J. Microbiol. Biotechnol. (2010), **20**(11), 1585–1591
doi: 10.4014/jmb.1003.03013
First published online 11 September 2010

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Immunological and Pathological Aspects of Respiratory Tract Infection with *Stenotrophomonas maltophilia* in BALB/c Mice

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Received: March 6, 2010 / Revised: June 27, 2010 / Accepted: July 26, 2010

A comprehensive study on the production of inflammatory mediators in the lungs of BALB/c mice following infection with *Stenotrophomonas maltophilia* was conducted. The levels of pro-inflammatory cytokines, tumor necrosis factor alpha (TNF-α), and interleukin-1β (IL-1β) were raised in the lungs of infected mice compared with control. The production of anti-inflammatory cytokine IL-10 was slightly delayed. Its peak level was on the 2nd day, whereas the peak of pro-inflammatory cytokines was observed on day 1 after intranasal challenge. This was accompanied by a rise in myeloperoxidase (MPO) and malondialdehyde (MDA) on day 1. The increase in MPO levels matched with histopathological observations, as neutrophils infiltration was detected on the first day. Alveolar macrophages (AMs) obtained from infected animals showed a higher rate of uptake and killing when exposed to bacteria *in vitro*, compared with similar experiments conducted with AMs from normal mice (control). This suggests that AMs were more efficient in cleaning the bacteria. The nitric oxide (NO) production however started early during infection but reached its maximum on the 3rd day. No mortality was observed among the infected animals, and infection was resolved by the 5th day post infection. No drastic changes in the lung tissue were observed on histopathological examination.

Keywords: Pro-inflammatory, anti-inflammatory, cytokines, *Stenotrophomonas maltophilia*, innate immunity

Stenotrophomonas maltophilia is a Gram-negative bacterium that is widespread in the environment. This organism has become important in recent years as an emerging opportunistic pathogen associated with nosocomial colonization and infection [5, 13, 22]. *S. maltophilia* is frequently isolated from clinical specimens and is implicated in catheter-related bacteremia and septicemia, urinary and respiratory tract infections, and endocarditis [5, 13, 22]. Infections occur in cystic fibrosis and burn patients and are common in individuals with impaired defenses who are susceptible to opportunistic infections. Several case control studies have drawn conflicting conclusions regarding the role of *S. maltophilia* in the pathogenesis of the infection process [7].

Activation of the innate immune response by bacteria is mediated by a group of molecules, such as lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acid, lipoarbinomannan, lipoproteins, and lipopeptides. The interaction between pathogen-associated molecular patterns of these molecules and pattern recognition receptors (*e.g.*, TLRs on resident airway macrophages and epithelial cells) result in the release of proinflammatory cytokines including tumor necrosis factor alpha (TNF-α) and interleukin-lβ (IL-1β) [9, 2, 23]. These cytokines recruit leukocytes and enhance the bactericidal activity of alveolar macrophages (AMs). The innate immunity and adaptive immunity are also activated by this process [15].

The inflammatory response in its controlled state has been shown to be beneficial to the host. On the contrary an exaggerated response can be detrimental to the host. Strain to strain variation in the generation of an inflammatory response in different animal models has been attributed to the difference in genetic factors and host responses [26]. These responses accordingly can affect the host in a positive or negative manner.

Myeloperoxidase (MPO) is a peroxidase enzyme most abundantly present in neutrophil granulocytes. It is a lysosomal protein stored in azurophilic granules of the neutrophil. The MPO assay was used to determine the levels of neutrophil infiltration into the lung [21]. Many investigators have found close relationship between the histological changes and MPO level, and that was very clear in flagellated bacteria as compared with aflagellated strains [12]. Malondialdehyde (MDA) is an organic compound, the structure of which is very complex. It occurs naturally and is a marker for oxidative stress. The level of

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MDA corresponds to some clinical disorders. For example, the high level of MDA was observed in corneas of patients suffering from keratoconus and bullous keratopathy [3]. MDA can also be found in tissue sections of joints from patients with osteoarthritis [27].

We provide evidence to support this observation on the basis of results obtained in an intranasal infection model in BALB/c mice, and compare these results with a recently published report from our laboratory in which LACA mice were used as a model [24].

