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Intranasal application of lentinan enhances bactericidal activity of rat alveolar macrophages against *Mycobacterium tuberculosis*

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To study the *in vivo* interaction between lentinan-stimulated alveolar macrophages and *Mycobacterium tuberculosis* were used rats intranasally infected with 2×10^8 CFU. Before infection animals were treated intranasally with lentinan at a dose of 1 mg/kg (administrated three times at 2 days periods). Samples of broncho-alveolar lavage fluid were obtained from rats at different intervals −3, 24 and 72 h after infection. The process of phagocytosis *in vivo* was observed by light and electron microscopy, as well as acid phosphatase cytochemistry methods. Bactericidal activity of alveolar macrophages following the same intranasal installation of lentinan was assessed by *in vitro* "killing" ability test against M. tuberculosis. Nitrite production by lentinan activated alveolar macrophages was measured 24 h after *ex vivo* culture of these cells. It was demonstrated that lentinan induces high level of alveolar macrophage activation manifested through enhanced bactericidal effect against *M. tuberculosis*, which correlates with the induction of reactive nitrogen intermediates, increased activity of lysosomal enzymes (acid phosphatase), and with effective phagolysosomal fusion followed by destruction of *Mycobacteria.*

1. Introduction

Tuberculosis continues to be a very large problem throughout the world and its elimination requires new knowledge and strategies. Better understanding of immunopathogenesis of tuberculosis (TB) is essential for the development of new tools for treatment and prevention (Enarson 2003).

Alveolar macrophages represent the first line of defence against *M. tuberculosis* in the lungs. They are responsible for the initial uptake of *M. tuberculosis*, mycobacterial killing, antigen processing and presentation, as well as recruitment of immune effector cells. Activation of innate immune response, such as recruitment of macrophages and induction of proinflammatory cytokines by *M. tuberculosis* occurs early after infection (Toossi 2000). The fate of *Mycobacteria* at the site of infection depends on the ability of the bacilli to survive and multiplicate intracellularly and the capacity of alveolar macrophages to inhibit their growth (Dannenberg and Tomashefski 1988; Dannenberg and Rook 1994). To become effective (bactericidal) against *Mycobacteria* macrophages need to be activated. Mechanisms involved in killing of *M. tuberculosis* within the phagolysosomes of activated macrophages include the production of reactive oxygen intermediates or reactive nitrogen intermediates (Arias et al. 1997; Chan et al. 1992; van Crevel et al. 2002).

Lentinan, a (1–3)-beta-D-glucan isolated from the Japanese mushroom *Lentinus edodes* (Shiitake), is a well known immunomodulator (Chihara 1992; Maeda and Chihara 1999). It is now recognized to act as an immunopotentiator by stimulating various kinds of immune cells including macrophages, NK-cells and lymphocytes (Chihara et al. 1969; Hamuro and Chihara 1984; Rowan et al. 2002). It is in an unique class of delayed-type-oriented assistants, in which macrophages play some parts (Rowan et al. 2002). In our previous study, lentinan has been shown to induce high-grade activation of broncho-alveolar cells after intranasal route of administration in rats (Markova et al. 2002).

Of special interest are its effects on respiratory tract cell defense mechanisms and their potential impact for preventing and treatment of pulmonary infection and especially of lung tuberculosis. Because macrophages are the most important cells in TB pathogenesis and the main effectors involved in uptake of *M. tuberculosis*, the purpose of our study was to investigate the effects of lentinan following intranasal application to rats on phagocytosis *in vivo* evaluated by light and electronmicroscopy, as well as on the killing ability of alveolar macrophages against *M. tuberculosis* at the early stage after infection.

2. Investigations and results

2.1. Electron and light microscopic observations

Transmission electron microscopy (TEM) observations of ultrathin sections of broncho-alveolar cell samples illustrated the interactions including the uptake, entry and fate of *M. tuberculosis* in the alveolar macrophages after intranasal application of Lentinan in rats (Fig. 1 and Fig. 3). At 3 h following an intranasal infection, alveolar macrophages from lentinan treated rats showed strong activation of cell surface with formation of many microvilli and pseudopodia (Fig. 1 A, B). Bacilli were observed in the intercellular space (Fig. 1 A), as well as in a process of

