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Lung Phagocyte Bactericidal Function in Strains of Mice Resistant and Susceptible to *Pseudomonas aeruginosa*

C. MORISSETTE, C. FRANCOEUR, C. DARMOND-ZWAIG, AND F. GERVAIS*

McGill Centre for the Study of Host Resistance, Montreal General Hospital Research Institute, Montréal, Québec, Canada

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The host response to *Pseudomonas aeruginosa* lung infection varies among inbred mouse strains. Mice of the BALB/c strain are resistant to *P. aeruginosa* lung infection, whereas mice of the DBA/2 strain are susceptible. This phenotypic variation correlates with a difference in the magnitude of the inflammatory response induced early following infection. In order to determine whether the ability of lung phagocytic cells to kill *P. aeruginosa* plays a role in the host response to the infection, we measured the in vitro bactericidal activity of resident and inflammatory alveolar and interstitial macrophages, using a temperature-sensitive mutant of *P. aeruginosa*. Lung macrophages obtained from resistant and susceptible animals displayed similar bactericidal activities, suggesting that the ability of phagocytes to kill *P. aeruginosa* does not play a crucial role in the outcome of infection. The bactericidal activity of lung phagocytes was also assessed in vivo following endobronchial infection with the temperature-sensitive mutant of *P. aeruginosa*. Resistant mice showed a rapid influx of polymorphonuclear leukocytes (PMNs) to the bronchoalveolar space which was shortly followed by an efficient clearance of the bacteria. Susceptible mice had a delay in both the inflammatory response to *P. aeruginosa* and the initiation of bacterial clearance. Susceptible mice have been shown to have a defect in tumor necrosis factor alpha production when infected intratracheally with *P. aeruginosa*. Intratracheal instillation of tumor necrosis factor alpha to susceptible mice at the time of infection significantly improved the recruitment of PMNs to the site of infection without affecting the process of bacterial clearance. Overall, these results suggest that both recruitment of a high number of PMNs to the lungs and an efficient activation process of the phagocytes are crucial for the prompt clearance of *P. aeruginosa*.

Pseudomonas aeruginosa is a ubiquitous opportunistic pathogen which often colonizes the lungs of patients with cystic fibrosis (CF) (2). These patients usually experience their first lung infections with *P. aeruginosa* around 10 years of age (20, 21). The incomplete eradication of the bacteria from the lungs leads to the establishment of a chronic *P. aeruginosa* infection (15).

The presence of *P. aeruginosa* in the lungs of CF patients induces a strong and sustained acute inflammatory response, which is characterized by an abundance of polymorphonuclear leukocytes (PMNs) in the air space of the endobronchial tree. The high morbidity and mortality rates due to *P. aeruginosa* lung infection in CF patients are most probably linked to the organ tissue damage caused by substances released by neutrophils following activation and during their phagocytic process (3). Although most CF patients become infected with *P. aeruginosa*, the severity of the pulmonary disease induced by *P. aeruginosa* can vary among patients even if these patients carry the same cystic fibrosis transmembrane conductance regulator (CFTR) mutation (25). The reason for such a variation in the time of onset as well as in the severity of the lung infection is unknown. Genes outside the CFTR locus can influence the host response to pulmonary *P. aeruginosa* infection (12, 13, 23, 24).

In order to study the genetic control of the host response to lung infection with *P. aeruginosa*, we have used a mouse model of endobronchial infection with *P. aeruginosa* entrapped in agar beads (17). Inbred strains of mice vary in their ability to control *P. aeruginosa* lung infection. Mice of the BALB/c strain were highly resistant to the establishment of lung infection with *P. aeruginosa*, whereas mice of the DBA/2 strain were very susceptible to this infection. Furthermore, the resistance or susceptibility to *P. aeruginosa* lung infection correlated with a difference in the magnitude of the endobronchial inflammatory response induced by the intratracheal instillation of *P. aeruginosa*. Mice of the BALB/c strain exhibited a prompter recruitment of inflammatory cells (PMNs and macrophages) to the site of infection than did mice of the DBA/2 strain. This inflammatory response was characterized by an early recruitment of PMNs, within the first 24 h of infection, which was followed by the influx of inflammatory macrophages starting on day 3 of infection.

Using the same model of infection, Gosselin et al. (11) analyzed the appearance of several inflammatory cytokines, namely, interleukin-1α, interleukin-1β, macrophage inflammatory protein 1α, and tumor necrosis factor alpha (TNF-α), early following intratracheal instillation of *P. aeruginosa* in both resistant and susceptible animals. These cytokines appeared within 3 to 6 h following infection in both resistant and susceptible animals. Interestingly, both the level of TNF-α in the bronchoalveolar lavage (BAL) fluid and the level of TNF-α gene expression in BAL cells were found to be significantly greater in mice of the resistant BALB/c strain than in mice of the susceptible DBA/2 strain. No difference was seen in any other cytokine measured, emphasizing the role of TNF-α in the host response to this specific lung infection.

Several studies have indicated that the phagocytes recruited to the site of infection are responsible for clearing *P. aeruginosa* from the lungs (1, 5, 7). Specifically, the greater number of inflammatory cells and the presence of a higher concentration of TNF-α observed at the infection site of resistant mice

* Corresponding author. Mailing address: Montreal General Hospital Research Institute, 1650 Cedar Ave., Montréal, Québec, Canada H3G 1A4. Phone: (514) 937-6011, ext. 4511. Fax: (514) 934-8332. Electronic mail address: mdfg@musica.mcgill.ca.

could reflect a better inflammatory process which leads to an efficient bactericidal activity and clearance of the bacteria. Since the difference in cytokine production was observed within hours following infection, it is possible that resident alveolar macrophages play an important role in the clearance of infection and/or in the recruitment of fresh phagocytes (PMNs and inflammatory macrophages) which would efficiently clear the invading microorganisms. In order to more precisely determine the role of alveolar macrophages in clearing *P. aeruginosa*, we compared the bactericidal activities against *P. aeruginosa* of resident lung macrophages, PMNs, and inflammatory lung macrophages in resistant and susceptible animals. The bactericidal activities of pulmonary phagocytes were determined in vitro and in vivo with a temperature-sensitive mutant (TSM) of *P. aeruginosa* which does not grow at 37°C. The use of this mutant enabled us to clearly determine the bactericidal activity of specific cell types.