Materials and Methods

Bacterial Isolate

An *S. maltophilia* (Sm2) clinical strain was used in this study. The isolate was a blood culture isolate, diagnosed and identified in PGI Institute, Chandigarh, India. Bacteria were preserved by lyophilization and were routinely cultured at 37°C on Luria–Bertani agar plates. Subcultures were made every week.

Mouse Model of Respiratory Tract Infection

Male BALB/c mice (4–6 weeks old, weighing 15–20 g) procured from the central animal house of Panjab University, Chandigarh, India were infected with *S. maltophilia*. The animals were kept in clean polypropylene cages and fed on standard antibiotic-free diet (JBD Agencies, Pvt. Ltd., India). The study was conducted following approval from the animal ethics committee of Panjab University, Chandigarh, India. Briefly, animals were given an inoculum of 10^9 CFU in 50 µl of phosphate-buffered saline (PBS) (0.2 M, pH 7.2), intranasally. To facilitate movement of inoculum to Lungs, mice were held in a vertical position for 2 min [29].

Experimental Protocol

Out of a group of 32 infected mice, 4 mice each were sacrificed at zero time, 4 h, 12 h, 1 day, 2 days, 3 days, 5 days, and 7 days post bacterial infection. Lungs were sampled to determine the bacterial count, quantify inflammatory mediators, and determine macrophage activity, and for histopathological examination of lung tissue.

The control group consisted of 24 mice, which were given PBS (0.2 M, pH 7.2) intranasally, with 3 animals being sacrificed at each time point. An additional 10 unsacrificed infected animals were used to monitor the death rate.

Bacterial Count

For bacterial quantitation, a standard plate count method was used. Lungs were homogenized in 1 ml of sterile normal saline. Serial 10-fold dilutions were made from the homogenate, and 0.1 ml of selected dilutions was plated on Luria–Bertani agar and incubated overnight at 37°C. On the following day, colonies were counted to determine the lung bacterial load and reported as bacterial count per gram of lung tissue.

Inflammatory and Anti-Inflammatory Mediators

Whole lungs were weighted and then homogenized in 3 ml of lysis buffer containing 0.5% Triton X-100, 150 mM NaCl, 15 mM Tris, and 1 mM MgCl (pH 7.4), using a tissue homogenizer. The homogenates were incubated in ice for 30 min and then centrifuged at 2,500 ×*g* for 10 min. The supernatant was collected, passed through a filter with 0.45 µm pore size (Polylabo, Strasbourg, France), and stored at –60°C for cytokine assay [18].

Cytokine Assay

TNF-α, IL-1β, and IL-10 levels were detected in lung homogenate supernatants of control and infected animals at different time intervals following bacterial infection. Mouse enzyme-linked immunosorbent assay kits were used to measure all the cytokines as per the manufacturer's instructions (BD OptEIA, San Jose, CA, USA and ANTIGENIX AMERICA Inc., Hunting sta., New York, USA).

Myeloperoxidase

Lungs were weighted and homogenized in 0.5 ml of 50 mM sodium phosphate, pH 6.0, containing 0.5% hexadecyltrimethylammonium bromide. Samples subsequently underwent multiple freezing and thawing and then sonication with a sonic dismembrator (Misonix Incorporated) set at 30%. Samples were cleared by centrifugation at 15,000 ×*g* for 15 min, and neutrophil myeloperoxidase activity was determined by incubating 50 µl of homogenate with 0.167 mg/ml *o*-dianisidine dihydrochloride and 0.0005% H_2O_2 in a reaction volume of 1.5 ml. Absorbance was determined at 460 nm and the activity calculated [10].

Measurement of Nitric Oxide (NO)

To confirm NO production in the lungs, the stable end product of the nitric oxide reaction was measured in the form of nitrite level in the lung homogenate by the colorimetric method of Tsai *et al.* [28]. To 0.1 ml of tissue sample taken in a tube, 0.4 ml of PBS and 2.0 ml of Griess reagent were added. Griess reagent containing 0.1% *n*-1-naphthylethylenediamine dihydrochloride was mixed in equal volume (1:1) with 1% sulfanilamide in 5% phosphoric acid. Then 2 ml of 10% trichloroacetic acid was added and the mixture was vortexed and incubated at room temperature for 20 min. The mixture was centrifuged for 15 min at 14,000 ×*g* for 15 min and the absorbance of the supernatant was taken at 540 nm.