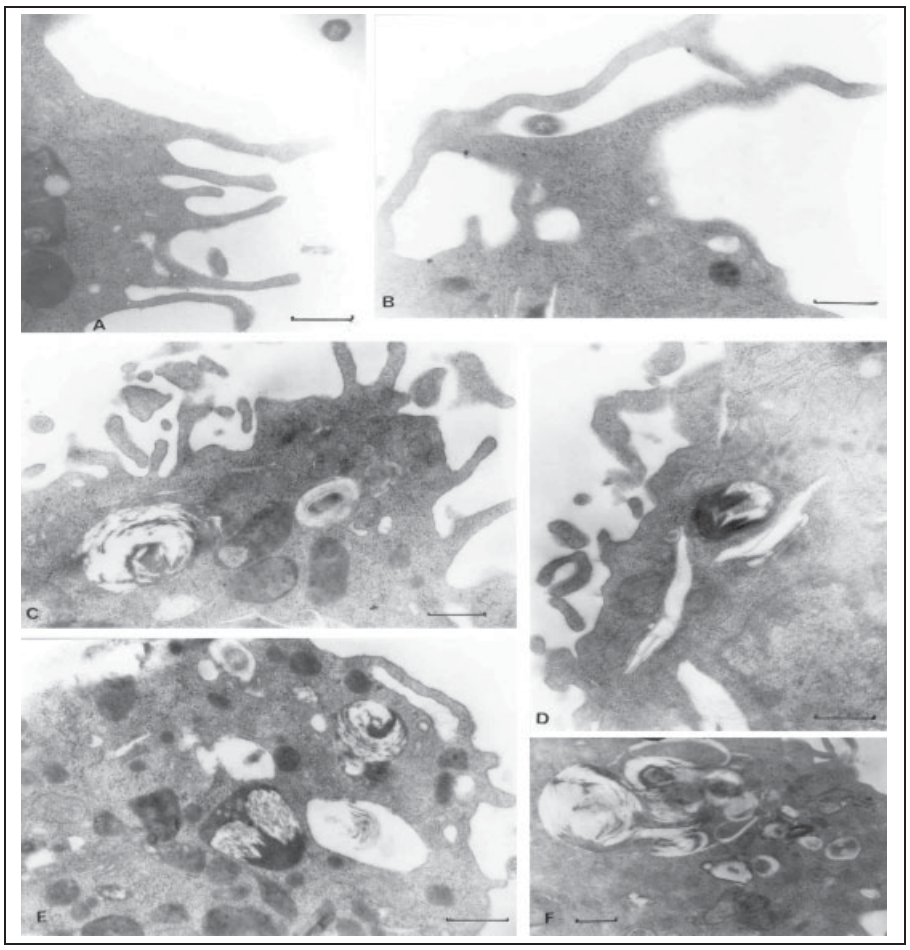

Fig. 1:
Alveolar macrophages after treatment with lentinan and subsequent infection with *M. tuberculosis*. A, B – at 3 h after challenge: Strong activated host cells; Outset of phagocytosis – attraction, adhesion and engulfment of *M. tuberculosis* by the macrophages. C, D, E, F – at 24 h after challenge: Fusion of lysosomes with phagosomes and forming of phagolysosomes with acid phosphatase localization in them

