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Altered bactericidal activity against *Staphylococcus aureus* in tuberculous bronchoalveolar lavage fluids

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Altered bactericidal activity against Staphylococcus aureus *in tuberculous bronchoalveolar lavage fluids. P. Selvaraj, N. Venkataprasad, V.K. Vijayan, P.R. Narayanan. ©ERS Journals Ltd 1994.*

ABSTRACT: We wished to evaluate the pulmonary defence capacity against common bacterial infections in patients with pulmonary tuberculosis.

Bactericidal activity against *Staphylococcus aureus* of bronchoalveolar lavage fluids (cell-free supernatants) of patients with active (n=13) and inactive pulmonary tuberculosis (n=8), and normal individuals (n=6), were studied.

The 2 and 4 h bactericidal activities were higher than the 0 h activity in lavage fluids of healthy subjects and patients with inactive pulmonary tuberculosis. Active tuberculous lavage fluids were equally competent in their bactericidal activity against *S. aureus* at 0 and 2 h, but a reduced *S. aureus* killing was seen at 4 h of incubation. Estimation of total phospholipid levels revealed no significant difference between the various lavage fluids. This reduced killing of *S. aureus* showed a relationship with the cellular components (lymphocytes and macrophages) of active tuberculous lavage fluids. A reduced killing was associated with no lymphocytic alveolitis, and an increased killing with lymphocytic alveolitis.

This study suggests that alveolar lining material of patients with active pulmonary tuberculosis has less bactericidal activity against bacterial infections, such as *S. aureus*.

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Alveolar macrophages are involved in the defence against pathogens and elimination of inhaled dusts. These cells reside in a lipid-rich alveolar lining fluid containing pulmonary surfactant, produced by alveolar type-II epithelial cells.

Pulmonary surfactant has been known to have immunoregulatory functions [1–4]. It has been shown that alveolar lining material promotes superoxide production, chemotaxis and the tumoricidal activity of monocytes and macrophages [1–3]. Moreover, whole and purified surfactant lipids of alveolar lavage material have been shown to suppress the human lymphocyte proliferation to mitogen [4].

Bacterial infection is associated with chronic respiratory diseases [5]. Bactericidal activity has been shown in crude preparations of lung lavage material [6, 7]. Several Gram-positive bacteria are susceptible to alveolar lining material. Phospholipids, especially the lysophosphatidylcholine of the surfactant, possess this antibacterial activity [7].

To understand the pulmonary defence mechanisms against common bacterial infections, such as *S. aureus*, in patients with pulmonary tuberculosis, we examined bactericidal activity in lavage samples from patients with active and inactive pulmonary tuberculosis, and healthy subjects. We used *S. aureus* as an indicator microorganism.

Materials and methods

Study subjects

Subjects included in this study were 13 patients with active pulmonary tuberculosis (7 nonsmokers and 6 smokers), 8 patients with inactive pulmonary tuberculosis (cured) (4 nonsmokers and 4 smokers), and 6 normal subjects (all nonsmokers).

Active tuberculosis patients (ATB)

These patients were drawn from among those attending the Tuberculosis Research Centre, Madras, during 1987, with respiratory symptoms and radiographic abnormalities suggestive of pulmonary tuberculosis. At the time of initial assessment, all patients (n=41) 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 13 patients whose sputa or lavage fluids had shown growth of *Mycobacterium tuberculosis*, and they were classified as active pulmonary tuberculosis patients.

Cured (inactive) tuberculosis patients (ITB)

These patients (n=8) had received antituberculosis treatment elsewhere, at least one year prior to our evaluation. They presented at our centre with recurrence of symptoms. Most of these patients (6 of the 8) had received a full course of antituberculosis treatment, mainly of long-term (12–18 months) duration, and two had received a short course of chemotherapy that included rifampicin. All had respiratory symptoms, particularly cough or breathlessness, varying from 6 months to one year duration, and radiographic abnormalities suggestive of healed tuberculosis in upper or mid-zones. These patients had sputum smears negative for AFB at the time of evaluation. Sputa and lavage cultures were also negative for *M. tuberculosis*.

