

Procoagulant activity of bronchoalveolar lavage fluids taken from the site of tuberculous lesions

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ABSTRACT: We wanted to determine the procoagulant activity (PCA) of bronchoalveolar lavage fluids, in order to understand the macrophage-mediated lung injury at the site of tuberculous lesion.

Alveolar lavage fluids taken from the site of a lesion (radiologically abnormal site (RAS)) and an unaffected site (radiologically normal site (RNS)) of active pulmonary tuberculosis (TB) patients (n=7) and inactive (cured) patients (n=9) were studied for their PCA producing potential. The observed results were not significant using Mann-Whitney test, and thus all increases/decreases reported below are trends/tendancies only.

An increased PCA was seen in 4 out of 7 cell-free lavage supernatants of active-TB taken from the site of lesion (RAS), compared to only 1 out of 9 in inactive-TB. The PCA producing potential of the alveolar macrophages of RAS and RNS of active-TB patients was enhanced when the alveolar macrophages were co-cultured with autologous peripheral blood lymphocytes under *in vitro* condition. Stimulation with purified protein derivative (PPD) of *M. tuberculosis* showed a variable (increased or decreased) PCA production. Peripheral blood monocytes and total mononuclear cells (monocytes + lymphocytes) of active-TB patients stimulated with or without PPD showed increased PCA production, compared with normal individuals and inactive-TB patients.

The present study suggests that increased production of PCA by the alveolar macrophages, in collaboration with lymphocytes and other cells at the site of tuberculous lesions will result in fibrin formation. The deposition of fibrin in the alveoli may lead to further lung injury.

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Lung parenchymal injury is one of the basic mechanisms involved in the pathogenesis of granulomatous lung diseases. The mechanism of macrophage-mediated tissue injury in the pathogenesis of the granulomatous lung diseases has been extensively reviewed [1]. A complex relationship has been observed between the macrophage and the coagulation-fibrinolytic pathways. Activation of the coagulation system and fibrin deposition has been associated with delayed type hypersensitivity and granulomatous inflammatory reactions. [1, 2]. Fibrin deposition in the alveolar space and in the interstitial tissue is a conspicuous feature of many types of inflammatory reactions [3-5]. Pathways of inflammation and coagulation are closely interrelated at the humoral and cellular levels [6]. Mononuclear phagocytes of various types have been found to display procoagulant activities (PCA), of which thromboplastin (tissue factor) has been shown to be one of the factors which is involved in the formation of fibrin [7]. Other inflammatory cells, such as neutrophils and lymphocytes, do not express PCA [8-11]. However, tissue factor has also been detected in

human fibroblasts [12], smooth muscle cells [13], and endothelial cells [14] under *in vitro* conditions. It has been shown that a variety of stimuli induce the synthesis of PCA in monocytes and macrophages [8, 10, 11, 15].

Pulmonary tuberculosis is a granulomatous disease of the lung caused by *M. tuberculosis*. Altered levels of alveolar macrophages and other inflammatory cells, such as lymphocytes, have been found in the bronchoalveolar lavage (BAL) fluids of tuberculosis patients [16-18].

The present study was carried out to understand the immunopathological changes occurring at the site of tuberculous lesions. PCA production by the activated macrophage has been recognized to be an *in vitro* measure of cell-mediated immunity. The production of PCA as a tissue factor by monocytes and macrophages, and the release of this factor as a consequence of macrophage and lymphocyte interactions that are involved in the pathogenesis of pulmonary tuberculosis has been studied.

Materials and methods

Subjects

Fibreoptic bronchoscopy and BAL were performed on 7 active pulmonary tuberculosis patients (6 nonsmokers and 1 smoker), and 9 inactive pulmonary tuberculosis patients (6 nonsmokers and 3 smokers).

Active tuberculosis patients (ATB). Patients attending the Tuberculosis Research Centre, Madras, with respiratory symptoms and radiographic abnormalities suggestive of pulmonary tuberculosis were evaluated for the study. At the time of initial assessment, all patients had six sputum smears negative for acid-fast bacilli (AFB). These patients had respiratory symptoms of less than 6 months duration and all had minimal radiographic abnormalities in one of the upper zones. None had received any antituberculosis treatment in the past. Among these patients, there were 7 patients whose sputa or lavage fluids had shown growth of *Mycobacterium tuberculosis* and they were classified as active pulmonary tuberculosis patients.

