Role of Pulmonary Alveolar Macrophages in Defense of the Lung against Pseudomonas aeruginosa

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Alveolar macrophages (AM) provide one of the first lines of defense against microbial invasion in the lower airways. The role of AM in the clearance of Pseudomonas aeruginosa in mice after intrapulmonary challenge was evaluated. AM were depleted by intranasal administration of liposome-encapsulated dichloromethylene diphosphonate. At 24 h following the instillation of liposomes, a sublethal dose of P. aeruginosa was inoculated intranasally. Spleen, liver, and lung tissue was then evaluated for viable bacteria and for histopathology. AM depletion of 78 to 88% did not affect the survival rate of infected mice or clearance of P. aeruginosa from the spleen, liver, or lung, compared to the control group, but the mice’s susceptibility to Klebsiella pneumoniae was greatly enhanced. The recruitment of neutrophils to the lung was also not affected. Freshly explanted AM were not competent to phagocytose unopsonized P. aeruginosa but were able to phagocytose zymosan particles. Further studies were conducted to assess the in situ phagocytic activities of AM. Three hours after the intranasal instillation of P. aeruginosa or other particles, bronchoalveolar lavage was performed. AM phagocytosis of zymosan particles and latex beads exceeded that of P. aeruginosa. Neutrophils were recruited to the lung in response to a high-dose bacterial challenge. These results suggest that AM do not play an important role in defense of the lung against P. aeruginosa.

Pseudomonas aeruginosa is a gram-negative, opportunistic pathogen that causes chronic infections in cystic fibrosis (CF) patients, immunocompromised individuals, and burn patients. Infection of the lung is the leading cause of morbidity and mortality in CF patients. Once acquired, this bacterium is difficult to treat and is rarely eradicated. Most strains of P. aeruginosa show substantial degrees of resistance to a wide variety of antimicrobial agents (12, 21).

Alveolar macrophages (AM), located at the interface between air and lung tissue, provide the first line of phagocytic defense against microbial invasion in the lower respiratory tract. Besides their phagocytic and microbialidical functions, AM also secrete numerous chemical mediators upon stimulation, thereby playing a role in regulating inflammatory reactions in the lung (14). The role of AM in defending the lung against various pathogens and in regulating inflammatory reactions has been assessed in studies in which AM were depleted. In rodent models, AM can be depleted to 5 to 30% of normal numbers by liposome-encapsulated dichloromethylene diphosphonate (LDMDP) delivered to the pulmonary tract (2, 4, 13, 15–17). The usual fate of liposomes is ingestion and digestion by macrophages. Due to its strong hydrophilic properties, DMDP is not able to escape from the cell when released intracellularly. Therefore, macrophages are selectively depleted without damaging other tissues or cells (27).

AM are the primary phagocytic cells in the uninflamed lower airways. Phagocytosis is categorized into opsonic and nonopsonic mechanisms. Opsonic phagocytosis is mediated by Fe and complement receptors. However, human and mouse AM show minimal expression of receptors for C3b/C3b (CR1/CR3/CR4). The receptors for immunoglobulin G, Fcγ receptors, are expressed in human AM but negligibly in mouse AM (3, 24).

Furthermore, the concentrations of complement and immunoglobulin in the lower airway are minimal; therefore, it is likely that AM utilize nonopsonic, instead of Fc or complement receptor-mediated, phagocytosis to eliminate pathogens such as P. aeruginosa from the lung. We and others have evaluated the in vitro response of macrophages to challenge with P. aeruginosa and other particles (1, 3, 8, 22, 29).

The main objective of this study was to further elucidate the in vivo role of AM in defense of the lung against P. aeruginosa challenge. AM were depleted by the intranasal (i.n.) instillation of LDMDP in mice. AM-depleted mice received a sublethal dose of P. aeruginosa or Klebsiella pneumoniae (as a positive control) by i.n. inoculation. The effects of AM depletion on bacterial clearance in spleen, liver, and lung and on polymorphonuclear leukocyte (PMN) recruitment to the lung were assessed. Secondly, the in vitro phagocytosis of unopsonized P. aeruginosa, zymosan, and latex beads by freshly explanted murine AM was compared to that by peritoneal macrophages (PM). Finally, assessment of the in situ phagocytic activities of AM was performed by challenging mice i.n. with unopsonized P. aeruginosa, zymosan particles, or latex beads and subsequently obtaining bronchoalveolar lavage (BAL) fluid to evaluate BAL cell numbers and the number of ingested particles per cell in the BAL-derived population.

