Effect of plasma lysozyme on live *Mycobacterium tuberculosis*

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The role of plasma lysozyme of normal healthy subjects (NHS, $n = 12$) and active pulmonary tuberculosis (ATB) patients ($n = 15$) on the innate immune mechanism was studied by the binding activity of lysozyme on live *Mycobacterium tuberculosis.* Plasma samples of NHS and ATB patients treated with live *M. tuberculosis* for 4 h and 24 h time points showed a significant decrease in the plasma lysozyme level when compared to the untreated samples (4 h, $P < 0.001$; 24 h, $P < 0.001$). Pretreatment of live *M. tuberculosis* with plasma of NHS and ATB patients showed a trend in the reduction of viability of live *M. tuberculosis.* Moreover, *M. tuberculosis* pretreated plasma of NHS showed a trend towards an increased spontaneous as well as antigen-induced lymphocyte response when compared to ATB plasma. The enzymatic action of the lysozyme and other enzymes on the cell wall may induce *M. tuberculosis* to release some antigenic components which may be immunogenic and induce lymphocyte proliferation. The present study suggests that lysozyme and other enzymes may play an important role in the first line defence, i.e. the innate immunity, against *M. tuberculosis* infection.

LYSOZYME is one of the hydrolytic enzymes of the lysosomal compartment of phagocytic cells such as monocytes/macrophages and neutrophils[1]. Lysozyme is secreted into the extracellular compartment and has been shown to be bactericidal. This enzyme along with other enzymes and proteins is involved in the first line of defence against any pathogenic infection. The biologic importance of lysozyme lies in its ability to hydrolyse the polysaccharide portion of bacterial cell walls. Lysozyme causes lysis of Gram-positive and some Gram-negative bacteria by specific cleavage of the β1-4 linkage between N-aceytylglucosamine and N-acetylmuramate in the peptidoglycan backbone of bacterial cell walls[2]. Lysozyme has been shown to specifically bind glucose-modified proteins bearing advanced glycation end-products (AGEs). Exposure to AGE-modified proteins has been shown to inhibit the enzymatic and bactericidal activity of lysozyme[3].

It has been shown that binding of lysozyme with lipopolysaccharide (LPS) affects the enzymatic activity of lysozyme as well as the immunostimulatory effect of LPS[4,5]. Since, carbohydrate moieties similar to LPS such as lipoarabinomannan and lipomannan have been shown in the cell wall of *Mycobacterium tuberculosis*, an attempt was made to find out whether lysozyme binds to live *M. tuberculosis*, and if so, whether binding of lysozyme affects the viability of *M. tuberculosis.*

Increased lysozyme level has been observed in mycobacterial diseases[6–8]. Although lysozyme levels have no diagnostic value *per se* in tuberculosis, this enzyme has been suggested as a non-specific diagnostic marker along with antibody levels in tuberculosis[8,9]. Since plasma lysozyme plays an important role in innate immunity, the goal of the present study was to find out the effect of plasma lysozyme on live *M. tuberculosis.* Moreover, the modulatory effect of plasma samples exposed to live *M. tuberculosis* on lymphocyte response has also been studied.

Patients who were sputum-positive for *M. tuberculosis* by smear and culture were known as active pulmonary tuberculosis (ATB) patients. Plasma samples of ATB patients ($n = 15$) and normal healthy subjects (NHS, $n = 12$) were used. These plasma samples were stored frozen at –20°C until required.

Based on our preliminary experiments (data not shown) on different concentrations (0.5, 1, 2 mg/100 µl) of heat killed *M. tuberculosis* $H_{37}Rv$, live *M. tuberculosis* $H_{37}Rv$ and *Micrococcus lysodeikticus* (Sigma Chemical Co, St. Louis, USA), 2 mg/100 µl plasma was selected for further experiments. Since, *M. lysodeikticus* is rich in polysaccharide moiety, this organism was used as a positive control. Two mg wet weight of live *M. tuberculosis* containing approximately $1.2–1.6 \times 10^8$ viable bacilli was suspended in 1 ml sterile distilled water. The suspension was taken in an 1.5 ml eppendorf tube and centrifuged for 5 min at 16,000 rpm. The supernatant was removed completely using a micropipette. To the packed volume, 100 µl plasma sample of either ATB patient or NHS was added, mixed well and incubated at 37°C for 4 h and 24 h. Similarly, *M. lysodeikticus* (positive control) was also taken and incubated with 100 µl plasma sample for 4 h and 24 h. Plasma samples without *M. tuberculosis* or *Micrococcus* served as controls. After 4 h and 24 h of incubation, the samples were centrifuged at 16,000 rpm for 5 min to pellet out *M. tuberculosis* and *M. lysodeikticus* and the plasma samples were checked for their lysozyme level. The stimulatory or suppressive effect of *M. tuberculosis*-treated plasma samples on the lymphocyte response was studied either with or without *M. tuberculosis* culture filtrate antigen. For the bactericidal activity, 3×10^6 bacilli per 100 µl of plasma of either ATB patients or NHS were taken, incubated for 4 h and 24 h and packed by centrifugation. Lysozyme level was also estimated. The packed bacilli were washed twice in sterile distilled water and resuspended in 500 µl of distilled water. Serial 10-fold dilutions were made from this suspension and plated on Middlebrook 7H11 agar plates in tripli-

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cate to find out the viable counts, and the exact colony forming units (CFU) were determined after 15 days of incubation at 37°C. Lysozyme level was estimated by single radial diffusion as described in detail earlier[7,10].