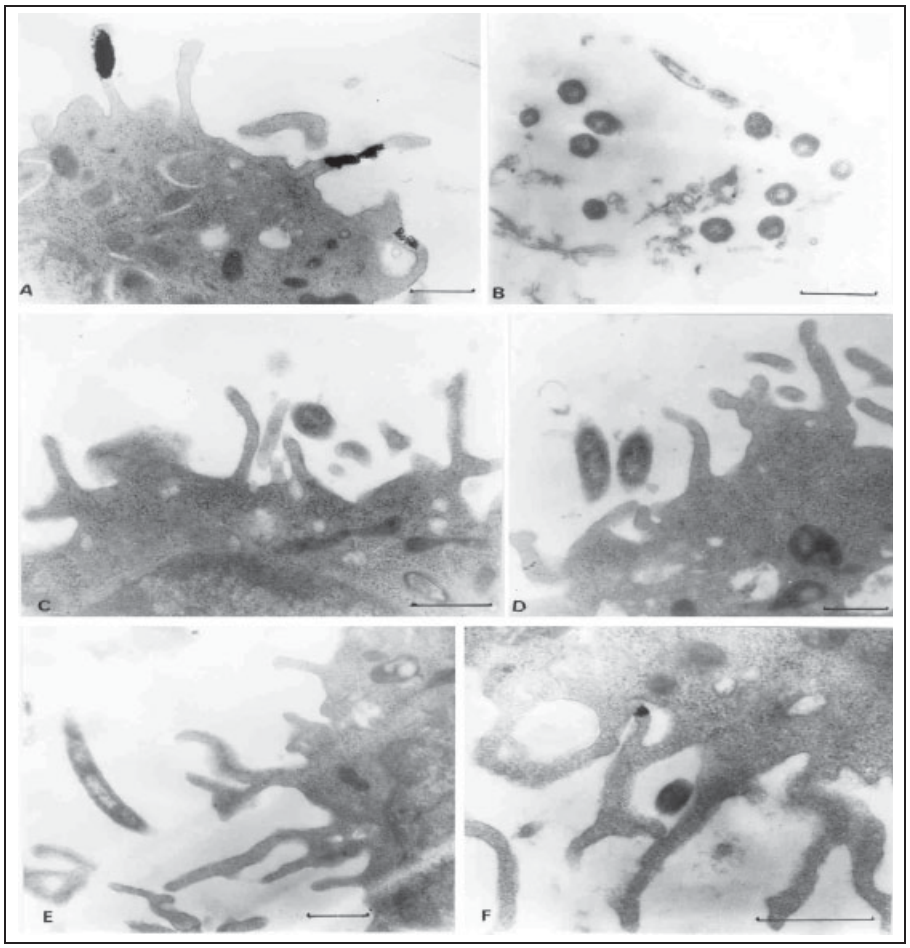

Fig. 2:
Alveolar macrophages after infection with *M. tuberculosis* without lentinan treatment. A, B – at 3 h after infection: Tubercle bacilli are located in intercellular space of macrophages with acid phosphatase in the membrane of pseudopodes. C, D, E, F – at 24 h after infection: Processes of attraction and adhesion of tubercle bacilli are at the beginning. E – Presence of single bacteria with elongated form in the intercellular space

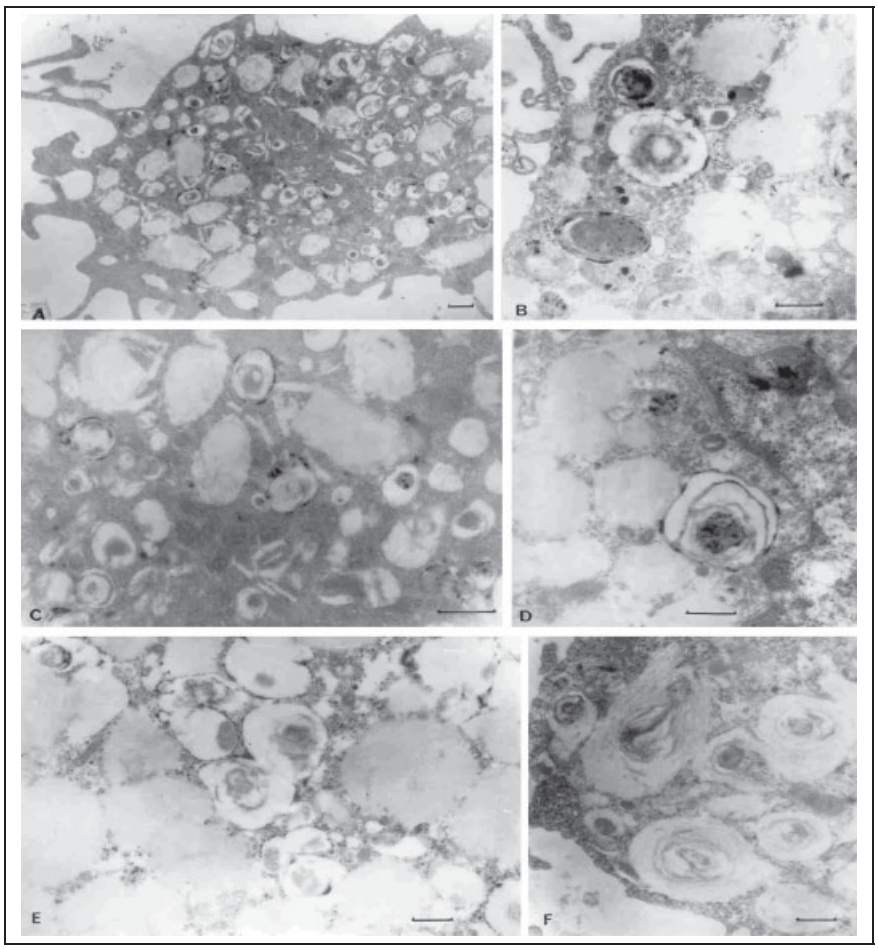

Fig. 3:
Alveolar macrophages after treatment with lentinan and subsequent infection with *M. tuberculosis* at 72 h after challenge: Vacuolization of cells with presence of many phagolysosomes and signs of strong acid phosphatase reaction in their membranes: Very active phagocytosis with digestion of tubercle bacilli