Normal subjects

These subjects (n=6) had no abnormalities in chest X-rays and pulmonary functions.

Fibreoptic bronchoscopy and bronchoalveolar lavage (BAL)

All subjects underwent fibreoptic bronchoscopy and BAL. The procedures for fibreoptic bronchoscopy and BAL have been described elsewhere [8, 9]. The BAL fluids were centrifuged at 1,500 rpm for 10 min. The cell-free supernatants were stored at -20°C. Alveolar cells were suspended in RPMI-1640 (Gibco, USA) tissue culture medium, supplemented with 10% foetal calf serum, and used for total and differential cell counts. All of the cell-free supernatants were concentrated threefold (3×) in a freeze-dryer (Birchover Instruments Ltd, UK) and used for bactericidal assay against *S. aureus* and phospholipid estimation.

Differential count

Ten microlitres of this cell suspension, containing 5×10^4 cells, was transferred to a clean dry glass slide and a smear was made. Smears of alveolar cells of the patients and normal subjects were stained, using modified Wright-Giemsa stain (Diff-Quick, American Scientific Products, USA). A total of 300 cells was counted for each smear.

Staphylococcus aureus

The *Staphylococcus aureus* strain used in this study was isolated from a tropical pulmonary eosinophilia patient [10], and was seen to be resistant to 100 μg·ml^{-1} lysozyme for 4 h. Log phase cultures of *S. aureus* were prepared using tryptic soy broth (Difco, USA), by incubating at 37°C for 18 h. The bacteria were washed three times in phosphate buffered saline (PBS), and counted in a bacterial-counting chamber (Thoma, Weber Scientific International Ltd, UK), and adjusted to 4×10^7·ml^{-1} PBS. Smears of this suspension were stained with Gram stain for confirmation.

Bactericidal assay against S. aureus

The method described by Coonrod and Yoneda [7] was followed. Briefly, 2×10^6 *S. aureus* in 50 μl PBS were mixed with 250 μl of threefold concentrated cell-free supernatants of the alveolar lavage samples or PBS (control), and incubated at 37°C for 0, 2 and 4 h. After the incubation periods, the samples were subjected to tenfold dilutions. One hundred microlitres of the diluted samples were plated on tryptic soy agar plates (Difco, USA) and streaked to enumerate the colony-forming units (cfu).

The plates were incubated at 37°C for 24 h, and the number of individual cfu were counted. For each sample, triplicates were made and the variation was less than 10%. The percentage bactericidal activity against *S. aureus* of the different lavage samples and for the various time-points were calculated based on the number of cfu.

Anti-staphylococcal antibody in the lavage samples

Staphylococcal agglutination test was performed to determine the presence of antibody against staphylococci in the lavage samples. Threefold concentrated lavage samples were serially diluted (twofold) in PBS. A 1% suspension of heat-killed *S. aureus* was added to each sample and incubated at 37°C. Readings were taken after 24 h.

Phospholipid estimation

Phospholipid estimation was carried out, using the method described for serum phospholipid [11]. Threefold concentrated (3×) lavage samples were used to estimate the phospholipid level, and expressed as mg·dl^{-1} of 1× lavage fluids (unconcentrated).

Briefly, 0.2 ml of the threefold concentrated lavage samples were transferred into 15×1.6 cm centrifuge tubes and 5 ml of 5% trichloroacetic acid added drop by drop whilst shaking. The samples were centrifuged for 20 min at 2,500 rpm. The supernatants were discarded, and 1 ml of digestion mixture (500 μl distilled water, 250 μl concentrated sulphuric acid and 250 μl perchloric acid (s.g. 1.75) was added to the deposit in each tube and heated gently for 30–45 min, until the liquids become colourless. The samples were allowed to cool. One millilitre of distilled water was added to each sample and boiled for 15 s to convert pyrophosphate to orthophosphate. One millilitre of 50% sodium acetate was added and made up to 10 ml with distilled water. Then, 1 ml of 2.5% ammonium molybdate and 1 ml of 1% metol in 3% sodium hydrogen sulphite solution was added and mixed well. After 15 min, the reaction mixtures were read at 700 nm in a spectrophotometer, against a reagent blank prepared by mixing 250 μl concentrated sulphuric acid, 1 ml sodium acetate, 1 ml molybdate, 1 ml metol and 8.75 ml distilled water.