MATERIALS AND METHODS

Bacterial strains and culture conditions. P. aeruginosa strain P1 is a nonmucoid, lipopolysaccharide (LPS)-rough derivative of a mucoid isolate obtained from a patient with CF. Strain c2908c was the initial colonizing isolate obtained from a CF patient. Strain PAK (LPS smooth) was obtained from W. Paranchych (Edmonton, Alberta, Canada), PA01 (LPS smooth) was from B. Holloway (Victoria, Australia), and FRD1 was from R. E. W. Hancock (Vancouver, British Columbia, Canada). K. pneumoniae (ATCC 43816) was purchased from the American Type Culture Collection (Manassas, Va.). Bacteria were kept frozen at −70°C in Mueller-Hinton broth with 8.0% dimethyl sulfoxide and grown fresh for each experiment. They were grown with shaking overnight in L broth at 37°C.

Animals. Specific-pathogen-free (SPF) male CD-1 mice weighing 35 to 37 g were obtained from Charles River Laboratories Canada (St. Constant, Quebec, Canada), and SPF female BALB/c mice weighing 20 to 22 g were obtained from...
In vivo phagocytosis assays. Anesthetized mice were challenged i.n. with 20 μl of *P. aeruginosa* (FRD1, P1, PAK, PA01, or c2908c), latex beads, or zymosan as described above. Three hours after instillation, BAL was performed. BAL-derived cells were centrifuged at 395 × g for 15 min at 4°C, resuspended, and treated with Diff-stained slides. The number of ingested bacteria was determined with a hemocytometer by trypan blue exclusion. Then 10^5 cells (in approximately 100 μl) were plated on acid-washed glass coverslips in 24-well plastic tissue culture trays. After 30 min of incubation at 37°C in 5% CO_2, 400 μl of F12K-10% FCS, 400 μl of F12K-10% FCS was added to the wells. After the addition of 15 μl of LPBS, LDMDP, or DMDP (600, 50, 5, and 0.5 μM), A549 cells were incubated at 37°C in 5% CO_2 for 24 and 48 h. At the end of these intervals, cells were washed twice with PBS fixed with methanol, stained with Giemsa, and mounted on microscope slides.

The morphology of untreated and treated epithelial cells was assessed microscopically.

### In vitro effects of LPBS, LDMDP, and DMDP on the morphology and phagocytic capacity of murine PM

Table 1 shows that substantially fewer bacteria were ingested by PM subjected to LDMDP than by control and LPBS-treated PM. The percentage of LDMDP-treated PM that ingested *P. aeruginosa* was lower than those of untreated and LPBS-treated PM.

### In vitro effects of LPBS, LDMDP, and DMDP on the morphology and phagocytic capacity of murine AM

These experiments were repeated three times, and the trend was identical for all. Due to large day-to-day biological variability, one representative experiment is shown (Fig. 2). In it, 100% of untreated (control) and LPBS-treated macrophages appeared to be healthy, whereas 61, 88, and 61% of AM showed unhealthy characteristics after incubation with LDMDP for 24, 48, and 72 h, respectively. All AM incubated with free DMDP for 24,
to 15% of cells incubated with 0.5 or 5 mM DMDP, 23 to 33% of cells incubated with 50 mM DMDP, and 80 to 85% of A549 cells incubated with 0.6 M DMDP).

In vivo depletion of AM by i.n. instillation of LDMDP. Figure 3 shows that 24 and 48 h after the i.n. instillation of 50 μl of LPBS or LDMDP, the number of AM recovered from LDMDP-treated mice was substantially lower than that recovered from LPBS-treated mice. At 72 h, the number of AM in the BAL from the LDMDP-treated group was slightly increased (data not shown). The histologic appearance of the lungs from LPBS- and LDMDP-treated mice was similar (data not shown).