MATERIALS AND METHODS

Animals. Male mice of the BALB/cAnNHsd, and DBA/2NHsd inbred strains were purchased from Harlan Sprague Dawley Inc. (Indianapolis, Ind.). The animals were maintained in a specific-pathogen-free environment until infection with *P. aeruginosa*, after which they were housed in a biohazard room. Mice between 10 and 16 weeks old were used.

Bacteria. Two strains of *P. aeruginosa* were used. Lung inflammatory response was induced by a heat-killed preparation of a mucoid isolate of *P. aeruginosa*. This strain originates from a clinical mucoid isolate of *P. aeruginosa* 508 kindly donated by Jacqueline Lagacé (Université de Montréal, Montréal, Québec, Canada) and subcultured as described previously (17). An overnight log-phase culture of *P. aeruginosa* 508 in 4% Proteose Peptone was expanded into a larger volume to 10^8 CFU/ml before being heat inactivated at 80°C. Entrapment of *P. aeruginosa* 508 in agar beads was done as previously described (17).

The second strain, a TSM of *P. aeruginosa*, was used for the determination of in vitro and in vivo bactericidal functions of phagocytes. The temperature-sensitive tight mutant (D/1/8) of a *P. aeruginosa* strain, unable to grow at temperatures above 36°C, was kindly provided by Anne Morris Hooke (Miami University, Oxford, Ohio) (18). An isolated colony of this bacteria was expanded in Trypticase soy broth (Fisher Scientific, Pittsburgh, Pa.) at 29 to 30°C under agitation and aliquoted in 20% glycerol as a stock bacterial suspension which was kept frozen at −80°C. An overnight log-phase culture was adjusted by nephelometry to 10^8 CFU/ml prior to its dilution to the required infectious dose.

Intratracheal instillation of *P. aeruginosa*. Mice were anesthetized following intramuscular administration of 0.2 to 0.3 ml of a 1:1 freshly prepared mixture of 2 mg of xylazine (Rompun; Bayvet Division, Chemagro Limited, Etobicoke, Ontario, Canada) per ml and 15 mg of ketamine hydrochloride (Rogarsetic; Rogar/STB Inc., London, Ontario, Canada) per ml. Intratracheal instillation was done as described by us previously (17).

BAL. Mice were sacrificed by CO_2 inhalation or cervical dislocation and BAL fluid was obtained according to a previously described technique (17). The bronchoalveolar cells were centrifuged and resuspended in the appropriate cell culture medium supplemented with 10% fetal bovine serum (FBS) (catalog no. 26140-038; GIBCO BRL).

Determination of *P. aeruginosa* CFU in lungs. Lungs were excised from the chest cavity and homogenized (30 s) in a 9.0-ml volume of sterile Hanks balanced salt solution or phosphate-buffered saline (PBS) kept on ice (homogenizer, model PT 10/35; Brinkmann Instruments, Mississauga, Ontario, Canada). Bacterial load was determined by plating serial 10-fold dilutions of lung homogenates onto Trypticase soy agar. The plates were incubated under aerobic conditions at 28 to 30°C for at least 24 h before the colonies were counted, and the data were expressed as $\log_{10}$ CFU per pair of lungs.

Purification of alveolar macrophages. Resident and inflammatory alveolar macrophages were purified from the BAL fluids of mice sacrificed by CO_2 inhalation. Inflammatory alveolar macrophages were obtained 5 days following intratracheal instillation of 50 μl of a 1:2 mixture of beads containing heat-killed *P. aeruginosa* in PBS. Mice were sacrificed by CO_2 inhalation. The bronchoalveolar cells were centrifuged and resuspended in Dulbecco's modified Eagle medium (D-MEM) (catalog no. 11965-050; GIBCO BRL) containing gentamicin (50 μg/ml; GIBCO BRL) and 10% FBS. Percent viability and total cell counts were determined by Trypan blue exclusion with a hemocytometer. Differential cell counts were determined on a CYTOSPIN preparation (Shandon Southern Products Limited, Astmoor, Cheshire, United Kingdom) following staining with a LeukoStat stain kit (Fisher Scientific). Macrophages were purified by adherence to plastic in 24-well plates for a 90-min incubation period at 37°C and 5% CO_2 with agitation. Nonadherent cells were discarded by consecutive washings, and adherent macrophages were incubated in RPMI–10% FBS overnight prior to being tested for their bactericidal function.

Purification of resident and inflammatory interstitial macrophages. Inflammatory interstitial macrophages were obtained 5 days following intratracheal instillation of 50 μl of a 1:2 mixture of beads containing heat-killed *P. aeruginosa* in PBS. Mice were sacrificed by CO_2 inhalation. For each mouse, the thoracic cavity was opened, the vena cava was sectioned, and the lungs were perfused by injection of a 10-ml volume of PBS-EDTA through the orbital plexus or into the heart. The lungs were excised following a BAL and cut in coarse pieces. Lung pieces obtained from two mice were minced in a 10-ml volume of D-MEM containing gentamicin, 5% FBS, collagenase (2 mg/ml; catalog no. C-7657; Sigma, St. Louis, Mo.), DNase I (50 μg/ml; catalog no. 104 159; Boehringer Mannheim, Laval, Québec, Canada), elastase (1 to 2 U/ml; catalog no. 1027891; Boehringer Mannheim), and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (ICN Biomedicals, Inc., Aurora, Ohio.). The tissue suspension was then gently agitated for 90 min at 37°C. Tissue aggregates were broken apart by sequential flushing through a 10-ml pipette and then 20-, 21-, and 22-gauge needle, and the cell suspension was collected and centrifuged (550 × *g*, 10 min). The erythrocytes were lysed by adding 10 ml of erythrocyte hypotonic lysis buffer to the cell pellet. Cells were then washed in 30 ml of D-MEM–gentamicin–5% FBS (550 × *g*, 10 min). The cell pellet was resuspended in 10 ml of medium, layered on Lympholyte-M (Cedarlane Laboratories Limited, Hornby, Ontario, Canada), and centrifuged (1,500 × *g*, 20°C, 20 to 25 min). Cells at the interphase of the medium and Lympholyte-M were collected and washed twice with a large volume of D-MEM–gentamicin–5% FBS and then resuspended in D-MEM–10% FBS. Total cell number, differential counts, and viability were determined as described for alveolar macrophages. Interstitial macrophages were purified by a 2-h adherence to plastic (37°C and 5% CO_2) with agitation. Nonadherent cells were removed by washing, and macrophages were incubated overnight in D-MEM–10% FBS prior to being tested for their bactericidal function.