A stock standard solution, 30 mmol·l^{-1} concentration of potassium dihydrogen orthophosphate (KH_2PO_4) was prepared, in double distilled water containing 2 ml concentrated sulphuric acid. A working standard of 0.12 mmol·l^{-1} was prepared from stock standard (30 mmol·l^{-1}) by diluting the stock standard 1 to 250 with distilled water. Five ml of working standard (0.12 mmol·l^{-1}) was added with 250 µl concentrated sulphuric acid, 1 ml molybdate, 1 ml sodium acetate, 1 ml metol and 3.75 ml distilled water, and read against the reagent blank. Lavage phospholipid phosphorous was calculated as described below:

$$\frac{\text{Reading of lavage sample}}{\text{Reading of working standard}} \times \frac{5 \text{ ml} \times 0.12 \text{ mmol}}{1000 \text{ ml}} \times \frac{1 \text{ ml}}{0.2 \text{ ml}} \times 1000 \text{ ml}$$

$$\frac{\text{Reading of lavage sample}}{\text{Reading of working standard}} \times 3 \text{ mmol}\cdot l^{-1} \text{ or } 9.3 \text{ mg}\cdot dl^{-1}$$

$$(3 \text{ mmol}\cdot l^{-1} = 93 \text{ mg}\cdot l^{-1})$$

For a standard curve, the stock standard (30 mmol·l^{-1}) was diluted from 1 to 150 to give a solution containing 0.20 mmol·l^{-1}. Zero, 1, 2, 3, 4 and 5 ml of this was added sulphuric acid, acetate, molybdate and metol as mentioned above, and made up to 10 ml with distilled water. These are equivalent to 0, 1, 2, 3, 4 and 5 mmol·l^{-1}. (3.1, 6.2, 9.3, 12.4 and 15.5 mg·dl^{-1}).

Statistical methods

The results are expressed as arithmetical or geometrical mean±SE, wherever applicable. Statistical analysis was performed using Students t-test.

Results

The mean percentage fluid during BAL was more than 50% of the saline infused in active pulmonary tuberculosis (ATB), inactive tuberculosis (cured) (ITB) patients and healthy subjects (table 1). The lavage fluids of ATB, ITB and normal subjects were equally competent in their *S. aureus* killing activity at 0 and 2 h. In normal and ITB lavage samples, increased killing activity was observed with increased incubation time. However, the bactericidal activity against *S. aureus* was reduced at 4 h in the ATB lavage fluids compared to normal and ITB (fig. 1).

In order to determine the effect of smoking on the bactericidal activity against *S. aureus*, the results at 4 h incubation were analysed separately for nonsmokers and smokers; this revealed that smoking has no significant effect on the bactericidal activity of the lavage fluids (data not shown).

Active tuberculous and inactive tuberculous lavage fluids had significantly increased total cell numbers, compared to normal subjects ($p<0.01$ and $p<0.05$, respectively). Furthermore, a decrease in the percentage macrophages ($p<0.01$) and increase in lymphocytes ($p<0.005$) was seen in the ATB lavage fluids compared to normal. This difference in the macrophage and lymphocyte count was