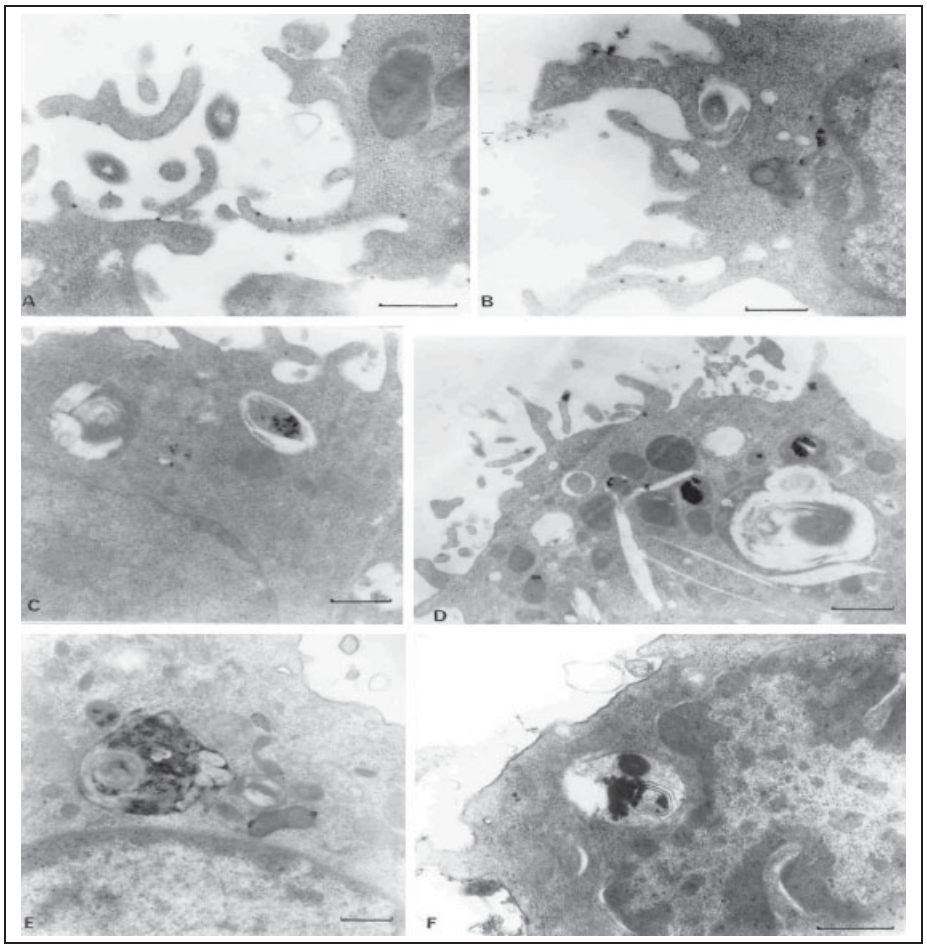

Fig. 4:
Alveolar macrophages at 72 h after infection with *M. tuberculosis*, without Lentinan treatment: A, B – The phagocytic process continues with adhesion, engulfment of *M. tuberculosis*, C – forming of phagosomes, D – attraction of lysosomes and E, F – phagolysosome fusion