Isolation of peritoneal macrophages. The resident peritoneal macrophages were obtained by peritoneal lavage with a 10-ml volume of cold RPMI–gentamicin (50 μg/ml)–10% FBS. The cell suspension was centrifuged and resuspended in RPMI–10% FBS. Macrophages were isolated by adherence to plastic following an incubation period of 90 min at 37°C and 5% CO_2. Inflammatory macrophages were collected similarly from mice undergoing a sterile inflammatory response, 5 days following an intraperitoneal injection of a 1-ml volume of 10% Proteose Peptone.

In vitro bactericidal assay against *P. aeruginosa*. The in vitro bactericidal assay was adapted from a previously described protocol (10). Briefly, 5×10^5 macrophages per well were distributed in 48-well flat-bottom tissue culture plates (Fisher Scientific). Adherent macrophages were infected with 2.5×10^6 bacteria (TSM of *P. aeruginosa*) (bacteria-to-cell ratio, 5:1). Macrophages were allowed to phagocytose the bacteria for 30 min at 37°C and 5% CO_2. Control wells consisted of cells alone and bacteria alone incubated for the complete duration of the experiment. The phagocytosis period was ended by removing the extracellular bacteria by two consecutive washings with warm RPMI. The bactericidal activity was determined with cells incubated in RPMI–10% FBS for another 90 min following removal of the extracellular bacteria. Phagocytosis and bactericidal activity were determined with cell lysates obtained after the 30-min phagocytosis period and the 90-min incubation period, respectively. Cells were lysed with a sterile solution of 0.01% (wt/vol) bovine serum albumin in distilled water (500 μl per well). Serial 1/10 dilutions of cell lysates were plated onto Trypticase soy agar. Bactericidal activity is expressed as the difference between the number of bacteria remaining in the cells after 90 min and the number of bacteria phagocytosed during the first 30 min.

In vivo clearance of *P. aeruginosa*. Systemic and pulmonary clearance of *P. aeruginosa* was determined in both resistant and susceptible strains of mice. Systemic clearance was assessed in mice challenged intravenously (i.v.) with 10^5 CFU of a log-phase culture of a TSM of *P. aeruginosa* (volume, 0.2 ml). The animals were sacrificed by CO_2 inhalation or cervical dislocation at 30 min following challenge (when uptake of the dose introduced occurred) or 4 h following challenge (when clearance occurred). For each animal, the liver and spleen were aseptically collected and homogenized in 8 and 9 ml of PBS, respectively. The bacterial load was determined by plating 1/10 serial dilutions of the homogenates onto Trypticase soy agar.

The pulmonary clearance was assessed following an intratracheal challenge with 10^5 CFU of a TSM of *P. aeruginosa* (volume, 50 μl) as described above. Mice were sacrificed at different time points following the endobronchial infection. Their lungs were excised aseptically without BAL, and the bacterial loads in the lung homogenates were assessed as described above. In parallel, BAL was performed on another set of infected mice to evaluate the magnitude of the inflammatory response triggered by the pulmonary instillation of the bacteria.

Statistical analysis. The statistical analysis of the results obtained for the in vitro bactericidal activities of the different macrophage cell types was performed by the *t* test (SYSTAT program; SYSTAT Inc., Evanston, Ill.). The comparison of the number of CFU obtained in the different strains of mice was done by the Mann-Whitney U test (SYSTAT program).

RESULTS

Mice of the resistant BALB/c and susceptible DBA/2 strains have different inflammatory response profiles when infected

TABLE 1. Lung bacterial load and PMN recruitment following intratracheal *P. aeruginosa* infection in mice of the resistant BALB/c and susceptible DBA/2 strains

Mouse strain	Bacterial load[a] (mean $\log_{10}$ CFU/pair of lungs ± SD) on day:			10^6 PMNs in BAL fluid[b] (mean ± SD) on day:		
	1	2	3	1	2	3
BALB/c	6.54 ± 0.3	6.47 ± 1.1	4.95 ± 0.9	1.46 ± 0.6	4.16 ± 2.2	4.85 ± 1.4
DBA/2	6.42 ± 0.3	7.93 ± 1.4	8.11 ± 1.7	1.47 ± 0.1	1.45 ± 0.7	2.52 ± 1.4

[a] Mice were infected intratracheally with ~3×10^4 CFU of *P. aeruginosa* 508 entrapped in agar beads as described previously (16). The bacterial loads in lung homogenates were determined on days 1, 2, and 3 of infection and are expressed as $\log_{10}$ CFU per pair of lungs. Seven to eighteen mice per time point were infected.

[b] The number of PMNs present in the BAL fluid was determined following total cell and differential counts of the BAL cells as described in Materials and Methods.

intratracheally with *P. aeruginosa* 508 entrapped in agar beads. As seen in Table 1, the decrease in the bacterial load in the lungs of infected mice of the BALB/c strain parallels a major influx of PMNs into the bronchoalveolar space. This influx of PMNs, although present in infected mice of the susceptible DBA/2 strain, is of lesser magnitude than that seen in mice of the BALB/c strain. Moreover, we can see that lung inflammatory cells, which consist predominantly of PMNs (>80%), control the bacterial growth in mice of the BALB/c strain whereas the fewer phagocytic cells present in the alveolar space of the susceptible mice do not display an efficient bactericidal activity. Indeed, results obtained with mice of the DBA/2 strain show that the bacteria still proliferate despite the recruitment of PMNs to the alveolar space. These results suggest that the magnitude of the inflammatory response and/or the activation of the inflammatory phagocytic cells to kill the invading bacteria could determine the outcome of *P. aeruginosa* infection in the lungs of different strains of mice.

