

Hydrogen peroxide producing potential of alveolar macrophages & blood monocytes in pulmonary tuberculosis

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Alveolar macrophages from patients with active pulmonary tuberculosis, inactive tuberculosis (treated patients), non-tuberculous lung disease and from normal healthy individuals were tested for their ability to produce hydrogen peroxide (H_2O_2) upon stimulation with phorbol myristate acetate (PMA) *in vitro*. PMA induced H_2O_2 production was significantly higher in all the groups of patients studied when compared to the normal subjects. Among the four groups studied, the spontaneous release of H_2O_2 was increased in the alveolar macrophages of smokers than non-smokers. There was no difference in the spontaneous and PMA induced H_2O_2 production between the non-smoker group of the active tuberculosis patients and the normal non-smoker group. Further, the blood monocytes of the active pulmonary tuberculosis patients and those of normal controls were equally competent in producing H_2O_2 , *in vitro*, upon stimulation with PMA. The study suggests that the increased production of hydrogen peroxide by alveolar macrophages is not specific for tuberculosis.

Alveolar macrophages are the major mononuclear phagocytes of the lung. These cells have been shown to play a significant role in clearance of smoke and other environmental dust from the lung and in the defence against pathogens. Macrophages appear to be important for bacterial phagocytosis and killing. Multiplication of *Mycobacterium tuberculosis* within the macrophages is the hall mark of the pathogenesis of tuberculosis. Studies indicate that macrophages are also involved in host resistance to *M. tuberculosis*^{2,3}. There are several reports on hydrogen peroxide (H_2O_2) mediated killing

of intracellular pathogens within the macrophages^{4,7}.

The present study was carried out to find out the H_2O_2 producing potential of alveolar macrophages and peripheral blood monocytes of pulmonary tuberculosis patients and normal individuals.

Material & Methods

Fibreoptic bronchoscopy was performed on 21 patients and 4 normal subjects. The patients were suspected cases of pulmonary

tuberculosis, attending Tuberculosis Research Centre with respiratory symptoms and radiographic evidence of lung diseases of varying duration. All the patients had at least six sputa smears negative for *M. tuberculosis* by microscopy. All patients' sputa were cultured for *M. tuberculosis* and depending upon the culture results, these patients were classified into the following groups :

- (i) *Active pulmonary tuberculosis* : These patients (9) had not received any previous anti-tuberculosis chemotherapy and had *M. tuberculosis* growth in one or more sputum specimens in culture.
- (ii) *Inactive pulmonary tuberculosis*: These patients (8) had received previous anti-tuberculosis chemotherapy and presently their sputa showed culture negativity for *M. tuberculosis*.
- (iii) *Non-tuberculous lung disease* : This group involved 4 patients with lung abscesses, bacterial pneumonia and pulmonary manifestations of *polycythemia vera*.
- (iv) *Normal subjects* : (4) who had normal chest X-ray and pulmonary functions and all of whom were non-smokers.

Fibreoptic bronchoscopy and alveolar lavage: The procedure of the fibreoptic bronchoscopy has been explained in detail⁸, and alveolar lavage has been described elsewhere⁹. In brief, the upper airways and trachea were anaesthetized with 4 per cent xylocaine spray. Fibreoptic bronchoscope was inserted either through

the mouth or nose. The tip of the bronchoscope was wedged into the third or fourth order bronchus in the affected lobe, middle lobe and lingula. The lobes were lavaged with sterile isotonic saline using a suction pressure of 50-100 mm of water. The entire bronchoscopy procedure was done under continuous cardiac and pulse-oximeter monitoring, and oxygen was supplemented during and after the procedure.

Blood samples of 13 active pulmonary tuberculosis patients and 8 healthy volunteers were also investigated for H₂O₂ production by the peripheral blood monocytes; 15 to 20 ml of blood was collected in a heparinized container from each subject.

Alveolar macrophages : Alveolar macrophages of the lavage fluids were separated from the mucus using a sieve mesh and the cells were used for hydrogen peroxide release assay. The dead cells ranged from 10 to 20 per cent, as judged by the trypan blue dye exclusion method. The percentages of neutrophils and eosinophils were 2-5 and <2 respectively.

Peripheral blood mononuclear cell separation: Mononuclear cells were separated using Ficoll-Hypaque¹⁰; the cells were washed thrice in RPMI medium and adjusted 'to 5 x 10⁶ cells/ml medium.

Non-specific esterase staining : Smears of Ficoll-Hypaque separated peripheral blood mononuclear cells were stained for non-specific esterase for determining the percentage of monocyte population according to the method of Horwitz¹¹. A total number of 300 cells were counted and the per cent monocytes (esterase positive cells) among them were determined.