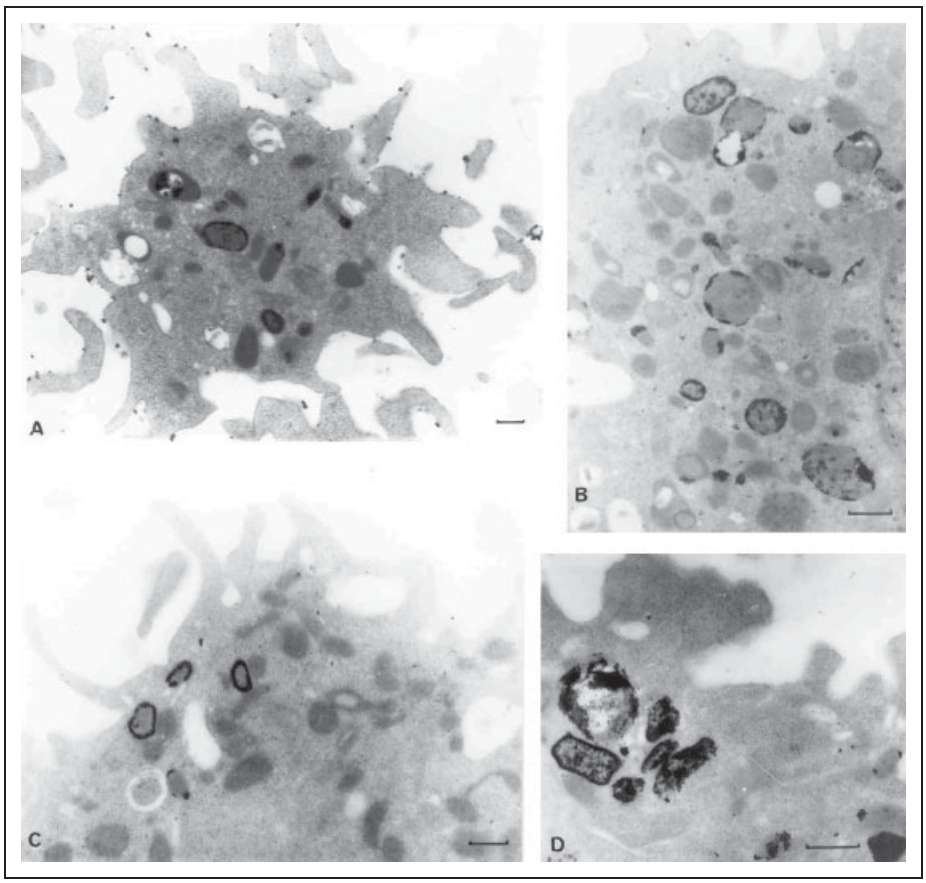

Fig. 5:
Alveolar macrophages after treatment with lentinan (control). Strong activated cell surface with localization of acid phosphatase on the pseudopodia membranes and in the lysosomes

adhesion and engulfment by alveolar macrophages (Fig. 1 B). At 24 h after challenge in this group were found processes of active phagocytosis (Fig. 1 C–F). The *Mycobacteria* were seen inside phagocytic vacuoles. Fusion of lysosomes with phagosomes, as indicated cytochemically by the presence of activated lysosomal acid phosphatase, resulted in the formation of large phagolysosomes (Fig. 1 C–F). Electron-dense material of acid phosphatase labeling was found inside the phagosomes (Fig. 1 D, E).
In contrast, in the second group of rats (without lentinan treatment), at 3 h after challenge (Fig. 2 A, B), the formation of alveolar macrophage microvilli with acid phosphatase location in some of them were seen (Fig. 2 A). The tubercle bacilli were located predominantly in the intercellular space (Fig. 2 B). In this animal group, the process of attraction and adhesion of tubercle bacilli was in the beginning at 24 h after challenge (Fig. 2 C, D, F). Single bacteria with elongated form were seen in the intercellular space near to the macrophage microvilli (Fig. 2 E), or were surrounded of them (Fig. 2 F). The samples taken at 72 h after challenge were characterized by significant differences in the pattern of phagocyte – *M. tuberculosis* interactions between both experimental groups (Fig. 3 and Fig. 4). In the group of lentinan treated rats (Fig. 3), bacilli were not observed free in the intercellular space. The process of very active phagocytosis was demonstrated by strong vacuolization of alveolar macrophages and intensive formation of phagolysosomes. *Mycobacteria* lost their ultrastructural identity and only cell debris or electron-dense material of acid-phosphatase labeling was found in the phagosomes (Fig. 3 A–F). At the same time (72 h) in the rats not treated with lentinan, free Mycobacteria were observed outside the macrophages (Fig. 4 A). The phagocytic processes were at the beginning stage and continued with attraction, adhesion (Fig. 4 A), engulfment (Fig. 4 B), and forming of phagosomes (Fig. 4 C, D) or single phagolysosomes (Fig. 4 E, F).
Micrographs of control alveolar macrophages, obtained from animals following the intranasal treatment only with lentinan, but without infection with *M. tuberculosis* (Fig. 5), showed strong activation of cell surfaces by forming numerous protuberances. Electron-dense material of the acid phosphatase labeling was found on the pseudopodia membranes (Fig. 5 A), on the lysosomal membranes and within the lysosomes of the cells (Fig. 5 A–F).
The phagocytosis *in vivo* was measured semiquantitatively by estimating percent phagocytosis (Fig. 6). We compared both groups and found that in lentinan treated rats 46% of

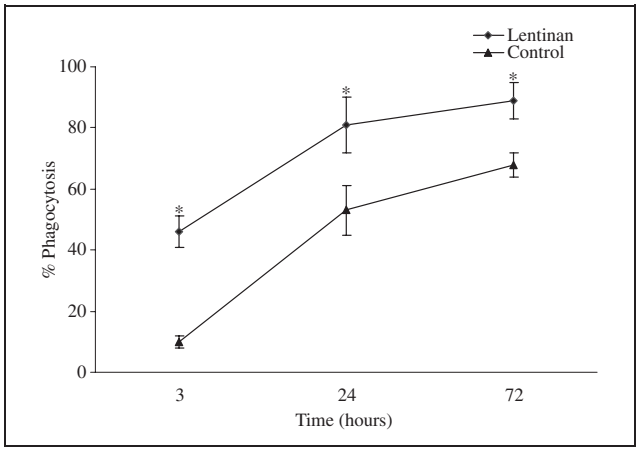

Fig. 6: Phagocytic activity of rat alveolar macrophages during intranasal (i.n.) infection with *M. tuberculosis* (10^8 cells). Treatment: ■ – lentinan was given i.n. befor infection three times at 2 days intervals and each dose consisted of 1 mg/kg; ▲ – Control (without) lentinan. The asterisk indicates statistically significant difference compared to control ($p < 0.01$).