In vitro lung macrophage ability to kill *P. aeruginosa*. Alveolar macrophages represent the first line of defense against invading respiratory pathogens. We examined whether the bactericidal activity of alveolar macrophages per se could account for the difference observed between resistant and susceptible mice in the control of the bacterial multiplication and the rate of bacterial clearance early following infection. Resident and inflammatory alveolar macrophages were obtained from both resistant and susceptible animals and tested for their in vitro bactericidal functions against *P. aeruginosa*. In addition, the bactericidal function of interstitial lung macrophages was tested. The bactericidal activity of lung macrophages was compared with the already well-known bactericidal function of resident and inflammatory peritoneal macrophages. A TSM of *P. aeruginosa* that does not multiply at 37°C was used for these assays. The use of such a mutant enables us to clearly determine the killing ability of a specific cell type. Indeed, this TSM, not being able to multiply at 37°C, allows us to better control the bacterial inoculum without having to consider any bacterial growth which would normally take place at 37°C during the assay period.

As seen in Fig. 1A, resident as well as inflammatory alveolar macrophages obtained from resistant and susceptible mice showed similar in vitro phagocytic and bactericidal functions. The killing abilities of resident and inflammatory interstitial lung macrophages from both resistant and susceptible animals were also comparable (Fig. 1B). Inflammatory lung macrophages did not show any significant improvement in their killing ability (compared with that of the resident lung macrophages) as is usually seen when peritoneal inflammatory and resident macrophages are compared. Peritoneal inflammatory macrophages had a significantly greater ability ($P = 0.0001$ to 0.015) to kill *P. aeruginosa* than did lung inflammatory macrophages (Fig. 1C), strongly suggesting that the ability of macrophages to kill *P. aeruginosa* varies according to the site of origin. However, no difference between resistant or susceptible animals was observed when the same type of macrophages were compared.

In vivo clearance of *P. aeruginosa*. Results obtained in the in vitro assay suggest that the bactericidal activity of lung macrophages per se cannot explain the phenotypic variation seen between different strains of mice in the outcome of *P. aeruginosa* lung infection. However, the in vitro assay is not an exact correlate of the in vivo situation, in which cytokines and chemokines act on the phagocytic and killing activities of the inflammatory cells. We thus determined the in vivo ability of alveolar phagocytic cells to clear a TSM of *P. aeruginosa* in both resistant and susceptible animals. The in vivo ability of lung phagocytic cells to clear the TSM of *P. aeruginosa* was compared with that of liver and spleen macrophages following an i.v. infection, in order to determine whether or not the clearance mechanism differs according to the type of infection induced. The TSM was again chosen for these experiments, to prevent any internal in vivo growth of the bacteria, enabling us to look solely at the ability of phagocytic cells to clear the bacteria.

As seen in Fig. 2, the levels of uptake of the bacteria by the liver and spleen in resistant and susceptible animals were similar at 30 min following i.v. infection. Both resistant and susceptible animals were found to have similar rates of clearance in the liver. However, a significant difference in the rate of bacterial clearance in the spleen was observed between resistant and susceptible animals. Mice of the BALB/c strain were able to efficiently clear the bacteria within 4 h of infection, while mice of the DBA/2 strain were less efficient at initiating an active killing ($P = 0.037$). Resident alveolar macrophages from resistant and susceptible mice did not display any killing ability in vivo, since no decrease in the bacterial load was seen in the first 4 h following intratracheal infection (Fig. 3). In contrast, early in infection the liver and spleen resident phagocytic cells from both strains can kill *P. aeruginosa* readily (Fig. 2). These results support the data obtained from the in vitro assay, suggesting that the phagocytic cell activity varies according to the organ site.

When mice were infected intratracheally with the TSM of *P. aeruginosa*, the ability to clear the bacteria was determined at different time points following instillation. Early time points (30 min and 2 and 4 h) were chosen to determine the ability of resident alveolar macrophages to kill *P. aeruginosa*, while later time points (6, 8, 18, and 24 h) were aimed at determining whether newly recruited PMNs were responsible for clearing the infection. Interestingly, the clearance of the TSM was initiated only following recruitment of PMNs to the alveolar space. As seen in Fig. 4A, killing of the bacteria was initiated between 4 and 6 h postinfection in mice of the BALB/c strain, the time at which PMNs begin to reach the alveolar space (Fig. 4B). On the other hand, the influx of PMNs to the alveolar space was delayed by approximately 2 h in mice of the DBA/2

