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Lung Defenses against *Pseudomonas aeruginosa* in C5-Deficient Mice with Different Genetic Backgrounds

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Lung defenses against *Pseudomonas aeruginosa* were investigated in C5-deficient strains of mice with different genetic backgrounds. We studied pulmonary clearance and cell responses after aerosol exposure to *P. aeruginosa* in C5-deficient B10.D2/oSnJ and DBA/2J mice and their closest C5-sufficient counterparts, B10.D2/nSnJ and DBA/1J mice. Different patterns of lung clearance and pulmonary cell responses were found for the two C5-deficient strains. C5-deficient B10.D2/oSnJ mice showed defective lung clearance of *P. aeruginosa* 4 h after challenge compared with C5-sufficient B10.D2/nSnJ animals. This finding was associated with a decreased number of polymorphonuclear leukocytes recruited into the airways during the same time. Interestingly, C5-deficient DBA/2J mice recruited higher numbers of polymorphonuclear leukocytes than did C5-sufficient DBA/1J mice by 4 h after aerosolization. Nevertheless, lung clearance of *P. aeruginosa* in DBA/2J mice was not as effective as in C5-sufficient DBA/1J mice, suggesting that other functions of C5 besides chemotaxism could be involved. Lung clearance of *P. aeruginosa* was also investigated in C5-deficient and -sufficient hybrids sharing the same genetic background (DBA/2J × B10.D2). The results suggested that murine lung clearance of *P. aeruginosa* is markedly affected by lack of C5 in a specific genetic background (B10.D2).

The complement system is, phylogenetically, one of the oldest nonspecific mechanisms of protection in vertebrates (7). The importance of this system in resistance to pathogens is demonstrated by recurrent microbial infections in hosts genetically deficient in complement components (1, 6). Several bacterial genera, including *Neisseria* spp., *Staphylococcus aureus*, and *Pseudomonas aeruginosa* have been isolated from patients with C3 or C5 deficiency (14, 22). Impaired defenses against bacterial infections have also been reported in experimental models of complement deficiency. Depletion of circulating complement components by treatment with cobra venom factor has been associated with defective pulmonary clearance of aerosolized *Streptococcus pneumoniae* and *P. aeruginosa* (9). Animals with genetic deficiencies in the complement components constitute another important tool for studying host defense mechanisms. Murine C5 deficiency, for example, is a genetic disorder inherited as an autosomal recessive trait (15), and it has been shown that C5-deficient mice have impaired lung defenses against aerosolized *S. aureus* (3) and intratracheally injected *P. aeruginosa* (13).

Resident and recruited phagocytes play a central role in the pulmonary clearance of bacteria. The magnitude of the cell responses generated by microorganisms in the lungs of experimental animals depends on the challenge bacteria and may be influenced by many factors, including the method of inoculation of the bacteria and the integrity of the complement system of the host (13, 16, 19). In addition, the genetic background may modify lung defenses against bacteria in different C5-deficient strains of mice (3). The purpose of the present work was to investigate pulmonary cell responses and ability to eradicate aerosolized *P. aeruginosa* in C5-deficient inbred strains of mice with different genetic backgrounds.

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MATERIALS AND METHODS

Mice. Five- to six-week-old DBA/1J and B10.D2/nSnJ C5-sufficient and DBA/2J and B10.D2/oSnJ C5-deficient inbred strains of mice were obtained from Jackson Laboratory (Bar Harbor, Maine). Hybrid B10.D2/nSnJ × DBA/2J and B10.D2/oSnJ × DBA/2J F_1 mice were bred in our laboratory. All animals were kept under the conditions described by the Guide for the Care and Use of Laboratory Animals, U.S. Department of Health, Education, and Welfare publication no. (NIH) 78–23.

Bacteria and culture conditions. *P. aeruginosa* immunotype 1 (Fisher-Devlin-Gnabasik) was propagated routinely on tryptic soy agar (BBL Microbiology Systems, Cockeysville, Md.) at 37°C. To prepare bacterial suspensions for nebulization, 20 ml of Trypticase soy broth (Difco Laboratories, Detroit, Mich.) was inoculated with a loopful from an overnight plate and incubated at 37°C and 300 rpm in a G-24 water shaker bath (New Brunswick Scientific Co., Inc., Edison, N.J.). Cultures were harvested in mid log phase by centrifugation at 12,000 × *g* for 10 min at 4°C. The pellet was suspended to a density of 10^9 CFU/ml in cold saline.