Inactive tuberculosis (cured) patients (ITB). Patients classified as suffering from active pulmonary tuberculosis (by BAL technique) were given antituberculosis treatment at our centre. All these patients ($n=9$) had received a short course chemotherapy of 6–8 months duration. BAL was repeated in these patients after chemotherapy. These patients had sputum smears negative for AFB at the time of evaluation. Sputa and lavage cultures were also negative for *M. tuberculosis*.

Fibreoptic bronchoscopy and bronchoalveolar lavage

All subjects in the present study were subjected to fibreoptic bronchoscopy and BAL. The procedure for fibreoptic bronchoscopy and BAL has been described previously [19, 20], and was performed at the site of lesion of the affected lobe (radiologically abnormal site (RAS)) and at an unaffected site (radiologically normal site (RNS)) from the unaffected lobe. The alveolar lavage fluids of the active and inactive pulmonary tuberculosis (cured) patients were centrifuged at $300\times g$ for 10 min. The cell-free supernatants and the alveolar cells were used separately for PCA assay.

Peripheral blood mononuclear cells

Peripheral blood samples drawn from the active and inactive pulmonary tuberculosis patients who underwent BAL and from normal laboratory volunteers were used. Mononuclear cells were separated using Ficoll-Hypaque, as described by BOYUM [21]. The cells were washed three times in RPMI-1640 tissue culture medium, with 2 mM L-glutamine (Flow Laboratories, Irvine,

Scotland, UK) containing 10% foetal bovine serum (FBS) (Intergen Purchase, NY, USA) and gentamicin $50\text{ }\mu\text{g}\cdot\text{ml}^{-1}$ (M.A. Bioproducts, Walkersville, Maryland, USA) and fungizone $2.5\text{ }\mu\text{g}\cdot\text{ml}^{-1}$ (Squibb, Princefon, NJ, USA). The cells were adjusted to $5\times 10^6\text{ cells}\cdot\text{ml}^{-1}$ culture medium.

Differential count

Smears of alveolar cells from the patients were stained with Diff-Quick (Halerco, Gibbstown, NJ, USA). A total of 300 cells was counted for each smear [17].

Nonspecific esterase staining

Smears of peripheral blood mononuclear cells were stained for nonspecific esterase for determination of the percentage of monocytes, according to the method of HORWITZ *et al.* [22]. A total of 300 cells was counted, and the percentage monocytes (esterase positive cells) among them was determined.

Design of the experiment

Five hundred microlitres of $1\times 10^6\text{ cells}\cdot\text{ml}^{-1}$ concentration of alveolar macrophages, or $5\times 10^6\text{ cells}\cdot\text{ml}^{-1}$ blood mononuclear cells were seeded in 24-well plates (Costar, Cambridge, MA, USA). The nonadherent cell population, mainly lymphocytes, was removed from the wells seeded with blood mononuclear cells by aspiration after one hour incubation at 37°C . Alveolar macrophages were used as such. The adherent mononuclear cells (monocytes - nonspecific esterase positive cells) and the alveolar macrophages were cultured for 72 h at 37°C , either with or without purified protein derivative (PPD) of *M. tuberculosis* (Central Veterinary Laboratory, Weybridge, Surrey, UK). In the second set of experiments $1\times 10^6\text{ cells}\cdot\text{ml}^{-1}$ alveolar macrophages were co-cultured with $4\times 10^6\text{ cells}\cdot\text{ml}^{-1}$ autologous lymphocytes (1:4 ratio) [23]. In the third set of experiments, $1\times 10^6\text{ cells}\cdot\text{ml}^{-1}$ alveolar macrophages and $4\times 10^6\text{ cells}\cdot\text{ml}^{-1}$ autologous lymphocytes were co-cultured with $20\text{ }\mu\text{g}\cdot\text{ml}^{-1}$ of PPD. In a similar way, $5\times 10^6\text{ cells}\cdot\text{ml}^{-1}$ peripheral blood mononuclear cells were cultured with or without PPD ($20\text{ }\mu\text{g}\cdot\text{ml}^{-1}$) for 72 h. The culture supernatants of all the cultures, were taken after 72 h of incubation at 37°C and centrifuged at $400\times g$ for 10 min, and the cell-free supernatants were assayed for procoagulant activity.