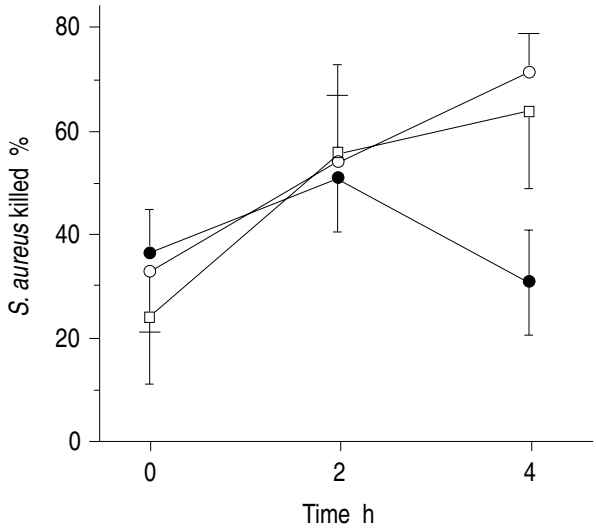

Fig. 1. – Bactericidal activity against *S. aureus* of bronchoalveolar lavage fluids (threefold concentrated) of: —□—: normal individuals (n=6); —●—: active tuberculosis patients (n=13); and —○—: inactive tuberculosis patients (n=8). Percentage *S. aureus* killing was measured at different time points. Data are presented as geometric mean±SE.

Table. 1. – Percentage fluid recovery, total and differential cell counts and phospholipid levels of alveolar lavage fluids of normal individuals, and patients with active and inactive tuberculosis (TB)

Group	Fluid recovery	Total cells	Differential counts				PL[$] levels
			Mac	Lymph	Neut	Eos	
	%	×10^7·l^{-1}	%	%	%	%	mg·dl^{-1}
Normals n=6	59±2	22±2	84±2	15±3	1±1	1±0	0.40±0.10
ATB n=13	58±3	51±9*	66±5**	31±5***	2±1	1±0	0.47±0.11
ITB n=8	53±4	56±16[#]	77±6	19±5	3±1	1±1	0.79±0.28

Data are presented as mean±SE. ATB: patients with active TB; ITB: patients with inactive TB; Mac: macrophages; Lymph: lymphocytes; Neut: neutrophils; Eos: eosinophils; PL: phospholipid level. Normal *vs* ATB: *: $p<0.01$; **: $p<0.01$; ***: $p<0.005$. Normal *vs* ITB: [#]: $p<0.05$. [$]: phospholipid level in 1× lavage fluid (unconcentrated).

not seen in ITB lavage fluids. A slightly higher level of total phospholipid in ATB and a considerable increase in ITB lavage fluids was seen compared to normal lavage fluids (table 1). Based on the percentage lymphocytes (and macrophages) of normal subjects (cut-off point 20% lymphocytes), the ATB and ITB patients were divided into no lymphocytic alveolitis (<20% lymphocytes) and lymphocytic alveolitis (>20% lymphocytes) groups. There was no difference in the total phospholipid levels between "no lymphocytic alveolitis" and "lymphocytic alveolitis" groups (table 2).

In normal subjects and ITB patients, a maximum bactericidal activity (67–72%) against *S. aureus* was observed at 0.4 and 0.79 mg·dl^{-1} (mean values) concentration of phospholipids in the lavage fluids (unconcentrated). Even though the phospholipid level (mean value) of ATB lavage was closer to normals, the bactericidal activity was low (32%) compared to normals. Increase in total phospholipid level had no effect on the bactericidal activity of the active tuberculous lavage fluids (fig. 2). The bactericidal activity against *S. aureus* of lavage fluids of normal subjects, ATB and ITB was not much influenced by the total phospholipid levels. However, the killing activity was influenced by the increased lymphocytes (and decreased macrophages) (fig. 3).

The lavage fluids of normal subjects and ITB patients with no lymphocytic alveolitis showed an increased killing of *S. aureus*. On the other hand, ATB lavage fluids with no lymphocytic alveolitis showed a considerably decreased bactericidal activity against *S. aureus*. An increased trend on bactericidal activity was seen in the ATB and ITB lavage fluids with lymphocytic alveolitis (>20% lymphocytes) (fig. 3).

All lavage fluids were screened for the presence of antibodies against *S. aureus*, and found to be negative, except for one inactive tuberculous and one normal lavage fluid (titre 1:2). The lavage fluids were also cultured to detect the presence of *S. aureus* and other bacteria. Only one ATB lavage fluid showed 26 cfu of *S. aureus*. One normal, one ATB and two ITB fluids showed growth of bacterial colonies other than *S. aureus*. However, the bactericidal activity against *S. aureus* of the lavage samples was not affected.