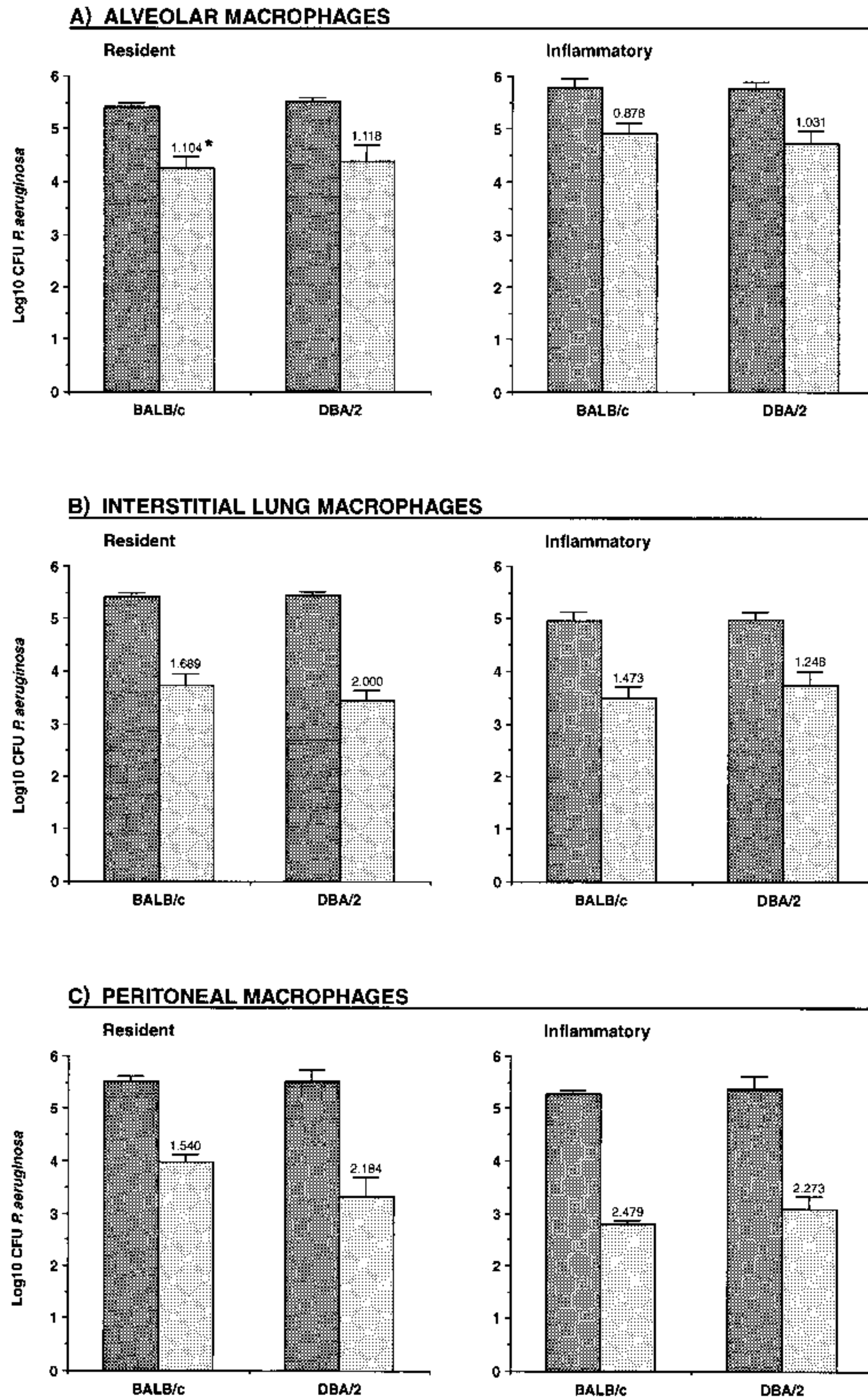

FIG. 1. In vitro phagocytosis and bactericidal activities of resident and inflammatory alveolar (A) and interstitial lung (B) macrophages were determined with a TSM of *P. aeruginosa*, as described in Materials and Methods. Their bactericidal activities were compared with those of peritoneal macrophages (control) (C). Resident and inflammatory alveolar and interstitial macrophages were obtained as described in Materials and Methods. The results represent the means ± standard deviations (error bars) of three experiments, each done in triplicate. The number shown above each error bar is the bactericidal activity expressed as the difference between the number of bacteria remaining in the cells after 90 min and the number of bacteria phagocytosed during the first 30 min. Dark-gray bars, phagocytosed bacteria; light-gray bars, remaining live bacteria.

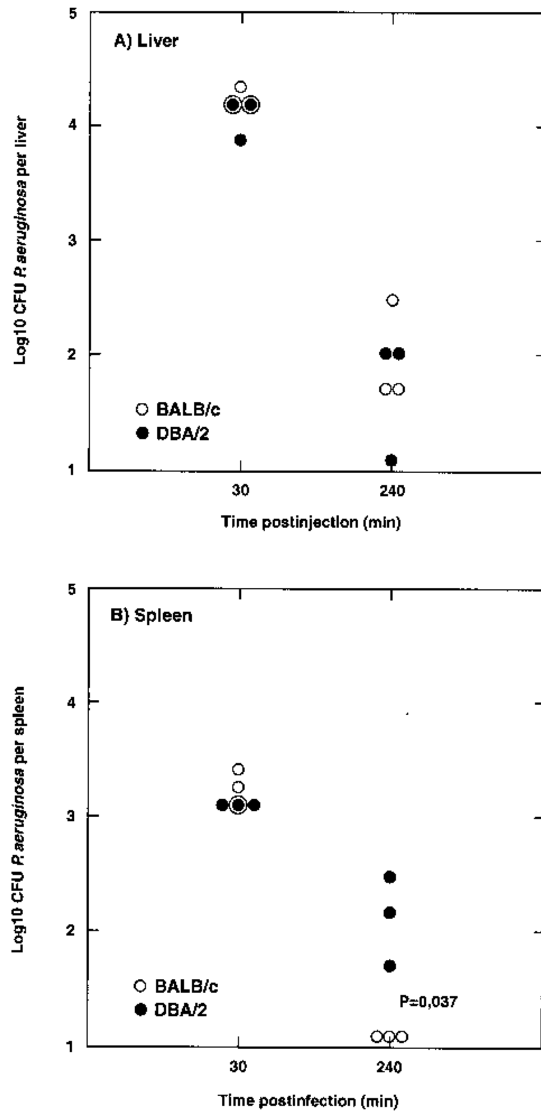

FIG. 2. Bacterial uptake by the liver (A) and the spleen (B) 30 min and 4 h following i.v. infection with the TSM of *P. aeruginosa*. The mice were infected i.v. and their organ bacterial loads were determined as described in Materials and Methods. Three mice per group were infected.

strain compared with that in mice of the BALB/c strain. Indeed, the bacterial load remained unchanged in mice of the DBA/2 strain for up to 6 h of infection, with no PMNs being recruited to the bronchoalveolar space of these mice. Figure 4B clearly shows that the patterns of the lung inflammatory responses in resistant and susceptible mice differ. Taken together, these results suggest that the initiation of bacterial clearance in resistant BALB/c mice correlates with the influx of PMNs in the alveolar space.

Effect of TNF-α on the lung inflammatory response in infected susceptible mice. TNF-α promotes the recruitment and activation of inflammatory phagocytes. Gosselin et al. (11) showed that resistant mice of the BALB/c strain had greater concentrations of TNF-α in their BAL fluids than did mice of the DBA/2 strain. Our observation of a delay in the recruitment of PMNs to the lungs of mice of the DBA/2 strain prompted us to determine whether or not TNF-α plays a role in the magnitude of the lung inflammatory response. Recombinant mouse TNF-α (500 U per mouse; Genzyme, Cambridge, Mass.) was instilled intratracheally in conjunction with the TSM of *P. aeruginosa* in mice of the DBA/2 strain to determine whether the presence of TNF-α at the infection site, at a concentration similar to that seen in resistant infected mice, could affect the early recruitment of PMNs to the airways. Mice of the DBA/2 strain which received TNF-α concomitantly to the TSM infection had more PMNs infiltrating the site of infection than did TSM-infected non-TNF-α-treated DBA/2 mice (values for the former and the latter, 11.3×10^5 and 3.9×10^5 cells, respectively [at 8 h postinfection], and 29.4×10^5 and 14.9×10^5 cells, respectively [at 18 to 24 h postinfection]). The magnitude of the inflammatory response obtained in TNF-α-treated infected mice of the DBA/2 strain was similar to that obtained in non-TNF-α-treated infected mice of the BALB/c strain. As seen in Table 2, DBA/2 mice inoculated with *P. aeruginosa* and TNF-α had an earlier influx of PMNs to the lungs. Interestingly, the bacterial clearance in TNF-α-treated mice of the DBA/2 strain was not initiated faster than it was in normal mice. Indeed, the ability to reduce the number of TSMs of *P. aeruginosa* in the lungs in the TNF-α-treated mice was only slightly improved compared with that in control mice. On the other hand, mice of the BALB/c strain started clearing the bacteria as soon as 6 h following infection.