Effects of AM depletion on P. aeruginosa clearance in vivo. There was no difference between AM-depleted and nondepleted BALB/c and CD-1 mice infected with P. aeruginosa in the clearance of viable bacteria (Fig. 4A through C). No viable bacteria were isolated from the spleens or livers of the infected mice at these time points (data not shown). The number of PMNs isolated from AM-depleted mice was not substantially different from that obtained from nondepleted mice for 5 days postinfection (data not shown). A histologic examination of the lungs of AM-depleted and control infected mice revealed increased numbers of PMNs compared to those of AM-depleted and control uninfected mice (data not shown).

Cytospin preparations of BAL cells from LPBS- and LDMDP-treated mice showed that recruited PMNs were competent to phagocytose P. aeruginosa. However, AM appeared not to have phagocytosed the bacteria (Fig. 5). Further studies with LPBS- and LDMDP-treated mice challenged i.n. with P. aeruginosa strain PA01 at various doses—1.32 × 10^7, 6.5 × 10^6, and 9 × 10^5 CFU—did not show any difference between the two groups of mice in bacterial clearance (data not shown).

Effects of AM depletion on K. pneumoniae clearance in vivo. Figure 4D shows a striking difference between AM-depleted and nondepleted BALB/c mice in the number of viable bacteria. Signs of disease were evident 48 h after the i.n. instillation of K. pneumoniae (100 CFU/mouse) in the LDMDP-depleted mice, while LPBS-treated mice appeared to be healthy. The spleens and livers of LDMDP-treated mice harbored high numbers of K. pneumoniae cells, but the same tissues from control animals were sterile.

In vitro phagocytosis of freshly explanted AM. Unopsonized P. aeruginosa was poorly ingested by freshly explanted murine AM from BALB/c and CD-1 mice in the presence or absence of D-glucose. Freshly explanted murine resident PM ingested unopsonized P. aeruginosa strain P1 only in the presence of D-glucose but ingested strain PAK with or without glucose.

### Table 1. In vitro phagocytosis of unopsonized P. aeruginosa by murine PM incubated with LPBS, LDMDP, and DMDP for 24 and 48 h

<table>
<thead>
<tr>
<th>Liposome</th>
<th>Avg. no. of ingested P. aeruginosa cells per macrophage after:</th>
<th>% of PM that ingested P. aeruginosa after:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>Control</td>
<td>19.7 ± 0.3</td>
<td>26.4 ± 6.5</td>
</tr>
<tr>
<td>LPBS</td>
<td>18.8 ± 1.5</td>
<td>27.4 ± 10.6</td>
</tr>
<tr>
<td>LDMDP</td>
<td>15.4 ± 0.4</td>
<td>8.2 ± 2.0</td>
</tr>
<tr>
<td>DMDP</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

*Assessment of nonopsonic phagocytosis of P. aeruginosa by PM was performed as described in the text. The experiments were repeated two or three times, with each sample in duplicate. Data are means ± SEM.

*Data are for P. aeruginosa P1 incubated with 10 mM D-glucose. Phagocytosis of P1 was negligible in the absence of D-glucose.
and PM phagocytosed similar numbers of zymosan particles (data not shown).

**In situ phagocytic activities of AM.** In untreated and uninfecte control mice, mononuclear cells comprised 99 to 100% of BAL cells and PMNs comprised less than 1% (Fig. 6A). Recruitment of neutrophils to the lung was substantially enhanced after infection with high doses of *P. aeruginosa*—PAK at $10^8$ CFU and P1, c2908c, and FRD1 at $10^7$ CFU. PMN recruitment to the lung was minimal in mice inoculated with zymosan particles and latex beads. Figure 6B shows the percentage of AM and PMNs with ingested particles in the BAL population. AM phagocytosis of *P. aeruginosa* (0 to 5%) was much lower than that of zymosan particles (23%) and latex beads (27%).