Malondialdehyde (MDA)

MDA is a metabolite resulting from lipid peroxidation, which was detected by the method of Ohkawa *et al*. [19] with slight modification. Briefly, 0.2 ml of lung homogenate supernatant was added to 0.2 ml of 8.1% sodium dodecyl sulfate, 4 ml of 1:11 H_2SO_4:H_2O (v/v), and 1.5 ml of 0.8% thiobarbituric acid (made in distilled water). The mixture was heated for 1 h at 100°C, and then 1 ml of water and 5 ml of butanol–pyridine [15:1 (v/v)] were added and the tubes were vigorously shaken. The upper organic phase was obtained after centrifugation for 10 min at 4,000 ×*g* and the absorbance was measured at 532 nm.

Protein Determination

The total protein content of the lung homogenate supernatant was determined by the Folin phenol method of Lowry *et al.* [14]. Briefly, 5 ml of working solution (working solution: 98 ml of 2% sodium carbonate in 0.1 N sodium hydroxide+1 ml of 1% $CuSo_4 \cdot 5H_2O$+1 ml of 2% sodium potassium tartrate) was added to 0.1 ml of sample and incubated for 10 min at room temperature. At the end of the incubation period, 0.5 ml of 1 N Folin reagent was added and mixed immediately. Tubes were incubated at 37°C for 30 min and the optical density was taken at 660 nm. A blank of distilled water was

also processed in the similar manner. A standard curved was prepared by using graded concentrations of BSA (10−500 μg/ml) to quantitate protein levels in the samples.

Phagocytosis

Phagocytosis was performed according to the method of Allen *et al*. [1]. Bacteria were harvested and suspended in PBS (0.2 M, pH 7.2) so as to obtain an optical density corresponding to 10^5 CFU/ml. Alveolar macrophages were obtained by the method of Sugar *et al.* [25] and their suspension was made in RPMI 1640 (High Media Laboratories Ltd., Mumbai, India). To check the uptake of bacteria, the normal mouse serum, macrophage cell suspension (10^4 cells/ml), and bacterial suspension (10^5 CFU/ml) were taken, vortexed, and incubated at 37°C in the presence of 5% CO_2. Aliquots were taken regularly at 0, 30, and 60 min of incubation and centrifuged. The viable count of bacteria in the supernatant was determined by plating appropriate serial dilutions on nutrient agar plates. The results were expressed as viable bacteria (CFU/ml) taken up by macrophages at respective sampling time intervals. For intracellular killing, a bacterial suspension (10^5 CFU/ml) was mixed with normal mouse serum and kept for 30 min at 37°C. Macrophages (10^4 cells/ml) were added to the above bacterial suspension, incubated and centrifuged. The cells were lysed at different time intervals (0, 1, 2, and 3 h) and different dilutions were plated on LB agar plates. The colony forming units (CFU) were counted after overnight incubation at 37°C.

Histology

Formalin-fixed, paraffin-embedded lung sections from two mice each were prepared after sacrificing at 4 h, 12 h, 1 day, 2 days, 3 days, and 5 days post infection. The sections were stained with hematoxylin and eosin for light microscopic examination [17].

The tissues were washed in distilled water for 30 min to remove the formalin. Then the tissues were dehydrated in different grades of alcohol (*i.e*., 70%, 80%, 90%, and 100%, or absolute alcohol) for 30, 40, 45, and 60 min, respectively. Tissues were then kept in toluene twice for 1 h and 40 min, respectively. These were dipped in molten paraffin wax. The wax was quickly cooled to prevent crystallization. Then 4−6 μm thick sections of the tissues were cut with a fine razor attached to a Spencer microtome. Wax-embedded tissue sections were kept in a water bath at 50°C to remove the wax. Sections were picked on separate slides coated lightly with Mayer's adhesive fixative. The sections on slides were dried by keeping in a hot air oven at 56°C for 20 min. The sections were then stained with hematoxylin and eosin stain and examined.