DISCUSSION

The phenotypic variation of the host response to *P. aeruginosa* lung infection appears to be linked to the ability of the host to mount a prompt and efficient inflammatory response. We have observed that resistant mice infected intratracheally with a mucoid strain of *P. aeruginosa* undergo a prompt recruitment of inflammatory cells to the alveolar space which is followed by a drastic decrease in the number of bacteria between day 2 and 3 of infection (17). This phenomenon of efficient inflammatory phagocyte recruitment to the lungs was slower and of lesser magnitude in susceptible animals. In this study, we aimed to determine whether the prompt clearance of

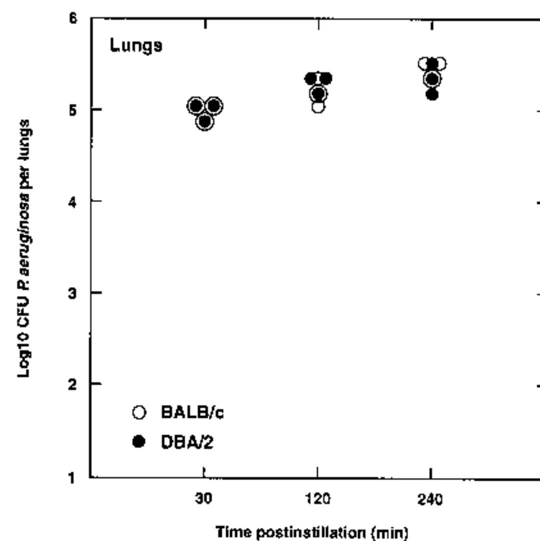

FIG. 3. Lung bacterial load found in animals resistant and susceptible to *P. aeruginosa* following intratracheal infection with the TSM of *P. aeruginosa*. Mice were infected intratracheally and their bacterial loads were determined at 30 min and 2 and 4 h following infection as described in Materials and Methods. Three mice per time point were tested.

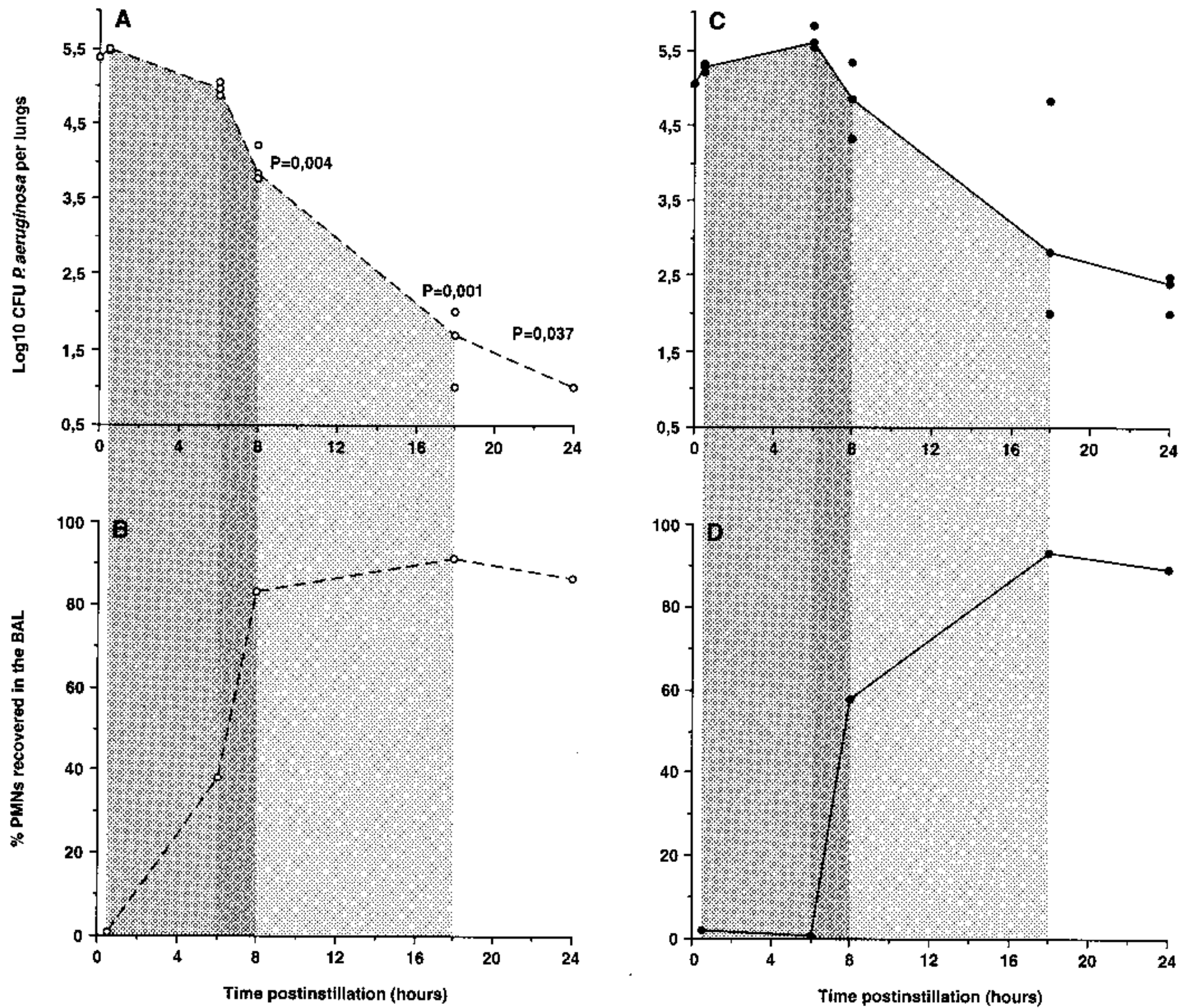

FIG. 4. (A and C) Lung bacterial load found in animals resistant and susceptible to *P. aeruginosa* following intratracheal infection with the TSM of *P. aeruginosa*. Bacterial load was determined 6, 8, 18, and 24 h following infection as described in Materials and Methods. Ten to twelve mice per time point were tested. Results show the median as well as the minimum and the maximum values obtained at each time point for one representative experiment. (B and D) Magnitude of PMN recruitment to the lungs at different time points following intratracheal infection. Cells were obtained by BAL and counted as described in Materials and Methods. Two mice per group were tested. The three shaded areas represent the three phases of infection (from left to right): recruitment of inflammatory phagocytes, initiation of killing, and clearance of bacteria. ○, BALB/c mice; ●, DBA/2 mice.