M. tuberculosis H_{37}Rv strain (TMC201) maintained by periodic passaging through guinea pigs and subcultured in Lowenstein–Jensen (L–J medium) was used. Approximately two-thirds of 3 mm external diameter (24 SWG wire loop) of bacillary mass from the L-J slope was taken and transferred to 0.2 ml of sterile distilled water in a 7 ml Bijou bottle containing 1–3 mm glass beads. This bacillary mass was considered to contain approximately about 4 mg wet weight of the organism. The Bijou bottle was kept in a mechanical shaker for a few minutes to produce a uniform suspension. To this suspension, 0.8 ml of sterile distilled water was added and mixed well and a bacillary count was made with a Thoma counter.

Heparinized (20 units/ml) peripheral blood samples drawn from NHS ($n = 9$) were subjected to ficoll-hypaque density gradient centrifugation as described by Boyum[11] and used for lymphoproliferative response. Lymphocytes were cultured/stimulated either with the plasma samples treated with live *M. tuberculosis* alone or plasma samples treated with live *M. tuberculosis* and *M. tuberculosis* culture filtrate antigen (10 µg/ml)[12].

The results are expressed as arithmetic mean ± standard error (SE). Statistical significance was done using Student's *t* test.

A decreased plasma lysozyme level was observed with plasma samples of both ATB patients and control subjects treated with either *M. lysodeikticus*, heat-killed or live *M. tuberculosis* than in untreated plasma samples. The decrease was proportional to the dose of the bacteria used (0.5, 1 and 2 mg/100 µl of plasma, data not shown).

Plasma samples pretreated with live *M. tuberculosis* (2 mg/100 µl) for 4 h and 24 h showed a significant decrease in the lysozyme levels of NHS (4 h $P < 0.05$; 24 h, $P < 0.001$) and ATB patients (4 h, $P < 0.001$; 24 h, $P < 0.001$; Figure 1). Live *M. tuberculosis* treated with plasma samples of either NHS or ATB patients showed decreased CFU (in log counts) both at 4 h and 24 h (0 h: PBS-CFU 6.5; 4 h: NHS-CFU 5.6 ± 0.1; ATB-CFU 5.7 ± 0.04; 24 h: NHS-CFU 5.0 ± 0.0; ATB-CFU 5.2 ± 0.1).

Plasma samples of ATB patients and NHS pretreated with live *M. tuberculosis* showed a trend towards a stimulatory effect on the lymphocyte response. Spontaneous lymphocyte response and the antigen-induced lymphocyte response of NHS were augmented by the plasma of NHS-treated with live *M. tuberculosis* for 4 h. Similar treatment of plasma samples with live *M. tuberculosis* for 24 h showed a moderate stimulatory effect on the lymphocyte response (Figure 2). Such an increase was not observed with plasma samples of ATB patients.

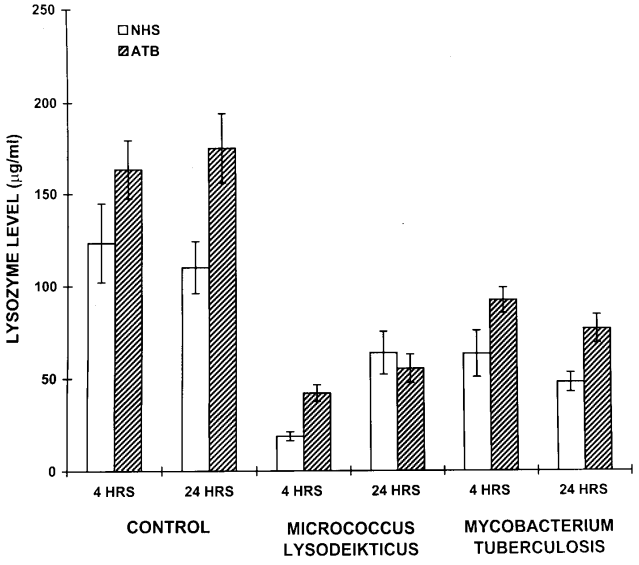

Figure 1. Binding activity of plasma lysozyme of normal healthy subjects (NHS) and active-TB (ATB) patients on *M. tuberculosis*. Each bar represents the arithmetic mean ± standard error (S.E.). Plasma lysozyme level before and after treatment with *M. tuberculosis* – NHS: 4 h, $P < 0.05$; 24 h, $P < 0.001$; ATB: 4 h, $P < 0.001$; 24 h, $P < 0.001$.