Statistical Analysis

All values were taken as the mean value and standard deviation calculated. The differences were analyzed by using Student's *t* test employing Origin 6.0 version Software. A value of $P<0.05$ was considered to be statistically significant.

RESULTS

Bacteriological Quantity

The results of the bacterial count in the lung homogenates of BALB/c mice after infection with *S. maltophilia* are shown in Fig. 1. Maximum bacterial count was achieved after one day of infection. Thereafter, the bacterial count started to decline dramatically and was cleared by the 5th day post infection. The bacterial isolate was cleared from the mice lungs within five days after bacterial infection. The present study proved that *S. maltophilia* is a low virulent bacteria, and the respiratory tract of normal mice will be cleared from bacteria by the normal activity of the immune system of the mucosal layer.

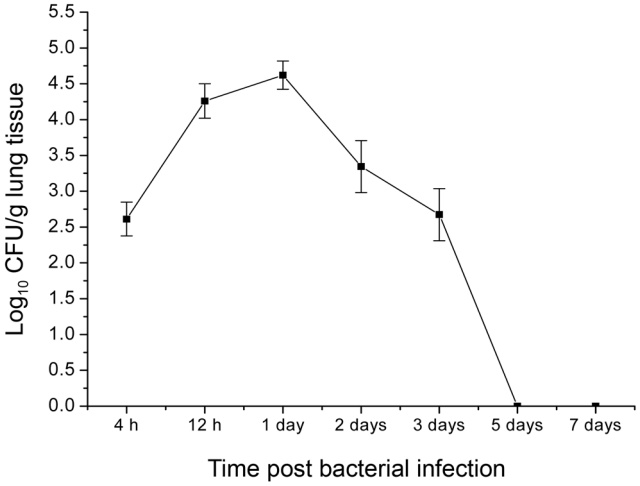

Fig. 1. Viable bacterial count ($\log_{10}$ CFU/g) in the lung homogenates of animals sacrificed at different time intervals post infection.

BALB/c mice were infected with 10^9 CFU in 50 μl (PBS) of *S. maltophilia* (Sm2) *via* the intranasal route. The presence of *S. maltophilia* was as early as 4 h post infection. The maximum bacterial count was at 24 h post infection. At the 5th and 7th days, the mice lungs were completely cleared from bacteria. Each value represents the mean±standard error of four independent experiments.

Pro-Inflammatory Cytokines

A significant increase in the levels of IL-1β and TNF-α was observed in the lung homogenates as early as 4 h post bacterial infection (Table 1). Their maximum level was seen on day 1, after which levels decreased with time. No significant difference was seen in their levels on the 5th and 7th days post infection compared with control. The infection with *S. maltophilia* stimulates the mucosal cells of the respiratory tract to produce the pro-inflammatory cytokines and these findings are concomitant with the bacterial count in mice lungs.

Anti-Inflammatory Cytokine

The IL-10 levels in lung homogenates are presented in Table 1. The level of this cytokine increased with time, and maximum elevation was detected after 2 days post bacterial infection. The level of IL-10 declined thereafter and came to its normal level by the 5th day. The level of anti-inflammatory cytokine has a negative relation with the level of pro-inflammatory cytokines.

Table 1. Levels of pro-inflammatory (IL-1β and TNF-α) and anti-inflammatory (IL-10) cytokines in the lung homogenates of BALB/c mice at different time intervals post infection with *S. maltophilia*.