bacteria observed in resistant mice of the BALB/c strain was due to a stronger bactericidal activity of the lung macrophages per se and/or a more efficient recruitment of inflammatory phagocytic cells to the infection site, which would readily kill the invading microorganisms.

Murine pulmonary macrophages consist of two populations, the alveolar and interstitial macrophages. Crowell et al. (7) recently showed that in mice, 37% of the pulmonary macrophages consist of interstitial macrophages. Both cell types were found capable of Fcγ receptor-mediated phagocytosis. We first

TABLE 2. Effect of TNF-α treatment on intratracheal infection of mice with the TSM of *P. aeruginosa*[a]

Mouse strain (treatment)	Bacterial clearance (change in $\log_{10}$ bacterial load) at time following infection					Inflammatory response (% PMNs) at time following infection				
	30 min	6 h	8 h	18 h	24 h	30 min	6 h	8 h	18 h	24 h
DBA/2 (TSM)	NA[b]	0.4 ± 0.2	−0.5 ± 0.4	−2.3 ± 0.9	−3.0 ± 0.3	0	0	58 ± 19	93 ± 1	89 ± 5
DBA/2 (TSM + TNF-α)	NA	0.3 ± 0.1	−0.2 ± 0.5	−2.8 ± 0.4	−3.9 ± 0.3	0	18 ± 13	80 ± 17	85 ± 16	89 ± 5
BALB/c (TSM)	NA	−0.5 ± 0.1	−1.6 ± 0.2	−4.0 ± 0.4	Cleared	0	38 ± 3	83 ± 3	91 ± 1	87 ± 2

[a] Mice were infected intratracheally with 10^5 CFU of the TSM of *P. aeruginosa* and were treated with 500 U of TNF-α or left otherwise untreated. Mice were sacrificed at different time points as described in Materials and Methods. The rates of bacterial clearance were determined with lung homogenates and are expressed as the change in $\log_{10}$ CFU seen between 30 min and different time points following infection. Three mice per group were tested. The inflammatory response was determined in parallel on a different set of infected mice. BAL fluid was obtained and the percentage of PMNs in the cells from the BAL was determined as described in Materials and Methods. Three to five mice per group were tested. All nonzero values are reported as means ± standard deviations.

[b] NA, not applicable.

evaluated both of these macrophage cell types for their ability to kill *P. aeruginosa* in vitro. The results showed that both resistant and susceptible animals displayed similar lung macrophage phagocytic and bactericidal functions, suggesting that the phenotypic variation of the host response to *P. aeruginosa* lung infection is not controlled by the bactericidal ability of tissue macrophages (both alveolar and interstitial). Our study also supported the functional heterogeneity of macrophages according to their site of origin. Indeed, alveolar and interstitial macrophages showed a weaker bactericidal activity than did peritoneal macrophages. Alveolar macrophages have been shown to be functionally and morphologically different from peritoneal macrophages. Their abilities to migrate, phagocytose, and produce arginase are less than those of peritoneal macrophages (29). They also produce more TNF-α than splenic macrophages and Kupffer cells following in vitro stimulation with lipopolysaccharide. These differences most probably result from specific organ conditions (19). However, we cannot exclude the possibility that alveolar and interstitial macrophages do not display strong in vitro abilities to kill *P. aeruginosa* because of the change in the oxygen concentration and/or in cytokine and chemokine environments when they are put in culture. These macrophages were however left overnight in culture prior to the assay to allow them to adapt to this new milieu.

The in vitro bactericidal functions in both resident and inflammatory macrophages were assayed to determine whether newly bone marrow-derived inflammatory macrophages reaching the alveolar space would display a killing ability greater than that of the resident alveolar macrophages. Inflammatory peritoneal macrophages have been shown to exhibit a greater bactericidal function than resident peritoneal macrophages in another bacterial system (10). In our system, resident and inflammatory alveolar macrophages did not differ in their ability to kill *P. aeruginosa* in vitro. A similar pattern was also found with interstitial macrophages.

Because of significant differences between the in vitro assay and the in vivo milieu where factors (e.g., cytokines and chemokines) induced by the infection are acting on cellular functions, we determined the in vivo abilities of resident alveolar macrophages as well as inflammatory PMNs to clear the TSM of *P. aeruginosa*. Resident alveolar macrophages did not display any in vivo bactericidal function, emphasizing their role as cytokine and chemokine producers that recruit PMNs to the infected airways rather than their role as phagocytic cells. We found that less than 3% of alveolar resident macrophages had phagocytosed bacteria during the first 4 h following intratracheal infection (data not shown). This result is similar to those of other studies in which a low percentage of rat alveolar macrophages were found to contain bacteria upon intratracheal infection with *P. aeruginosa* (5). This supports the model proposed by Dunkley et al. (9) in which resident alveolar macrophages would act as cytokine producers rather than as bactericidal effector cells.

The impact of the organ milieu on the ability of phagocytic cells to eliminate the invading microorganism was determined by comparing the clearing process taking place in the lung compartment with that occurring in the liver and the spleen following systemic inoculation. Only the first 4 h following infection were studied in order to assess the ability of resident macrophages to kill *P. aeruginosa*. Two major phenomena were observed. First, phagocytic cells present in the liver and the spleen readily killed the bacteria whereas alveolar macrophages had not yet initiated any clearing process in the lungs. The presentation of *P. aeruginosa* to macrophages between the two routes of infection can differ and lead to a more efficient killing when mice are infected i.v. Second, we observed a significant difference in the clearing process from the spleen but not from the liver for the two strains of mice. This strain variation could be related to the difference in the ability of bacterial and cellular products to induce macrophage activation and/or to PMN recruitment.