Assay for procoagulant activity (PCA)

The procoagulant activity of the cell-free supernatants of the lavage fluids and the spontaneous and PPD-stimulated culture supernatants of alveolar macrophages, monocytes, alveolar macrophages co-cultured with autologous lymphocytes, and peripheral blood mononuclear

cells were measured. The PCA of the supernatants were assessed using a standard one-stage clotting assay [24, 25]. Briefly, 100 μ l of the supernatants were prewarmed at 37°C for 3 min, together with 100 μ l of 0.025 M calcium chloride, in 76 \times 9 mm glass tubes. Citrated normal human plasma, 100 μ l, prewarmed to 37°C, was then added and the clotting time determined by the manual tilt method. All supernatants were assayed in duplicate. A value for the PCA was derived by comparison of the clotting times with the coagulant activity of standard rabbit thromboplastin (Sigma Chemical Co., St. Louis, MO, USA). One hundred microlitres of standard was assigned a value of 1,000 milliunits (mU) of PCA (10,000 mU·ml⁻¹). The activity of the standard was also assayed in duplicates and found to be linear on a log/log plot of clotting time against units of PCA, over the range 0.1–1,000 mU. The PCA of the supernatants were expressed as mU·ml⁻¹ for cell-free supernatants of the BAL fluids. For *in vitro* culture supernatants of alveolar macrophages, monocytes and mononuclear cells the PCA is expressed as mU·10⁻⁵ cells.

Statistical methods

The results are expressed as arithmetic mean \pm standard error (SEM). Statistical analysis was performed using Mann-Whitney U-test (nonparametric test).

Results

All increases/decreases reported below were found to be not significant using the Mann-Whitney test, and thus indicate trends or tendencies only. The percentage fluid recovery, the total and differential cell count analysis of the alveolar lavage fluids taken from the site of the pulmonary lesion (RAS) and an unaffected site (RNS) of active and inactive (cured) pulmonary tuberculosis patients revealed no difference at the fluid recovery and the cellular level (table 1). Five of the 7 active, and 5 of the 9 inactive TB patients had no lymphocytic alveolitis (<20% lymphocytes).

The cell-free supernatants of the active TB BAL fluids taken from the site of lesion (RAS) showed an increase in the procoagulant activity (mean \pm SEM 130.2 \pm 52.3 mU) when compared to the lavage fluids taken from RAS of inactive-TB patients (24.8 \pm 19.7 mU).

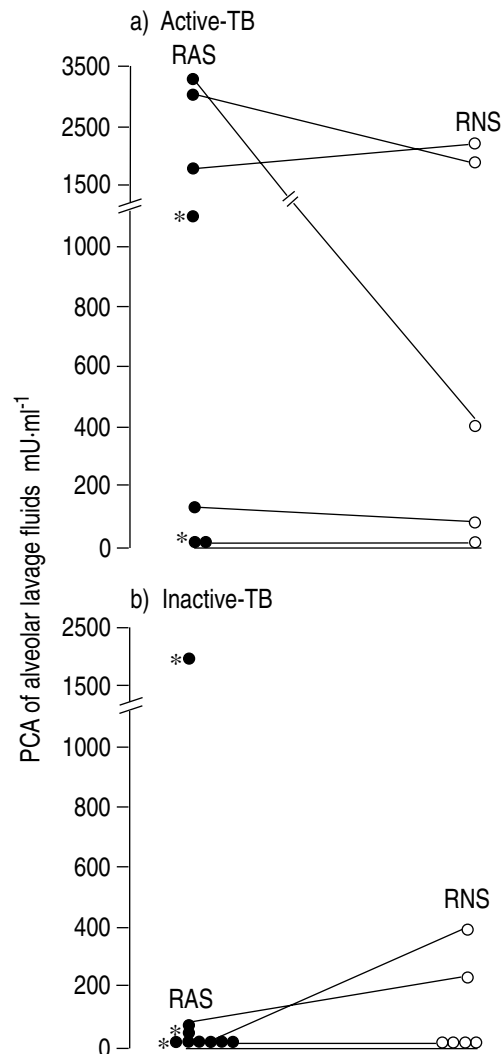


Fig. 1. — Procoagulant activity (PCA) of cell-free supernatants of bronchoalveolar lavage fluids taken from the site of lesion (radiologically abnormal site (RAS)) and an unaffected site other than the infected lobe (radiologically normal site (RNS)) in a) patients with active and b) inactive pulmonary tuberculosis, respectively. *: only one sample (RAS) available; corresponding (RNS) sample not determined. ●: RAS; ○: RNS.