Table. 2. – Percentage lymphocytes and total phospholipid levels in the lavage fluids of normal subjects and patients with no lymphocytic alveolitis and lymphocytic alveolitis

	No lymphocytic alveolitis <20% lymphocytes		Lymphocytic alveolitis >20% lymphocytes	
	Lymph %	PL$^{\$}$ mg·dl^{-1}	Lymph %	PL$^{\$}$ mg·dl^{-1}
Normals	15±3 (n=6)	0.40±0.10 (n=6)	Nil	Nil
ATB	11±3 (n=4)	0.64±0.33 (n=4)	40±5 (n=9)	0.39±0.07 (n=9)
ITB	8±3 (n=4)	0.68±0.48 (n=4)	30±3 (n=4)	0.90±0.36 (n=4)

Data are presented as mean±SE. $^{\$}$: phospholipid level in 1× lavage fluid. Numbers in parentheses represent the number of individuals studied. For definitions of abbreviations see legend to table 1.

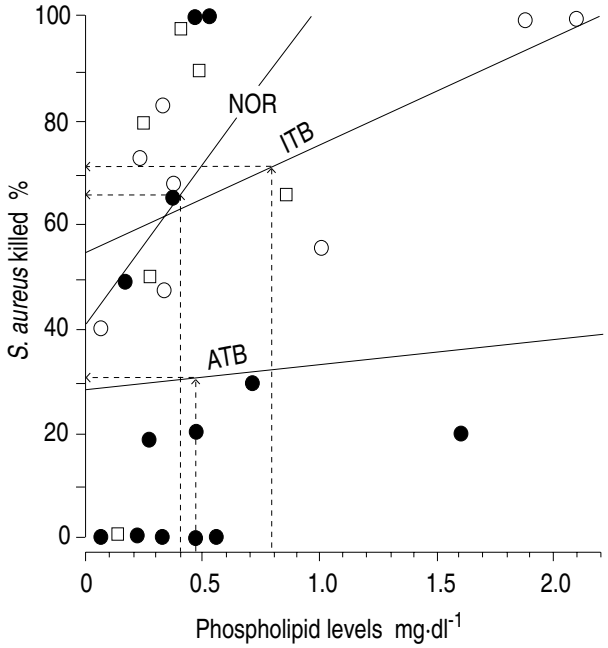

Fig. 2. – Effect of phospholipid levels on *S. aureus* killing activity of:- □ normal (NOR); ●: active tuberculous (ATB); and ❍: inactive tuberculous (ITB) lavage fluids. Percentage *S. aureus* killed at 4 h time-point is presented. Arrow marks with dotted lines represent the mean values of total phospholipid levels and the corresponding bactericidal activities against *S. aureus*.

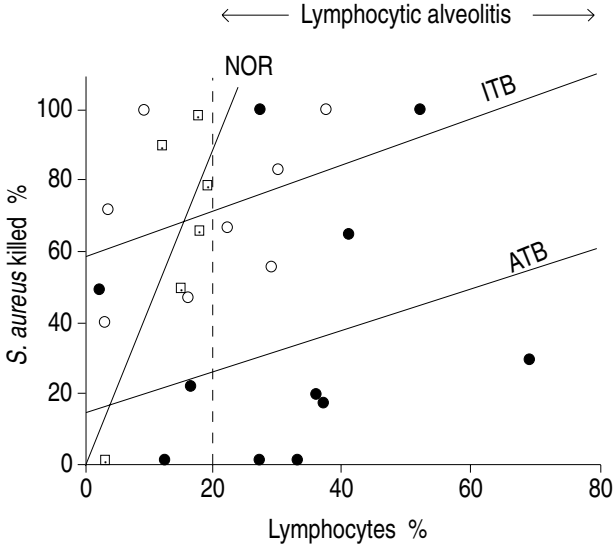

Fig. 3. – Relationship between lymphocytes and *S. aureus* killing activity with correlation lines (simple linear regression). Bactericidal activity at 4 h time point is presented. □: normal (NOR); ●: patient with active tuberculosis (ATB); ❍: patient with inactive tuberculosis (ITB).

