been demonstrated to upregulate iNOS expression and enhance NO production in murine macrophages [8, 23], this has not been found in human monocytes/macrophages [24–26]. However, NOS has been shown to be induced in human monocytes through an IgE-dependent mechanism or by the sequential treatment with IL-4 and IFN-γ [27, 28]. Human monocyte-derived macrophages were also demonstrated to generate NO after exposure to selected avirulent strains of *Mycobacterium avium complex* in combination with TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulation [29], though treatment with the cyto-kines alone failed to elicit significant NO synthesis. MARTIN and EDWARDS [30] reported that *in vitro* matured human macrophages released substantial amounts of nitrite, appar-ently without stimulation. In addition, the product of myco-bacterial lipoarabinomannan (LAM) can also induce NO production from murine macrophages [31, 32]. Thus in active pulmonary TB, human AM might be activated to upregulate their NO synthetic capacity by interaction with cytokines and with mycobacterial components.

The threefold increase in the magnitude of iNOS activity in AM and a threefold increase in AM numbers contributed to a mere 2.5 fold increase in exhaled NO levels in patients with pulmonary TB compared to that of control subjects suggesting NO produced from AM is not the main cellular source of the basal level of NO in TB patients and control subjects. Enhanced production of NO from AM in TB patients may amount to only 1.5 fold of the basal production from other cellular sources, such as airway epithelial cells, vascular endothelial cells, fibroblasts, chondrocytes, smooth muscle cells and nerve endings [4, 15].

The finding that patients with more extensive disease had a lesser capacity to generate NO from AM might suggest that in humans too, the generation of RNI may limit mycobacterial growth.

Our results show that the increase in exhaled NO in patients with active pulmonary TB disappears following anti-TB treatment and the resolution of radiographical changes. Thus, the measurement of exhaled NO in patients with pulmonary TB may monitor the disease activity in a noninvasive fashion. It is unclear, however, whether this fall in exhaled NO is representative of the resolution of the inflammation or of the bactericidal effect of anti-TB treatment. Thus, whether it may be practically useful in assessing response to anti-TB therapy.

In summary, our results demonstrate that inducible nitric oxide synthase of human alveolar macrophages from active pulmonary tuberculosis is upregulated, likely leading to a higher output of nitric oxide, which can be detected in the exhaled air. Our *in vivo* results were consistent with the role of alveolar macrophages in the cellular immunity against the mycobacterial infections and suggest a biological role of nitric oxide in human diseases.

As nitric oxide may not only destroy pathogens but also damage the host cells and tissue, the possible implication of reactive nitrogen intermediates and its derivatives such as peroxynitrite, hydroxyl radicals in tissue damage in active pulmonary tuberculosis needs further investigation.

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