Nebulization procedure. The animals were exposed to the bacterial aerosol for 30 min in a chamber built in our laboratory to the specifications of a previously described design (24). Exposures were carried out at 24°C and at −2 mm of water pressure.

Pulmonary clearance. Animals were sacrificed either immediately (t_0) or 4 h (t_4) after aerosol exposure, and the lungs were carefully excised and homogenized in 5 ml of ice-cold distilled water using a Potter-Elvehjem tissue homogenizer (Arthur H. Thomas Co., Philadelphia, Pa.). The homogenates were cultured quantitatively on tryptic soy agar, and the numbers of CFU at t_0 and t_4 were determined.

Lung lavages. Groups of animals were sacrificed at various times after aerosolization, and the lungs were lavaged twice with the appropriate volume of sterile saline at 37°C, accord-

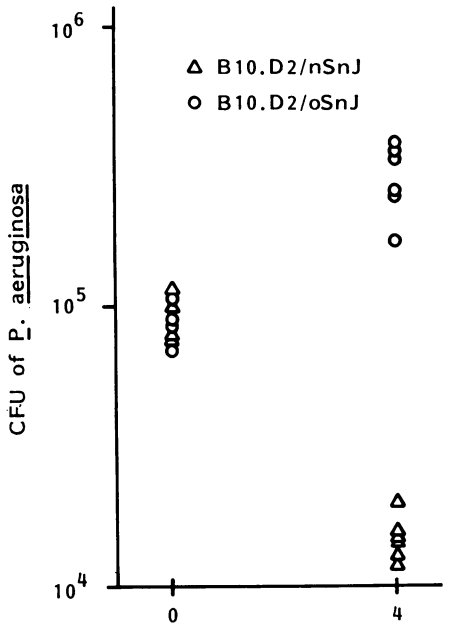

FIG. 1. Individual values of *P. aeruginosa* CFU per lung in C5-sufficient B10.D2/nSnJ and C5-deficient B10.D2/oSnJ inbred strains of mice after aerosol exposure.

ing to a procedure described elsewhere (20). The two cell suspensions from each animal were pooled, and each pool was studied separately. Cell suspensions were kept on ice until all determinations were completed. Cells were counted in a hemacytometer, and viability, determined by trypan blue exclusion, was in all cases >95%. Cells were pelleted by centrifugation at 150 × *g* at 4°C, washed, and suspended in phosphate-buffered saline at pH 7.3. Cytocentrifuge smears from each cell suspension were prepared; the slides were fixed with 100% methanol and stained with Giemsa stain.

Statistical analysis. The numbers of CFU and polymorphonuclear leukocytes (PMN) were compared by using the Student *t* test for unpaired samples. Values of $P < 0.01$ were considered significant.

RESULTS

The ability of the lungs to eliminate *P. aeruginosa* was investigated in two different C5-deficient strains of mice and their closest C5-sufficient relatives. No significant difference was found in the initial deposition of bacteria between B10.D2/nSnJ and B10.D2/oSnJ strains ($1.1 \times 10^5 \pm 0.16 \times 10^5$ and $1.20 \times 10^5 \pm 0.13 \times 10^5$ CFU per lung, respectively) (Fig. 1). Four hours after aerosol exposure, however, C5-deficient B10.D2/oSnJ mice showed defective clearance of *P. aeruginosa*. The number of CFU at 4 h in C5-deficient mice had increased to $5.5 \times 10^5 \pm 0.98 \times 10^5$, whereas in C5-sufficient B10.D2/nSnJ animals the number of CFU remaining after 4 h was $1.68 \times 10^4 \pm 0.19 \times 10^4$ (Fig. 1). This difference was significant at $P < 0.01$. Similar bacterial deposition was also found for DBA/1J and DBA/2J mice at t_0 ($1.00 \times 10^5 \pm 0.11 \times 10^5$ and $0.98 \times 10^5 \pm 0.08 \times 10^5$ CFU, respectively) (Fig. 2). Four hours after nebulization, there were lower numbers of CFU in the lungs of C5-sufficient DBA/1J mice than in the lungs of C5-deficient DBA/2J mice ($2.80 \times 10^4 \pm 0.31 \times 10^4$ vs $5.1 \times 10^4 \pm 1.3 \times 10^4$), although this difference was not significant. Survival of the animals after aerosol challenge was 100% (10/10) after 7 days for all strains of mice investigated; no sign of illness or different behavior was noticed.