The in vivo ability of lung inflammatory phagocytic cells to clear the TSM of *P. aeruginosa*, as determined between 6 and 24 h following intratracheal instillation of the bacteria, points toward the PMNs as the cells responsible for a prompt clearance of *P. aeruginosa* from the lungs. This is in agreement with several reports demonstrating that PMNs were responsible for clearing *P. aeruginosa* infection. Indeed, mice rendered granulocytopenic by cyclophosphamide treatment did not survive a *P. aeruginosa* infection because of their inadequacy to recruit PMNs (1). A model of the host response to *P. aeruginosa* lung infection has recently been suggested: alveolar macrophages would first be activated, and PMNs would then be recruited and would undergo an activation process to kill *P. aeruginosa* (6, 9). The fact that we observed a delayed lung inflammatory response in susceptible mice puts even more emphasis on the role of PMNs in this proposed model. Our results suggest that genetic resistance to *P. aeruginosa* in mice of the BALB/c strain depends on both the recruitment of a high number of PMNs to the site and the activation process of the inflammatory phagocytes.

The parallel observation between the in vivo clearing process of the bacteria and the incoming of the newly recruited PMNs strongly suggests that PMNs play a major role in the resolution of the infection. The lag in the response to *P. aeruginosa* infection seen in mice of the DBA/2 strain may result from a difference in the kinetics of their cytokine and chemokine production, leading to a different pattern of adhesion molecule expression. The fact that mice of the DBA/2 strain are $C5a^-$ probably cannot explain the delay in cell recruitment. Indeed, several reports have clearly shown that gram-negative bacteria, such as *P. aeruginosa*, induce recruitment of PMNs to the lungs via a CD18/CD11-dependent activation process rather than via C5a activation (8, 26, 27). We propose that in mice of the DBA/2 strain, alveolar macrophages do not produce a specific cytokine(s) at a level required for the induction of increased expression of adhesion molecules which would then allow for the extravasation of the PMNs to the airspace. One cytokine to consider in this hypothesis is TNF-α, which was shown to be deficient early following *P. aeruginosa* intratracheal infection of mice of the DBA/2 strain (11).

TNF-α has been shown to promote PMN and macrophage recruitment to the site of infection via the increase of adhesion molecules on endothelial and epithelial cells as well as on PMNs (16, 26, 28). TNF-α has also been shown to play a role in the activation process of PMNs. Indeed, TNF-α induces PMN phagocytic activity, increases the production of superoxide anions and H_2O_2 by PMNs, and stimulates degranulation as measured by the release of myeloperoxidase, β-glucuronidase, and lysozyme (14, 22). When we treated mice of the DBA/2 strain with a dose of TNF-α similar to that produced by infected mice of the BALB/c strain, we observed an improvement of the magnitude of the inflammatory response (at a level comparable to that obtained in BALB/c mice) which took place between 4 and 8 h after infection. There was however no striking improvement of the clearance process in these TNF-α-treated mice. Although the total numbers of PMNs present at the infection site in BALB/c and TNF-α-treated DBA/2 mice were similar, *P. aeruginosa* was cleared more efficiently in BALB/c mice than in TNF-α-treated DBA/2 mice. A single dose of TNF-α at the time of infection could not completely

change the phenotypic trait of susceptibility in these mice. It improved the lung inflammatory response without inducing a more efficient bactericidal activity. However, the presence of TNF-α in the lungs could trigger a cascade of events and initiate the synthesis and/or the release of other factors, such as cytokines and chemokines, which would play a central role in the efficient recruitment of PMNs to the airspace.

Buret et al. (5) previously showed that intratracheal inoculation of recombinant TNF-α at the time of infection in naive rats improved bacterial clearance by enhancing the phagocytic activity of bronchoalveolar neutrophils without inducing any change in the profile of recruitment of inflammatory cells to the bronchoalveolar space during the first 3 h following challenge. Several aspects could explain these discrepancies. Mice could have a response to TNF-α as well as an inflammatory response profile different from those seen in rats. Moreover, normal DBA/2 mice appear to have a defect in TNF-α production upon intratracheal infection with *P. aeruginosa*. This was not the case in Buret's study, in which rats most probably had normal TNF-α production upon infection. Their approach was to provide an increased amount of TNF-α as seen following *P. aeruginosa* infection of immunized rats. The TNF-α normally produced in naive rats following infection may be at a level high enough to induce a maximal inflammatory response. The presence of an extra amount of TNF-α would therefore not increase an already maximal inflammatory response but would rather improve phagocyte ability to kill *P. aeruginosa*. Gosselin et al. (11) treated systemically resistant mice of the BALB/c strain with anti-TNF-α and showed a decrease in the ability of these mice to clear the bacteria after 3 days which was not associated with a decrease in the magnitude of the inflammatory response. Determining the magnitude of the inflammatory response on day 3 of infection might have been too late to see a difference which is usually observed earlier following infection.

This model of intratracheal *P. aeruginosa* infection in resistant and susceptible strains of mice allowed us to more accurately define the parameters of the early host response to the invading bacteria which are crucial in the outcome of the disease. It appears that the resistant host would benefit from a prompt recruitment of PMNs that readily become activated to kill *P. aeruginosa*. On the other hand, a delayed recruitment of PMNs and a weaker PMN bactericidal function would allow the bacteria to grow, leading to the establishment of an infection in the susceptible host. CF patients infected with *P. aeruginosa* have a strong inflammatory phagocyte recruitment consisting mainly of PMNs. Moreover, high concentrations of TNF-α have been found in their BAL fluids (4). The presence of such high levels of TNF-α most probably activates PMN functions and secretion leading to lung tissue damage. A defect in the host response to *P. aeruginosa* infection, coupled with other compromising conditions, such as the ones encountered in the lungs of CF patients, could favor the early establishment of chronic lung infection seen in some CF patients. In other CF patients, who would benefit from an efficient host response to *P. aeruginosa*, the first episodes of *Pseudomonas* lung infection would be cleared or controlled, thus retarding the establishment of a chronic infection.

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