	Control	Zero time	4 h	12 h	1 day	2 days	3 days	5 days	7 days
IL-1β pg/g lung tissue	131.4 ±11.7	139.6 ±13.79 NS	1,363.3 ±255.4 *P*<0.005	1,933.3 ±454.8 *P*<0.005	2,078.3 ±343.6 *P*<0.001	807 ±118.7 *P*<0.005	406.6 ±162.89 *P*<0.05	156.6 ±51.6 *P*>0.05 (NS)	142.6 ±34.07 *P*>0.05 (NS)
TNF-α pg/g lung tissue	109.3± 12.3	103.6 ±15.17 NS	398.8 ±59.5 *P*<0.005	521.8 ±80.46 *P*<0.001	548.1 ±75.2 *P*<0.001	287.3 ±21.9 *P*<0.001	200 ±10 *P*<0.001	126.7 ±38.7 *P*>0.05 (NS)	109 ±19 *P*>0.05 (NS)
IL-10 pg/g lung tissue	69.1± 8.4	65.6± 6.02 NS	76± 7.54 *P*>0.05 (NS)	87.57± 11.11 *P*<0.05	114.66± 6.19 *P*<0.005	172.3± 17.61 *P*<0.001	161.26± 32.79 *P*<0.01	70.63± 18.18 *P*>0.05 (NS)	52.13± 12.93 *P*>0.05 (NS)

All data represent mean±standard error of four independent experiments. *$P<0.05$, compared with the control (PBS-treated group).

Myeloperoxidase

The MPO activity was checked in the lung homogenates of mice post bacterial infection as an indicator of neutrophil infiltration in lung tissue. The results presented in Fig. 2 show significant MPO activity after 12 h, which remained elevated up to the third post-infection day. The high level of MPO was combined with the maximum level of bacterial count of *S. maltophilia* in mice lungs.

Malondialdehyde

The extent of tissue destruction in experimental mice was assessed on the basis of MDA estimation. Following infection, a significant increase in MDA activity was detected after the 12th h, with its peak on day 1 (Fig. 3). The MDA levels declined thereafter and reached the normal level by the 5th day. The elevation of MDA was related with the bacterial survival in mouse lung.

Nitric Oxide

Fig. 4 shows the nitrite level in mouse lungs on different post infection days. The level of nitrite concentration increased with time. The maximum level of nitrite was on the third post-infection day and then the nitrite level declined sharply. No significant difference was seen in its level on the 5th day compared with control. The increase in NO level was not related with bacterial activity but found after bacterial decline. This indicator proves the role of immunological reactions in stimulating the NO secretion.

Phagocytic Activity of Alveolar Macrophage

The alveolar macrophage uptake was checked *in vitro*. The macrophages were obtained from mice suffering from *S. maltophilia* (Sm2) induced pneumonia at different time intervals post bacterial infection. The results in Fig. 5 show that the rate of engulfment of bacteria was significantly high

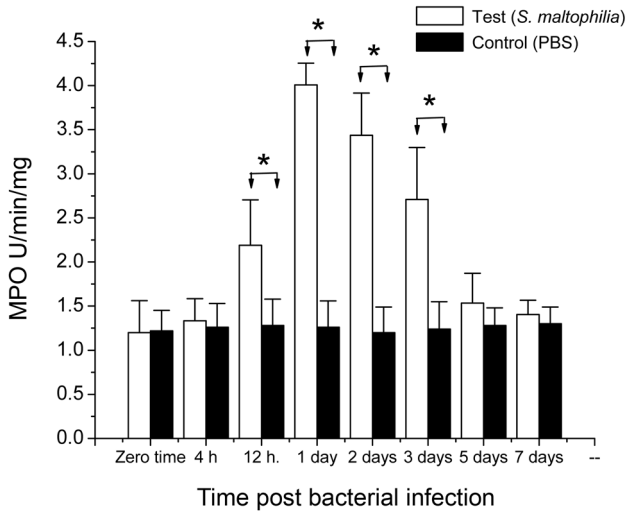

Fig. 2. Myeloperoxidase (MPO) levels in lungs homogenates of mice at different time intervals following infection with *S. maltophilia*.
*Significantly different from control (PBS-treated group) ($P<0.05$). All data represent mean±standard error of four independent experiments.