Fig. 1 shows that 3/7 of the active TB group had PCA values <200 mU·ml⁻¹, similar to the inactive TB group. No difference was observed in the PCA level between RAS (130.2 \pm 52.3 mU) and RNS (86.1 \pm 44.1 mU) of active and inactive tuberculosis patients (fig. 1).

Table 1. — Percentage fluid recovery, total and differential cell counts of alveolar lavage fluids taken from the site of tuberculous pulmonary lesion and an unaffected site

Group	Site of lesion (RAS)						Unaffected site (RNS)					
	% Fluid recovery	Total+ cells	Differential counts				% Fluid recovery	Total+ cells	Differential counts			
			Mac	Lymph	Neut	% Eos			Mac	Lymph	Neut	% Eos
Active-TB*	64 \pm 8	22 \pm 3	77 \pm 3	17 \pm 3	4 \pm 1	2 \pm 1	69 \pm 6	15 \pm 4	72 \pm 3	20 \pm 2	5 \pm 1	2 \pm 1
Inactive-TB†	61 \pm 4	21 \pm 5	83 \pm 3	12 \pm 3	3 \pm 1	2 \pm 1	64 \pm 7	19 \pm 5	74 \pm 4	19 \pm 4	5 \pm 1	1 \pm 0

+: 10⁶ cells·dl⁻¹; *: RAS n=7 patients, and RNS n=5 patients; †: RAS n=9 patients, and RNS n=6 patients. Mac: macrophages; Lymph: lymphocytes; Neut: neutrophils; Eos: eosinophils.

Table 2. – Effect of purified protein derivative (PPD) of *M. tuberculosis* on the PCA producing potential of alveolar macrophages and peripheral blood adherent (monocytes) and nonadherent (lymphocytes) mononuclear cells

Subjects	Alveolar macrophages PCA in mU*				Peripheral blood PCA in mU*†		
	Site of lesion (RAS)		Unaffected site (RNS)		Adherent mononuclear cells	Adherent mononuclear cells + PPD	Non-adherent mononuclear cells
	AM	AM + PPD	AM	AM + PPD			
Active-TB	15.8±6.3 (n=5)	32.1±22.6 (n=5)	21.8±20.8 (n=5)	32.7±31.8 (n=5)	15.9±12.9 (n=6)	12.1±9.2 (n=6)	8.2±3.8 (n=6)
Inactive-TB	8.9±7.6 (n=6)	14.6±10.7 (n=6)	13.5±8.0 (n=6)	22.5±14.8 (n=6)	0.8±0.4 (n=8)	2.6±1.1 (n=8)	0.9±0.4 (n=8)
Normals	ND	ND	ND	ND	0.22±0.13 (n=7)	0.4±0.3 (n=7)	ND

Mean±SEM. Numbers in parentheses represent the actual number of subjects studied. ND: not determined; AM: alveolar macrophages; PCA: procoagulant activity. *: PCA in mU per 10^5 alveolar macrophages (AM) or peripheral blood adherent (monocytes) cells; †: PCA in mU per 5×10^5 peripheral blood nonadherent (lymphocytes) cells. More than 95% of the nonadherent cell population were lymphocytes.