H₂O₂ assay : Total alveolar cells were taken for the assay. Hundred microliter containing 0.1×10^6 alveolar cells, was plated in 96 well flat bottom plate. For peripheral blood, 0.5×10^6 blood mononuclear cells were seeded in 96-well flat bottom plates (Falcon) and non-adherent cells were removed by aspiration after one hour at 37°C. The adherent mononuclear cells (non-specific esterase positive cells) were used for H₂O₂ assay. The H₂O₂ producing potential of these cells were studied.

The H₂O₂ assay method described by Pick and Mizel¹² was followed. Briefly, the plates with the cells were washed with phenol red free Hank's balanced salt solution (HBSS). 100 µl of HBSS containing 19 units/ml horse radish peroxidase (Type-III, Sigma), 1 µg/ml phorbol myristate acetate (PMA) (Sigma) and 0.02 per cent phenol red (Sigma) were added to each well. After incubating the plate at 37°C for 1 h the enzyme reaction was arrested with 10 µl of 1M NaOH. Absorbance at 605 nm was determined spectrophotometrically for the assay mixture pooled from five wells. For each equipment, a standard was set up in the range of 0-10 nmol/ml of H₂O₂.

Statistical methods : The results are expressed as mean \pm SE and statistical analysis was done using the Student's t-test.

Results

Among the three groups of patients and normal individuals studied, the alveolar macrophages of the three patient groups produced more H₂O₂ than the normal individuals ($P < 0.05$). On the other hand, there was no significant difference in H₂O₂ production by alveolar macrophages among active TB, inactive TB and non-TB patients (Fig. 1).

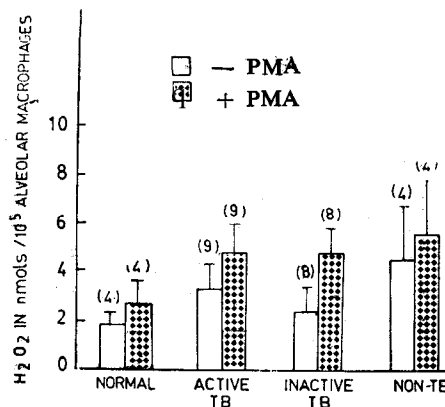


Fig. 1. Hydrogen peroxide production by the alveolar macrophages. Figures in parentheses indicate the actual number of cases studied and the results are expressed as Mean \pm SE.

The 3 groups of patients and the normal individuals were divided into smokers and non-smokers, and the H₂O₂ producing capacity of these alveolar macrophages were analyzed (Fig. 2A). The spontaneous and the PMA induced H₂O₂ production in smokers were significantly higher than the alveolar macrophages of non-smokers (spontaneous H₂O₂ $P < 0.01$; PMA H₂O₂ $P < 0.05$). However, the ability of the alveolar macrophages to PMA stimulation was higher in the non-smoker group (2.5 folds) than the smoker group (1.3 fold). This analysis was based on the following formula :

$$\frac{\text{PMA stimulated H}_2\text{O}_2 \text{ release}}{\text{Spontaneous release of H}_2\text{O}_2}$$

Alveolar macrophages of normal individuals (non-smokers) and those of the non-smokers among active tuberculosis patients were equally competent in their H₂O₂ release. Further, the active tuberculosis smoker group produced an increased H₂O₂ than the active tuberculosis non-smoker group (Fig. 2B).

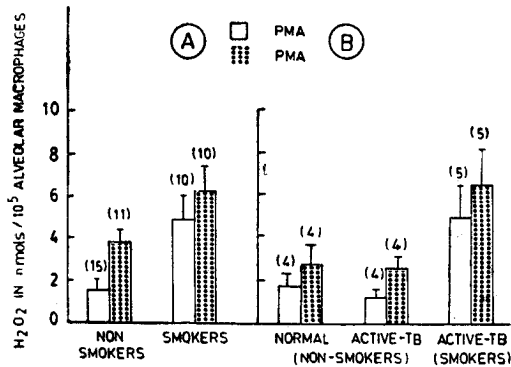


Fig. 2A. Spontaneous and PMA stimulated hydrogen peroxide production by the alveolar macrophages of non-smokers and smokers. Actual number of cases studied are presented in the parentheses. Mean \pm SE. B. Hydrogen peroxide release by the alveolar macrophages of normal individuals, active TB non-smoker group and active-TB smoker group.