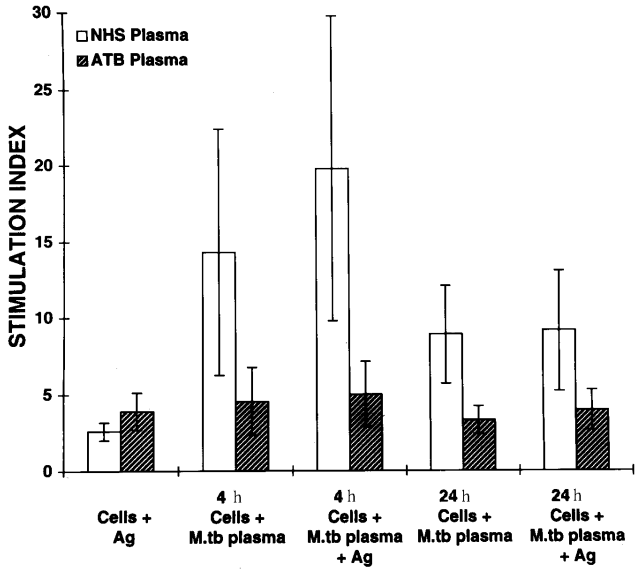

Figure 2. Immunomodulatory effect of *M. tuberculosis* pretreated plasma of normal healthy subjects (NHS) and active-TB (ATB) patients on spontaneous as well as *M. tuberculosis* culture filtrate antigen-induced lymphocyte response of normal subjects. Lymphocyte response was studied in 9 healthy subjects. Each bar represents mean ± S.E. Cells, peripheral blood mononuclear cells; Ag, *M. tuberculosis* culture filtrate antigen; *M.tb* plasma, Plasma pretreated with *M. tuberculosis*.

Plasma of NHS and ATB patients pretreated with live *M. tuberculosis* showed a significant reduction in the plasma lysozyme level. This shows that plasma lysozyme binds to live *M. tuberculosis*. Increased lysozyme level in any infectious disease, including mycobacterial diseases, is well established. The action

of lysozyme is through binding and cleavage of cell wall polysaccharide by the enzymatic action. Even though *M. tuberculosis* cell wall contains mainly cord factor and other lipids, it also contains polysaccharides such as lipoarabinomannan and lipomannan. In ATB, the bacterial load harbouring in the system may be high, which may induce the system to produce more lysozyme to encounter the *M. tuberculosis* pathogen.

In the present study, a trend towards a decreased CFU of *M. tuberculosis* pretreated with plasma of either NHS or ATB patients was observed. One of the mechanisms for this decreased CFU may probably be due to the damage/injury caused by the enzymatic action of lysozyme on the carbohydrate moieties of the cell wall of *M. tuberculosis*, along with other enzymes and proteins of the plasma (which may also be detrimental to *M. tuberculosis*).

The stimulatory effect of 4 h plasma pretreated with live *M. tuberculosis* may be due to the release of some of the carbohydrate moieties such as lipoarabinomannan, lipomannan and some other cell wall components due to the action of lysozyme and other enzymes on live *M. tuberculosis*, which may be immunogenic and stimulatory to lymphocytes.

The present study suggests that lysozyme in the plasma as well as in cells/tissues may be detrimental to *M. tuberculosis* and to other pathogens. Moreover, lysozyme along with other host enzymes and proteins may play an important role in innate immunity against *M. tuberculosis* infection.

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Noctiluca blooms in Port Blair Bay, Andamans

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Moderate to intense blooms of the dinoflagellate *Noctiluca scintillans* occurred in Port Blair Bay, Andamans during June and July of 2000. The blooms occurred thrice in succession after short periods of waning. The bloom that came about second in the sequence, between 11 and 20 July 2000, gave us an opportunity to investigate the changes in water characteristics associated with the event. Results indicate that variations in dissolved oxygen concentration were fairly small, whilst nutrient levels and plankton compositions were dramatically altered. Primarily, the bloom led to exclusion of other phytoplankton, also limiting zooplankton prevalence to essentially copepods. The bloom resulted in exceptionally high levels of chlorophyll *c*, which exceeded levels of chlorophyll *a* by several folds. Nutrient levels were characterized by decrease of nitrate during active periods of the bloom, and sharp increase in phosphate, particularly during the waning phase.

BLOOMS of the dinoflagellate *Noctiluca scintillans* have been reported from Indian waters by Prasad[1] in Palk Bay, Subrahmanyan[2] and Katti *et al.*[3] in the Arabian Sea, Sargunam, Rao and Nair[4] in Kalpakkam coastal waters and more recently, by Naqvi and coworkers[5] off Cochin. In only one case has fish kill been reported[5], although the authors were cautious not to overrate the severe fish mortality as a direct consequence of the *Noctiluca* bloom.

An intense bloom of *Noctiluca* was first noticed in the waters of Port Blair Bay during the second week of June 2000. The bloom imparted light to the vivid green colour of the coastal waters. The bloom persisted for several days, waning eventually by the end of the month. In course of time, the bloom reappeared twice during July 2000. This communication addresses the changes in coastal water characteristics associated with the event, with particular emphasis on the bloom that occurred between 11 and 20 July 2000.

The Port Blair Bay extends as a narrow stretch in a northeast to southwest direction (Figure 1). Six station locations spread over the entire Bay, as indicated in the figure, were considered for the primary sampling done on 12 June 2000. Further investigations were limited to

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