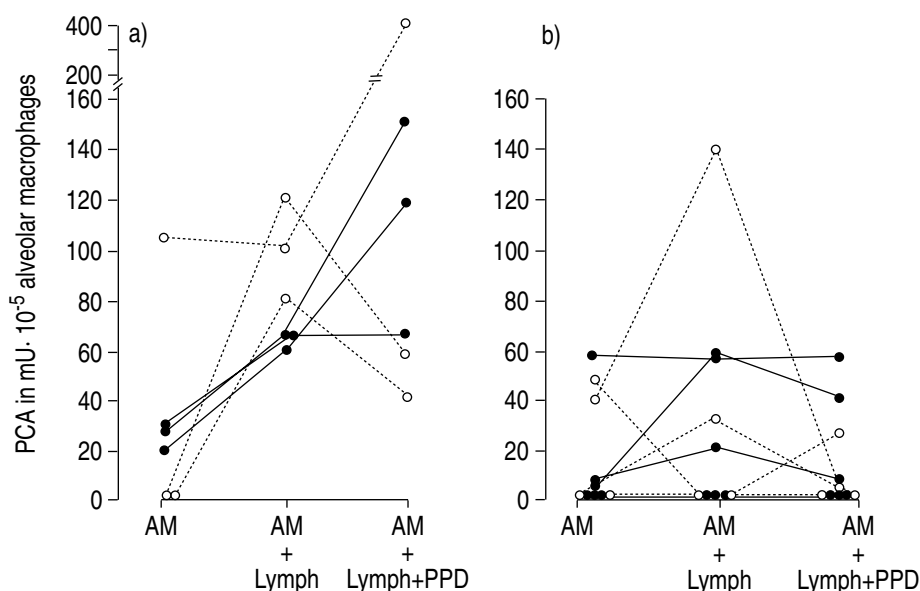


Fig. 2. – Effect of autologous lymphocytes and purified protein derivative (PPD) ($20 \mu\text{g}\cdot\text{ml}^{-1}$) on the PCA producing potential of alveolar macrophages (AM). The PCA producing potential of alveolar macrophages taken from the site of lesion (RAS) and unaffected site (RNS) of: a) 3 active; and b) 6 inactive (cured) pulmonary tuberculosis patients were studied independently and compared. For abbreviations see legend to figure 1. ●—●: RAS; ○---○: RNS.

The procoagulant activity releasing potential of the alveolar macrophages harvested from patients with active or inactive tuberculosis was similar. Furthermore, stimulation of alveolar macrophages with PPD showed a moderate increase in the PCA of alveolar macrophages taken from RAS and RNS of active-TB and inactive-TB patients (table 2).

When the alveolar cells from patients with active tuberculosis were co-cultured with autologous lymphocytes (1:4 ratio) an increase in the PCA was evident, compared to the PCA released by the alveolar macrophages alone (fig. 2). An increased PCA response was seen with the macrophages of RNS when compared to RAS recruited macrophages of active-TB patients

(RAS 63.7 ± 1.9 ; RNS 100 ± 11.5). However, alveolar macrophages of RAS and RNS of active-TB patients co-cultured with autologous lymphocytes and PPD showed a variable (enhanced or suppressed) PCA release, compared to non-PPD stimulated cells (fig. 2a). In inactive-TB patients, cultures of alveolar macrophages with autologous lymphocytes, with or without stimulation with PPD showed variable responses (fig. 2b).

The PCA of the peripheral blood monocytes of active-TB or inactive-TB patients was comparable to that of the alveolar macrophages of either active or inactive stage of tuberculosis (figs. 2 and 3).

The spontaneous and PPD-induced procoagulant activity of the peripheral blood adherent mononuclear cells