Table: Activation of rat alveolar macrophages after intranasal application of lentinan administered 3 times at 2 days intervals at a dose of 1 mg/kg

Parameters→	Nitrite production (μM)		"Killing" ability of alveolar macrophages against *M. tuberculosis* (Killing index − KI**)	
Groups ↓	Uninfected macrophages	*M. tuberculosis*-infected macrophages	2 h	24 h
Lentinan	16.1 ± 2.3*	27.5 ± 4.8*	4.5 ± 1.7	13.3 ± 2.4*
Control (saline)	9.5 ± 1.9	15.8 ± 3.8	3.7 ± 0.8	5.5 ± 0.6

KI** was defined as ratio of initial bacterial number at zero time and bacterial number after 2 h and 24 h incubation with macrophages.
The table shows the average of three independent experiments (± SEM). Alveolar macrophages used for each experiment were collected from five animals. The asterisk indicates statistically significant difference compared to saline control ($p < 0.01$)

alveolar macrophage population was engaged in the phagocytic process at the initial time point (3 h after challenge), while in the control rats this value was only 10%. Further at 24 h and 72 h after challenge the percent phagocytosis in lentinan treated group was 81% and 89% versus 53% and 68% for the control group.

2.2. Bactericidal activity of lentinan stimulated macrophages

The results of alveolar macrophage response to intranasal treatment with lentinan in rats was assessed by production of RNI and "killing" ability test against *M. tuberculosis*. In the Table the effects of lentinan in comparison to the control (saline) after intranasal administration in rats are summarized. In contrast to saline control groups, macrophages collected from the lungs of animals previously inoculated with lentinan were greatly stimulated as assessed by *in vitro* tests of nitrite production (16.1 ± 2.3* versus 9.5 ± 1.9 for saline control without addition of *Mycobacteria* and 27.5 ± 4.8* versus 15.8 ± 3.8 for saline with addition of *Mycobacteria in vitro*). Macrophages from lentinan treated rats showed increased killing ability against *M. tuberculosis in vitro*. "Killing" index values found after 24 h incubation were 13.3 ± 2.4* for lentinan activated alveolar macrophages and 5.5 ± 0.6 for saline treated macrophages.

3. Discussion

Biologically active branched beta-glucans and especially lentinan have been of special interest, because of their ability to stimulate non-specific protection against viral, bacterial, fungal and parasitic infection (Rasmussen and Seljelid 1991). They exert an adjuvant effect when used with antibiotics and vaccines and strengthen mucosal immunity (Sakurai et al. 1992). Immunotherapy with lentinan as an adjunct to conventional chemotherapy may represent an important addition to the modern treatment of pulmonary tuberculosis.

Based on the ability of lentinan to activate alveolar macrophages (Markova et al. 2002), our investigations were focussed on the effects of lentinan on the early interactions between *M. tuberculosis* and alveolar macrophages *in vivo* and the initiation of host cell mycobactericidal response. The electronmicroscopical observations indicated that after intranasal administration of lentinan, alveolar macrophages showed typical signs of macrophage surface activation-formation of undulating ruffled membrane, microvilli and pseudopodia, as well as cytochemically manifested activation of acid phosphatase. As was demonstrated by electron and light microscopy, the rate of *M. tuberculosis* uptake by lentinan activated alveolar macrophages and the percent of phagocytosis-engaged alveolar macrophage population were apparently enhanced. Phagolysosomal fusion labeled with acid phosphatase localization in the lysosomes and phagolysosomes, and destruction of *Mycobacteria* were seen early in lentinan-treated rats. In contrast, these processes were not observed at the same time points and the phagocytosis was still at the beginning stage in the control group. Because the survival of *M. tuberculosis* is linked to evasion of phagolysosomal fusion (Hickey 2000) the ability of antitubercular drugs to stimulate the phagosome-lysosome fusion is of special importance. Following the entry into the alveolar macrophages, *Mycobacteria* may be destroyed by alveolar macrophages, in which case no real infection will take place, or alternatively they may be not immediately killed (van Crevel et al. 2002).