**DISCUSSION**

AM depletion did not compromise the capacity of BALB/c or CD-1 mice to clear *P. aeruginosa* from the lung. However, AM depletion was noted by others to profoundly compromise pulmonary host defenses against *P. aeruginosa* and other respiratory pathogens (4, 15, 16). AM depletion results in reduced killing of *Mycoplasma pulmonis* in C57BL/6 mice (15). Forty-eight hours after *K. pneumoniae* inoculation, mice with 65% AM depletion show increased bacterial counts in lungs compared to control infected mice (4). However, *K. pneumoniae* is more virulent than *P. aeruginosa*; 100 CFU of the former was used to infect mice (4), whereas a much higher dose ($10^6$ CFU) of *P. aeruginosa* can be cleared from the lungs of normal mice. The discrepancy between our results with *P. aeruginosa*-infected AM-depleted mice and those with *Klebsiella* (4) and *M. pulmonis* (15) may be due to differences in infecting agents. This difference was confirmed by the experiments that we performed with *K. pneumoniae*. On the other hand, decreased bacterial clearance has been observed in CD-1 mice depleted of AM and infected with *P. aeruginosa* (16). The discrepancy between our results and those of Kooguchi et al. (16) may be due to the method of LDMDP administration, the infecting dose of bacteria, and the strain of *P. aeruginosa* used. Liposomes can be disrupted by nebulization (9), thereby releasing their contents into the endobronchial space. We chose to deliver LDMDP by i.n. inoculation to avoid the potentially toxic effects of free DMDP. The enhanced susceptibility of LDMDP-treated mice to pulmonary *P. aeruginosa* infection observed by Kooguchi et al. (16) may have resulted from the nonspecific effect of DMDP on multiple components of the respiratory tract. Our i.n. instillation likely conserved the integrity of the liposomes and delivered them specifically to AM with subsequent selective depletion. This effect was supported by our studies with *K. pneumoniae*.

Previous in vitro studies have shown that AM actively phagocytose liposomes; AM display the morphological traits characteristic of ongoing phagocytic activity, such as grossly irregular perimeters and extensive pseudopod formation (20). The effects of LDMDP on the phagocytic competence of PM and AM were more apparent than those exerted by LPBS, suggesting that a significant decrease in the phagocytosis of *P. aeruginosa* by macrophages was not due to liposomes alone. The results of these in vitro experiments assessing the effects of LPBS, LDMDP, and DMDP on AM and PM correlated with those of an in vitro study of rat AM incubated with LPBS, LDMDP, and DMDP (2). LDMDP-treated AM and PM had
shriveled nuclei and cytoplasm, suggesting that they had undergone apoptosis (4, 18, 27). AM and PM treated with free DMDP were lysed, resulting in a loss of cytoplasm, indicating that the mechanisms by which DMDP and LDMDP affect macrophages are not the same. A549 respiratory epithelial cells incubated with high concentrations of free DMDP showed the same change in morphology as the macrophages. However, the concentration of DMDP used in in vitro assays was higher than that incorporated into LDMDP. During liposomal preparation, only 1% of DMDP is encapsulated into LDMDP (28). No change in the morphology of epithelial cells incubated with LPBS and LDMDP was observed, suggesting that these liposomes administered in vivo in mice would not affect the respiratory epithelium in mice; their effects appear to be limited to macrophages.

Recruitment of PMNs to the lung was not impaired in LDMDP-treated mice. PMNs did not appear to be affected by LDMDP or LPBS, consistent with the results of in vitro and in vivo experiments demonstrating that neutrophils are not affected by LDMDP (4). PMNs, but not AM, actively phagocytosed P. aeruginosa, as shown in BAL preparations from both groups of mice, indicating that PMNs actively participated in bacterial clearance. In the uninfected or untreated lung, PMNs comprise a small proportion of cells in the lower respiratory tract and macrophages constitute 85 to 98% of cells (15, 26, 29). However, the number of PMNs increases in lungs challenged with P. aeruginosa by intratracheal inoculation (19).