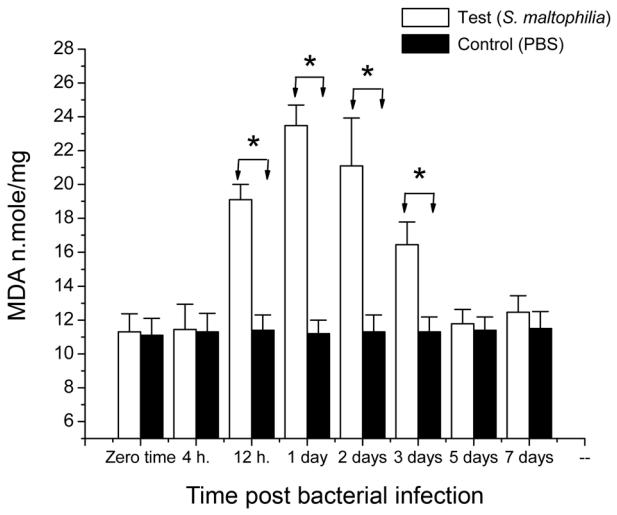

Fig. 3. Malondialdehyde (MDA) levels (n.mol/mg protein) in lung homogenates of mice at different time interval following infection with *S. maltophilia*.
*Significantly different from control (PBS-treated group). ($P<0.05$). All data represent mean±standard error of four independent experiments.

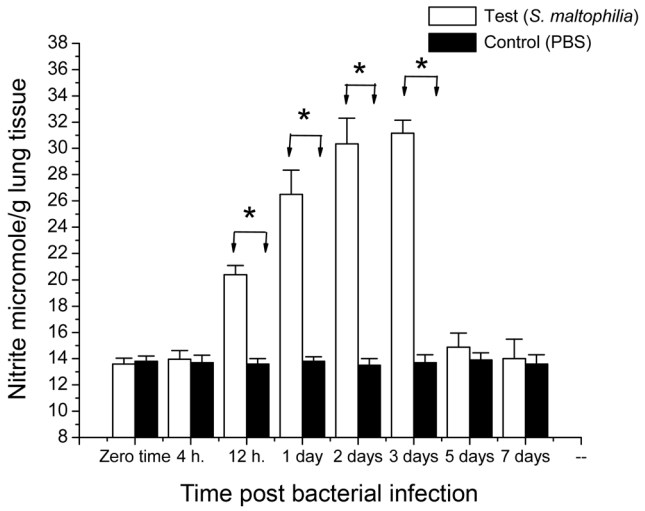

Fig. 4. Nitrite levels (μmol/g) in lung homogenates of mice (BALB/c) with *S. maltophilia* pneumonia.
*Significantly different from control (PBS-treated group) ($P<0.05$). All data represent mean±standard error of four independent experiments.

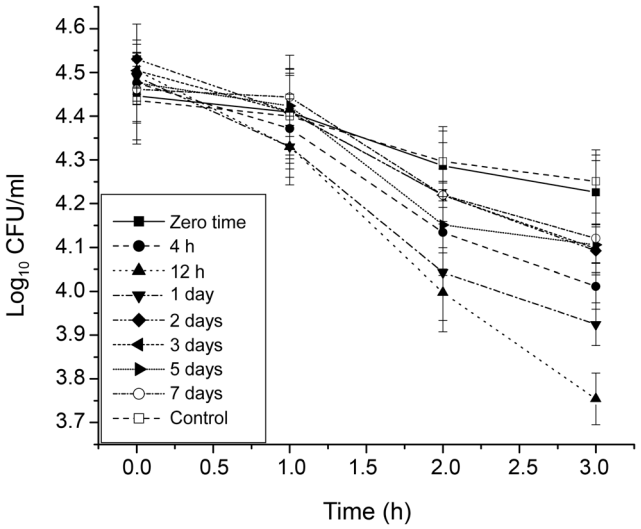

Fig. 6. Intracellular killing by AMs harvested at different time intervals following bacterial infection.
The significant killing ($P<0.05$) was found in all cases compared with control (PBS-treated group) except at zero time. All data represent mean± standard error of four independent experiments.

in all cases compared with the control ($P<0.05$). The maximum bacterial uptake was seen by AMs obtained from animals during the initial stage of infection (4 h, 12 h, and 24 h) and the minimum when macrophages were obtained from 7-day-old infected mice (Fig. 5).

Similar trends in intracellular killing of *S. maltophilia* by AMs were observed. AMs obtained from infected animals on different intervals of post infection days showed increased intracellular killing compared with the control ($P<0.05$). The maximum intracellular killing was observed by AMs obtained at 12 h of bacterial infection. The minimum intracellular killing was seen by AMs obtained on the seventh post-infection day (Fig. 6).