Discussion

The cell-free supernatants of alveolar lavage samples of healthy subjects, ATB and ITB patients showed an increasing bactericidal activity with increasing time of

incubation. However, the bactericidal activity against *S. aureus* of ATB was reduced at 4 h of incubation, compared to the normal and ITB lavage samples (fig. 1). The lavage fluids of normal and ITB were more efficient in their killing activity against *S. aureus* than ATB. This increased killing effect of normal and ITB lavage fluids was not due to anti-staphylococcal antibody, since almost all samples were negative for a detectable level of antibodies. Furthermore, the killing effect of the ITB lavage fluids was not due to anti-staphylococcal activity of rifampicin. The ITB patients included in this study had received antituberculosis treatment at least one year prior to our evaluation.

It has been shown that crude preparations of lung lavage material possess intrinsic bactericidal activity [6, 7]. Moreover, the phospholipid-protein content of the surfactant produced by the type-II epithelial cells of the lung has been shown to contain this bactericidal activity. The bactericidal activity against *S. aureus* of the normal and ITB lavage samples observed in this study may be due to the phospholipid-protein content of the surfactant. It has been well established that palmityl lysophosphatidylcholine (lysophospholipid) possesses anti-bacterial activity against several Gram-positive bacteria [7]. Altered levels of phospholipids have been shown in smoking [12], and diseases such as bacterial pneumonia and idiopathic pulmonary fibrosis [13–15], and respiratory distress syndrome [13, 16]. Although smoking has been shown to alter the surfactant level, the present study showed no significant effect of smoking on the bactericidal activity of ATB and ITB lavage fluids.

The reduced bactericidal activity against *S. aureus* of the ATB lavage was not due to the presence of *S. aureus* in the lavage fluids. Out of 13 samples, one sample was positive for *S. aureus* and the rest were negative. Even though reduced levels of surfactant containing phospholipids have been shown in ATB lung [17], in the present study there was no difference in the level of total phospholipid contents of normals, ATB and ITB lavage fluids. In normal individuals, since the integrity of the alveolar space is maintained, the phospholipid production by the type II epithelial cells are probably regulated. A similar kind of phospholipid production can also be expected in the ITB individuals. In ATB patients, there may be macrophage lysis due to *M. tuberculosis* multiplication, or lysis of *M. tuberculosis* infected macrophages by cytotoxic T-lymphocytes [18]. Phospholipid contents of the lysed host cells [11, 19], and killed *M. tuberculosis* [20], in the alveolar space would, thus, result in increased total phospholipid contents of the alveolar lining material. The present study suggests that the reduced bactericidal activity in the lavage fluids of ATB could not be due to the total phospholipid level of the lavage fluids. An altered level of lysophospholipid which (possesses antibacterial activity) in the total phospholipid may be one possible factor involved in the reduced bactericidal activity against *S. aureus*.

On the other hand, the bactericidal activity was influenced by the cellular components of the lavage fluids (lymphocytes and macrophages). A reduced bactericidal activity against *S. aureus* was seen in ATB lavage fluids with no lymphocytic alveolitis (<20% lymphocytes). However, a trend towards increased bactericidal activity was seen in the ATB and ITB lavage fluids with lymphocytic alveolitis (>20% lymphocytes). Several human respiratory diseases have been shown to be associated with pulmonary lymphocytic infiltration and alveolitis [21]. The association of bactericidal activity and the level of cellular components could be due to the interaction of lymphocytes and alveolar macrophages with alveolar type-II epithelial cells, resulting in the production of a decreased level of the lysophospholipid in the total phospholipid content of the surfactant.

The present study, thus, reveals that the reduced bactericidal activity against *S. aureus* of the alveolar lining material of ATB lavage may be a transient effect. When the disease (active tuberculosis) progresses, the level of pulmonary defence against bacterial infections, such as *S. aureus*, may be lowered. Infiltration of cellular components, such as lymphocytes and other cells, into the alveolar microenvironment may probably be involved in regulating the pulmonary defence mechanism against such infections. Treatment with antituberculosis drugs restores the pulmonary defence mechanism against bacterial infections, such as those caused by staphylococci.

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