Poor nonopsonic phagocytosis of P. aeruginosa by freshly explanted murine AM correlated well with previously published data on freshly explanted and cultivated murine AM, where the former, but not the latter, showed negligible phagocytosis of unopsonized P. aeruginosa (8, 29). However, the ingestion of zymosan particles and latex beads by freshly explanted AM demonstrated that they were phagocytically competent. The phagocytic activities of freshly explanted AM from BALB/c and CD-1 mice were similar, suggesting that the poor phagocytic capacity of uncultivated AM is not mouse strain specific. The difference in phagocytic competency between freshly explanted AM and PM may be due to their differential glycolytic activities. AM reside in the oxygen-rich environment

FIG. 4. Effects of AM depletion in BALB/c and CD-1 mice on P. aeruginosa and K. pneumoniae clearance in vivo. BALB/c mice, 24 h after the i.n. instillation of LPBS (C) or LDMDP (F), were challenged i.n. with P. aeruginosa FRD1 ($6.0 \times 10^6$ CFU) (A) and PA01 ($2.7 \times 10^5$ CFU) (B). CD-1 mice were challenged with PA01 ($6.9 \times 10^6$ CFU) (C), and BALB/c mice were challenged with K. pneumoniae ($1.0 \times 10^2$ CFU) (D). Depletion of AM was confirmed by BAL counts and cytospin preparations on the day of infection. At 3 h (day 0), 24 h (day 1), 48 h (day 2), and 120 h (day 5) postinfection, bacterial counts in lung homogenates were determined. Data are expressed as means ± SEM of CFU (log$_{10}$) per gram of lung tissue from three mice at each time point. †, mice were euthanized due to signs of disease.
of lung airways (13% O₂), whereas PM are in the relatively anaerobic peritoneum (6.5% O₂); therefore, their metabolic capacity and dependence upon oxidative phosphorylation versus glycolysis are quite dissimilar, as would be their capacity to transport glucose and to ingest bacteria in a glucose-dependent fashion (6, 8, 22). The association of glucose transport and nonopsonic phagocytosis of *P. aeruginosa* has been previously reported (1, 8, 29). In addition to the implicated role of glycolysis, signal transduction also plays a role in macrophage phagocytic activities (10, 11). Taken together, the differences

FIG. 5. Differential determination of leukocytes from BAL performed on *P. aeruginosa*-challenged mice pretreated with LPBS or LDMDP. BAL differentials were calculated for LPBS-treated mice (A) and LDMDP-treated mice (B) 3 h after their i.n. inoculation with *P. aeruginosa* strain PA01 (6.9 × 10⁶ CFU). Magnification, ×1,125. Arrowheads indicate bacteria within PMN.
in AM and PM phagocytic competency may be due to their differential abilities to transport and utilize glucose and initiate the signalling pathways.

In situ results indicated that AM were phagocytically competent for control particles but were unable to ingest unopsonized \textit{P. aeruginosa}, consistent with data from in vitro assays showing poor nonopsonic phagocytosis by freshly explanted AM. Buret et al. (5) have demonstrated that the phagocytosis of \textit{P. aeruginosa} by AM in rats infected with \textit{P. aeruginosa} decreases substantially at 4 h postinfection from that at 30 min after infection, despite an increase in AM numbers. However, the number of PMNs increases dramatically at 4 h postinfection from that at 30 min postinfection (5). The decreased phagocytic activity of AM in rats at 4 h postinfection reported by Buret et al. may be due to the recruitment of other immune cells, such as PMNs, which migrate to the infected sites and are involved in bacterial clearance, or to suppression by gamma interferon (23). Bactericidal activity is higher in neutrophils than in unactivated macrophages, due to the robust capacity of the former to produce reactive oxygen radicals (7). The number of PMNs elicited in response to latex bead and zymosan challenge was minimal. These results suggest that the recruitment of PMNs is specific and correlates with an orchestrated response.

In summary, depletion of the majority of AM in mice did not result in decreased clearance of \textit{P. aeruginosa} or impairment of PMN recruitment. AM were phagocytically competent but inefficient in ingesting unopsonized \textit{P. aeruginosa} in vitro and in situ, whereas PMNs recruited upon bacterial challenge phagocytosed unopsonized \textit{P. aeruginosa}. These results suggest that the role of AM in defense against \textit{P. aeruginosa} challenge may rely upon their capacity to recruit other phagocytic cells rather than on their intrinsic phagocytic function.

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