In the second case, *Mycobacteria* use these host cells as an intracellular niche, avoiding destruction by lysosomal enzymes, ROI (reactive oxygen intermediates) and RNI (reactive nitrogen intermediates) (Ulrichs and Kaufmann 2003). *M. tuberculosis* can persist in a dormant state for a long period of time and when the balance between pathogen and host immune system is disturbed, an active infection can be established (Ulrichs and Kaufmann 2003).

In our experiments alveolar macrophages were shown to increase nitrite production after treatment with lentinan, which correlated with the increased bactericidal activity of these cells against *M. tuberculosis in vitro*. Nitrite production (e.g., indicative of nitric oxide production) by alveolar macrophages following *in vivo* treatment with lentinan was determined in response to stimulation *in vitro* with *M. tuberculosis*, or without stimulation. In both cases, the production of nitrite was higher after lentinan application then that in the control group. Mechanisms of mycobacterial killing include the ability of macrophages to produce ROI and RNI, as well as to enhance the activity of digestive lysosomal enzymes (Adams et al. 1997; Chan et al. 1992; Cooper et al. 1990; Sturgill-Koszycki et al. 1994; van Crevel et al. 2002). The generation of nitric oxide by activated macrophages is believed to be involved in the control of mycobacterial infection in the murine system (Chan et al. 1992) and it is induced upon stimulation of the macrophages with cytokines such as gamma interferon (Ding et al. 1988; 1988; Stuehr and Marletta 1987). Lentinan has been shown to act *in vivo* as a multicytokine inducer. Secretion of cytokines such as IL-1, CSF, MIF, IL-3, IL-6 and IFN γ is stimulated by lentinan (Maeda and Chihara 1999).

Observations have been made for beta-glucans activated macrophages infected *in vitro* with *M. tuberculosis*, where

the growth of *Mycobacteria* was inhibited probably due to cellular stimulation, or competitive inhibition of uptake of bacteria via CR3 receptors (Hetland and Sandven 2002). The studies of some authors (Abel et al. 1989; Cropz and Austen 1985; Oka et al. 1996) provide data about the ability of lentinan to bind to monocytes and macrophages, through beta-glucan or CR1 and CR3 receptors and suggest that Lentinan triggers macrophage activation through receptor-mediated mechanisms.

Our results suggest, that lentinan-mediated activation response of alveolar macrophages involves increases in the phagocytic effectiveness, lysosomal acid phosphatase activity and production of RNI. Activated macrophages kill intracellular *Mycobacteria* faster than unstimulated ones, probably by combination of mechanisms including prevention of microbial escape from phagolysosomes and enhanced phagolysososmal fusion.

The activation of alveolar macrophages is obviously due to mechanisms taking place *in vivo*, before challenge with the whole organisms, reacting to stimulation with lentinan. The triple intranasal application of lentinan resulted in the effective killing of *Mycobacteria* and this could be explained generally by lentinan's ability to enhance cell-mediated immune response in which macrophages play an important role.

In conclusion, the modulation of the early *M. tuberculosis*-alveolar macrophage interactions by lentinan may have a potential impact on the subsequent pathogenesis, and development of tuberculosis.

4. Experimental

4.1. Lentinan

Lentinan used in this study was obtained from Ajinomoto Co.Tokyo (Japan). Before use, lentinan was dissolved in saline.

4.2. Bacterial strain

Mycobacterium tuberculosis strain 5143 was isolated from patient sputum and kindly provided by Microbiology Laboratory, Sofia State Hospital for Tuberculosis. The strain was cultivated on Löwenstein-Jensen medium, following standard technique – for 45 days at 37 °C. The strain was mouse passaged prior to its use in the experiment.

4.3. Experimental animals

Male and female Wister rats weighing 200–250 g, (10–12 weeks old) were used. The animals were obtained from National Center for Laboratory Animals, Sofia. The experiments were performed with permission of Bulgarian Animal Use Committee.

4.4. Experimental infection

A total of 30 rats were divided into two groups. The first group was inoculated intranasally (i.n.) with lentinan, 3 times at 2 days intervals with a dose of 1 mg/kg starting from 6 days before bacterial infection. An application of this dose was not lethal and non toxic for rats. The second group (control) was i.n. given 0.2 ml of saline at the same intervals. Both groups of rats were infected i.n. with 2×10^8 CFU of *M. tuberculosis*. Rats were decapitated after a slight ether anesthesia at 3, 24 or 72 h after infection and samples of broncho-alveolar lavage fluid were obtained for light and electron microscopical study.