The macrophage activity had a close relation with bacterial survival in mouse lung, and the highest activity of macrophage was found as early as the maximum number of bacteria in lung.

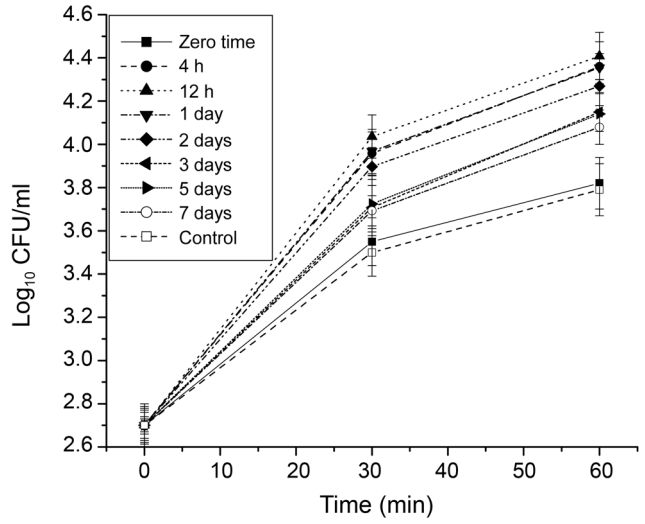

Fig. 5. Phagocytic uptake of *S. maltophilia* (Sm2) by AMs obtained at different time intervals post *S. maltophilia* intranasal administration in BALB/c mice.
Significant uptake of *S. maltophilia* ($P<0.05$) was found in all cases compared with control (PBS-treated group) except at zero time. All data represent mean±standard error of four independent experiments.

Histopathological Examination

Histopathological examination of lung tissue of BALB/c mice intranasally infected with *S. maltophilia* (Sm2) showed normal pathology in the lungs of animals obtained after 4 h of infection (Fig. 7a). The alveoli maintained zigzag walls and alveolar septa were thin. By 12 h post infection (Fig. 7b), small foci of inflammation along with a zone of neutrophils outside the bronchi were seen. On day 1 (Fig. 7c), medial focus inflammation and neutrophils infiltration were detected outside and inside the bronchus. On the 2nd and 3rd days, neutrophils infiltration outside the bronchi was reduced (Fig. 7d and 7e). In the control (Fig. 7f), a normal histological picture of lungs was seen. On the 5th day, no histological changes were seen. On all different days, the alveoli and bronchi were normal. The histological study proved the low pathogenisity of *S. maltophilia* in normal mouse lung.

DISCUSSION

In all reports from different geographical areas, the respiratory tract is reported to be the common site of infection with *S.*

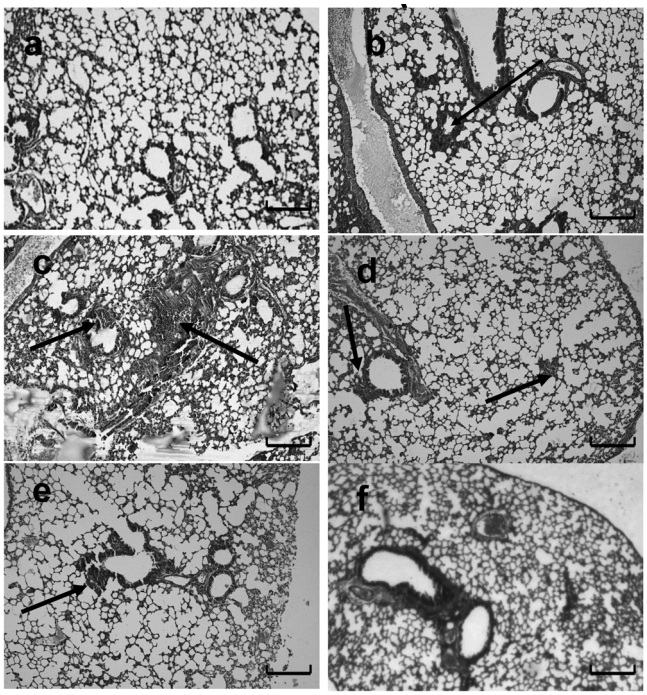

Fig. 7. Histopathological examination of mouse (BALB/c) lung obtained at different time intervals post bacterial infection.
a: at 4 h; b: at 12 h; c: on 1st day; d: on 2nd day; e: on 3rd day; f: control (PBS -treated group) (Bars, 200 μm).