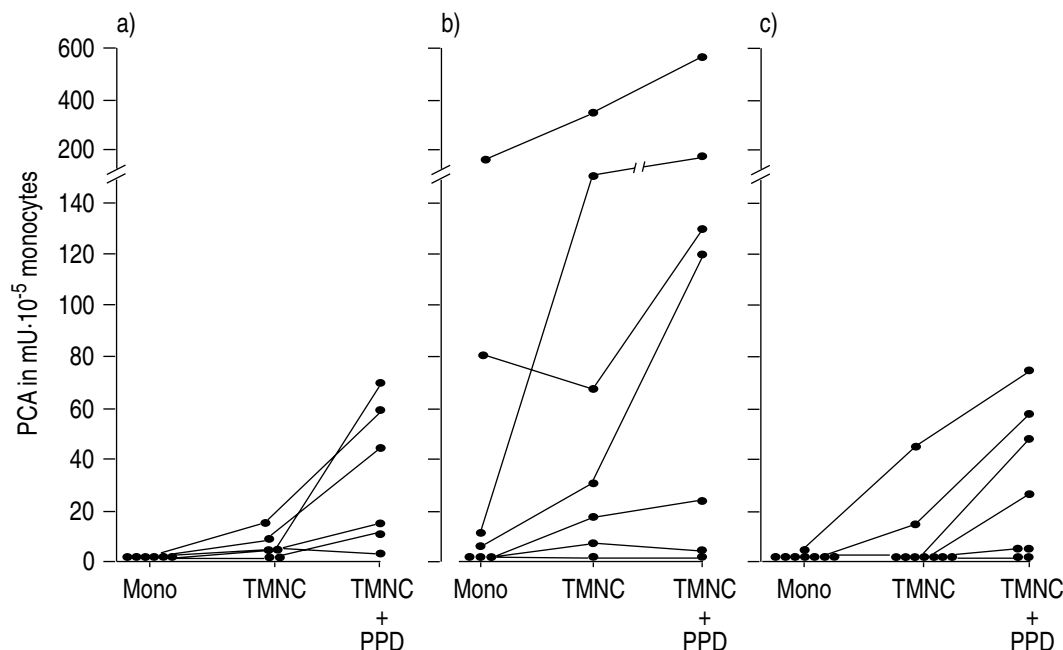


Fig. 3. — The spontaneous and PPD (20 $\mu\text{g}\cdot\text{ml}^{-1}$) induced procoagulant activity of peripheral blood monocytes (Mono) and total mononuclear cells (monocytes and lymphocytes) (TMNC) of: a) normal; b) active; and c) inactive (cured) pulmonary tuberculosis patients. PPD: purified protein derivative; PCA: procoagulant activity.

was higher in cells derived from active-TB patients than from the inactive-TB patients and normal individuals (table 2). When the total mononuclear cells of active-TB patients were stimulated with PPD, 5/7 patients showed an increase in their PCA production. This appears similar to the responses in inactive-TB and normals (fig. 3).

Discussion

Bronchoalveolar lavage fluids and lung alveolar macrophages from humans have been shown to contain procoagulant activities [25–29]. Furthermore, it has been shown that the PCA of the lavage fluids are membrane vesicles consisting exclusively of alveolar macrophage membrane material [25, 29, 30]. In the present study, an increased procoagulant activity was seen in the cell-free supernatants of active-TB lavage fluids taken from the site of lesion (RAS) compared to the lavage fluids from inactive-TB patients. This increase was not significant, however, and thus indicates a trend only. The same is true for the other differences observed in this study.

The increased procoagulant activity of the cell-free supernatants of the lavage fluids may be due to the PCA containing microvesicles that are released at the site of lesion (RAS) by the alveolar macrophages and other cells, such as fibroblasts [12], to promote coagulation.

Alveolar lavage fluids taken from RAS and RNS of active-TB patients showed no difference in their PCA level. This suggests that the alveolar macrophages which are residing at the site of lesion (RAS) and at the unaffected site (RNS) are equally sensitized to the antigens of *M. tuberculosis*, and capable of releasing

PCA into the microenvironment of the alveolar space. In contrast, lavage fluids taken from radiologically abnormal site of inactive-TB patients showed a low level of PCA. This low level of PCA in the BAL fluids of inactive-TB patients may be due to an unresponsive state of the alveolar macrophages, or to a low proportion of *M. tuberculosis* sensitized macrophages, at the sites of RAS and RNS.

Alveolar macrophages taken from the site of lesion (RAS) and unaffected site (RNS) of both active-TB and inactive-TB patients produced a low level of spontaneous and PPD-induced PCA *in vitro*. However, alveolar macrophages co-cultured with autologous lymphocytes (1:4 ratio) showed a variable (increased or decreased) spontaneous and PPD-induced PCA production. This reveals that the PCA-releasing potential of alveolar macrophages is influenced by lymphocytes, and that lymphocyte co-operation seems necessary for the increased PCA production. It has been shown that T-lymphocytes [23] and T-lymphocyte factors [31] are important for the increased production of PCA by the monocytes and macrophages. Further, the monocyte procoagulant-inducing factor (MPIF) has been shown to be a T-helper cell derived cytokine, that may play a collaborative role in the expression of cell-mediated immune responses [32].

The PCA releasing potential of the peripheral blood monocytes, monocytes co-cultured with lymphocytes as mononuclear cells with or without stimulation with PPD, was also comparable to that of the alveolar macrophages of the active-TB and inactive-TB patients. Peripheral blood monocytes and mononuclear cells of normal subjects produced a low level of PCA. Mononuclear blood cells with or without stimulation with PPD showed a higher PCA production in active-TB

patients than in inactive-TB patients and normal subjects. This suggests that the peripheral blood monocytes and the lymphocytes of active tuberculosis patients are sensitized to PPD antigen. The monocytes and lymphocytes of inactive-TB patients and normal subjects are in an unresponsive state or less sensitized to the stimulating antigen.

The present study suggests that the alveolar macrophages taken from the site of lesion (RAS) and the unaffected site (RNS) of the active-TB patients are equally competent in their PCA producing potential. Increased production of procoagulant activity by the alveolar macrophages of active-TB patients during inflammation may result in fibrin formation, a cell-mediated immune reaction. The fibrin deposition in the alveolar space and interstitial tissue may lead to further lung injury.

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