4.5. Electron microscopy and cytochemical acid phosphatase detection

The examination included samples of bronchoalveolar fluid, taken at the intervals indicated above. The cellular elements were isolated in ice-cold 0.85% NaCl, centrifuged for 20 min at 1500 rmp, washed in 0.1 M cacodylate buffer supplemented with 0.1% $MgSO_4$ and 4.5% sucrose, pH 7.4, and fixed in 2.5% glutaraldehyde in the same buffer for 2 h at 4 °C. After washing with the same buffer, the macrophages were incubated for 1 h, at 37 °C, in medium containing 3% *p*-nitrophenylphosphate (Merck), 0.12% lead nitrate and 7.5% sucrose, dissolved in 0.2 M acetate buffer, pH 5.5. Cytochemical controls were incubated with the omission of substrate *p*-nitrophenylphosphate or incubated in full medium with addition of 0.01 M NaF as inhibitor. The samples were post-fixed in 1% OsO_4 in symm-kollidin buffer (Fluka), pH 7.2 for 2 h at 4 °C. After short-time dehydration in graded ethanol series and propylene oxide, the cells were embedded in Epon-Araldite (Fluka) for polymerization at 56 °C, 48 h. Ulthrathin sections were cut on a Reichert-Jung Ultracut microtome, stained with uranylacetate and lead citrate, and observed with a Zeiss 10C electron microscope at 60 kV.

4.6. Evaluation of phagocytosis in vivo

The uptake of mycobacteria *in vivo* was controlled quantitatively by light microscopic determination of "percent phagocytosis". Percent phagocytosis was the percentage of alveolar macrophages containing one or more bacteria attached or ingested. Smears of bronchoalveolar fluid samples were prepared at all time points and stained by Ziehl-Neelsen and the number of alveolar macrophages containing at least one bacterium in total 100 alveolar macrophages was counted.

4.7. Harvesting of alveolar macrophages

For *in vitro* tests were harvested broncho-alveolar cells by a standard lavage procedure of rat lungs following treatment with lentinan (first group), or with saline (second group-control). After centrifugation, the cells were washed, resuspended in saline and counted by a Cobas Helios cell counting system (Hoffman La Roche). The alveolar macrophages were enriched by adherence to plastic. Non adherent and dead cells were removed by washing with PBS. Purity of the cells (> 85%) was confirmed by α-naphthyl-acetate esterase staining. Viability of the cells (> 90%) was determined by trypan blue dye exclusion.

Alveolar macrophages used for *in vitro* experiments were collected from 5 animals in each group. Experiments were repeated three times.

4.8. "Killing" ability test in vitro

"Killing" ability test of alveolar macrophages against *M. tuberculosis* was carried out according to the method of Vissar et al. (1995). A mixture of 1×10^6 macrophages/ml and 1×10^7 bacterial cells/ml were incubated in a volume of 500 µl with rotation at 37 °C in separate tubes for each time point. At time zero and after 2 h and 24 h, 550 µl of ice-cold saline was added to the suspension to stop phagocytosis. Killing indicated by the decrease in the total number of bacteria was assessed by adding 100 µl samples of bacterium – cell suspension to 1000 µl of ice-cold distilled water and after freezing and thawing the suspension was mixed for 1 min to lyse the cells. The number of viable bacteria was determined by plating 10-fold serial dilutions on Löwenstein-Jensen medium. "Killing" index (KI) was defined as ratio of initial bacterial number at time zero and the bacterial number after 2 and 24 h incubation with alveolar macrophages. Experiments were repeated three times.

4.9. Detection of reactive nitrogen intermediates (RNI)

The production of RNS was measured in alveolar macrophages obtained from the lentinan treated rats and from the control animals. Macrophages (1.5×10^6 per well) were added to wells of a 6-well tissue culture plates and were incubated for 45 min at 37 °C to allow adherence of macrophages to the plate surface. Nonadherent cells were then removed by rinsing the plates with PBS. Macrophages were infected with *M. tuberculosis* at a multiplicity of infection of 10 : 1. The production of RNS was detected by quantitating the amount of nitrite released by macrophages in obtained incubation mixtures using the Griess reagent (Stuehr and Marletta 1985).

Culture supernatants were centrifuged (1500 rpm, 10 min, 4 °C). One hundred microliters of supernatant were mixed with 100 µl of the Griess reagent and incubated at 25 °C for 10 min. The OD_{550} was read, and the nitrite values were calculated using a standard curve prepared by using $NaNO_2$. Triplicate samples were examined at 24 h postinfection with *M. tuberculosis* in two independent experiments and the values were then expressed as mean and standard errors of the mean. The results are expressed as µM released nitrite.

4.10. Statistical analysis

The results were expressed as mean values ± SEM. In order to assess the significance of differences within experiments Student's t-test was used. Statistical significance was defined as $p < 0.05$. Experiments were repeated three times.

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