maltophilia [19, 22]. Using a mouse model of pneumonia in BALB/c mice, in this study we were able to show that this clinical isolate has the ability to establish itself in the respiratory tract of an immunocompetent host. However, early clearance of this organism from the lungs of infected animals without any treatment shows an inability of this organism to produce invasive disease, though it can adhere to epithelial cells [4]. These results confirm the earlier observation made in patients suffering from *S. maltophilia* infection [19]. According to this clinical study, the presence of *S. maltophilia* in the respiratory tract represents colonization rather than disease. It is likely that a small number of organisms in the respiratory tract does not go beyond colonization of the upper respiratory tract. However, a higher inoculum, as used in this study, may lead to persistence of the organisms in the lower respiratory tract of an immunocompetent host, which may be cleared with time. Although infection with *S. maltophilia* was cleared on its own, the presence of bacteria in the lungs did result in inflammation. A rise in MPO and MDA levels, along with pro-inflammatory cytokines (TNF-α and IL-1β) and NO, is suggestive of induction of the inflammatory immune response. Airway inflammation, the hallmark of pneumonia, is required to clear the bacteria from the airways, but accumulation of activated neutrophils and macrophages and their products can be detrimental to normal lung function. However, in this study, no drastic signs of inflammation were observed on histopathological examination of the lung tissue. On the contrary, when this organism was used to induce pneumonia in LACA mice, it did result in changes in the lungs, leading to interstitial pneumonia [24]. These results show that although both strains of mice showed persistence of the organisms in the lungs, the differences in histopathological changes observed in the lungs of the two different mouse strains may be attributed to a difference in the inflammatory response. This suggests that whether inflammatory response generated as a result of bacterial presence in the lungs will be detrimental to the tissue and contribute towards immunopathology of infection is dependent on the host genetic factors, as has been suggested earlier [26].

The results of the present and many previous studies suggest that *S. maltophilia* is a low virulent bacteria, in that it causes very little pathological disorders in the host but it stimulates the immune system strongly, which is why we suggest the pathological marker that associate with the infection with this bacteria belong to the immunopathological properties of respiratory tract following infection of *S. maltophilia* [22, 29]. The infection of *S. maltophilia* was marked with infiltration of PMN cells and this finding was related with a high level of MPO. The increase in pro-inflammatory cytokines for some time has a negative side effect, assigned by tissue problems and tissue stress that resulted in a high level of MDA [21, 27].

AM, on the contrary, showed an increased ability to engulf and kill bacteria *in vitro*, compared with AM from control animals. This may be attributed to the immunostimulation by bacterial antigens like lipopolysaccharide (LPS) and flagellin, which bind to specific receptors on AM (CD14 and TLR5) [6, 11]. This may lead to activation of macrophages with enhanced ability to phagocytose and produce nitric oxide. NO has been reported to be secreted by AMs, epithelial cells, endothelial cells, fibroblast, and lymphocytes [8, 30, 31]. The early release of NO in this study might have restricted the bacterial growth, as the role of NO as an effecter molecule in the killing of microorganisms has been observed [16, 28]. Since AMs are considered as the first line of defense, their increased antibacterial potential along with neutrophils influx might have helped the host to clear the organisms. These results suggest that *S. maltophilia* may act as a pathogen in hosts with altered macrophage function. This observation is supported by previous reports, as the incidence of *S. maltophilia* has been shown to be high in specific patient populations with comorbid illness such as cystic fibrosis, immunosuppression, organ transplant, and malignancies [5].

It can be concluded from this study that *S. maltophilia* can activate the innate immune system mediated by AMs, and that host genetic factors might be the contributory factor in preventing *S. maltophilia* to cause severe respiratory bacterial infection in an immunocompetent host.

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