The H₂O₂ producing potential of the blood monocytes of active pulmonary tuberculosis patients and the normal healthy subjects were compared. The blood monocytes of normal subjects and tuberculosis patients were equally competent in their H₂O₂ producing capacity ($P > 0.1$; Fig. 3).

Discussion

The present study was carried out to understand the hydrogen peroxide producing potential of the alveolar macrophages and the blood monocytes of active pulmonary tuberculosis patients. The study revealed that the alveolar macrophages of active pulmonary tuberculosis, inactive tuberculosis (treated patients) and non-tuberculous lung disease patients produced an increased H₂O₂ than the normals (Fig. 1). It is possible that this increase in H₂O₂ production may be due to an activated state of the alveolar macrophages *in vivo*. Enhanced production

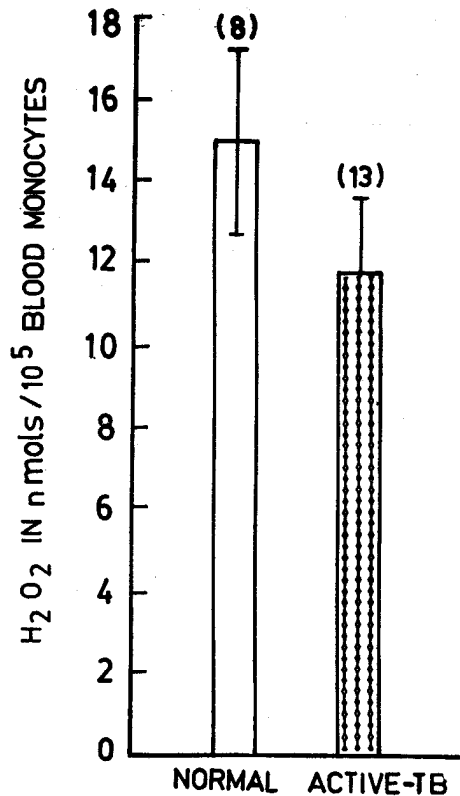


Fig. 3. PMA induced hydrogen peroxide production by the peripheral blood monocytes of active pulmonary tuberculosis patients and the normal healthy volunteers. Numbers in the parentheses represent the actual number of individuals studied. Mean \pm SE.

of H₂O₂ is known to be one of the characteristics of macrophage activation^{5,13,14}. It has been proposed that immunologically activated alveolar macrophages may play a major role in tubercular resistance³.

Irrespective of the various groups of patients included in this study, the spontaneous and PMA induced H₂O₂ were high in all of them suggesting that, in addition to antigens of tubercle bacilli, infection due to other pathogens and other factors such as

smoking etc. could have led to an increase in the production of H₂O₂ by alveolar macrophages. Fig. 2A shows that non-smokers produce less spontaneous and PMA stimulated H₂O₂ than the smokers. There was no difference in the spontaneous and PMA induced H₂O₂ release between the active tuberculosis non-smoker group and the normal non-smoker group. Since the patients included in the study were all smear negative subjects harbouring less number of bacilli (paucibacillary state), it is likely that adequate stimuli would not be available for the activation of the macrophages in the affected lobe of the non-smoker group of the active tuberculosis patients (Fig. 2B). This correlates well with the recent study carried out in murine model. It has been shown that low level of either heat killed or live *M. lepraemurium* stimulated a very low level of H₂O₂ in the resident peritoneal macrophages. This study suggests that low level of bacteria may not trigger a respiratory burst and this may allow the infection to become established¹⁵.

The ability of the alveolar macrophages of the non-smokers to release H₂O₂ on PMA stimulation was 2.5 fold increased, whereas in smokers, the increase was 1.3 fold suggesting that the alveolar macrophages of the smokers were in a stimulated/activated condition. Hence, *in vitro* stimulation with PMA showed less release of H₂O₂. It has been suggested that smokers' alveolar macrophages are in an activated condition *in vivo*^{16,17}, which was further substantiated by Baughman *et al*¹⁸, who showed an increased spontaneous production of H₂O₂ in smokers. Also, human alveolar macrophages from smokers release more H₂O₂ on phagocytic stimulation than those from non-smokers and have greater anti-bacterial activity^{19,20}.

The present study indicates that the blood monocytes and alveolar cells of active pulmonary tuberculosis patients and normals were equally competent in producing H₂O₂ to PMA stimulation. However, in this study, the alveolar cells were obtained from the affected lobe as well as middle lobe and lingula, thus causing a dilution of the cells recovered from the affected lobe. Hence, it may be advisable to look for the metabolic and microbicidal properties of macrophages exclusively of the affected lobe of the lung for the exact role of macrophages in the pulmonary tuberculosis.

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