To study the cellular response to aerosolized *P. aeruginosa*, the animals received inocula similar to those used in lung clearance experiments (1×10^5 to 2×10^5 CFU per lung). Lung lavages were performed 2 and 4 h after aerosolization. Four animals of each strain, without aerosol treatment, were included as controls. No differences in the cellular pattern in untreated animals were found among the strains. In all cases, the number of PMN recovered was less than 250, i.e., less than 1% of the lavageable leukocytes. The PMN response to aerosolized *P. aeruginosa* in B10.D2 strains is shown in Fig. 3 (upper panel). Two hours after aerosol exposure, the number of PMN recovered from C5-deficient B10.D2/oSnJ mice was lower than, although not significantly different from, the number of PMN recovered from C5-sufficient B10.D2/nSnJ mice ($0.38 \times 10^5 \pm 0.09 \times 10^5$ and $1.08 \times 10^5 \pm 0.41 \times 10^5$ cells, respectively). At t_4, this difference became significant ($P < 0.01$); C5-deficient mice recruited $4.92 \times 10^5 \pm 0.55 \times 10^5$ PMN or 57% of the number of PMN recovered from the lungs of C5-sufficient animals ($8.57 \times 10^5 \pm 0.61 \times 10^5$). No differences in the number of pulmonary mononuclear cells were found in B10.D2 strains at any time sampled. The pulmonary cell response after *P. aeruginosa* aerosolization of DBA strains showed a different pattern (Fig. 3, lower panel). Two hours after aerosol exposure, the numbers of PMN recovered from the lungs of C5-deficient DBA/2J and C5-sufficient DBA/1J mice were similar ($0.52 \times 10^5 \pm 0.10 \times 10^5$ and $0.45 \times 10^5 \pm 0.13 \times 10^5$, respectively). Two hours later, C5-deficient DBA/2J mice showed a threefold increase ($P < 0.01$) in the number of PMN recruited compared with the number of cells lavaged from C5-sufficient DBA/1J mice ($9.36 \times 10^5 \pm 0.98$

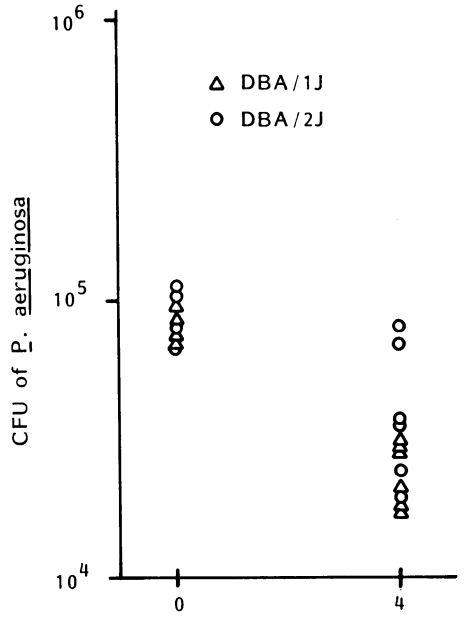

FIG. 2. Individual values of *P. aeruginosa* CFU per lung in C5-sufficient DBA/1J and C5-deficient DBA/2J inbred strains of mice after aerosol exposure.

$\times 10^5$ and $2.64 \times 10^5 \pm 0.24 \times 10^5$, respectively). Again, the number of mononuclear cells was similar for both strains at all times sampled.

C5-deficient and -sufficient hybrids were obtained by crossing DBA/2J females × B10.D2/oSnJ males and DBA/2J females × B10.D2/nSnJ males, respectively. Murine C5 deficiency is an autosomal recessive failure (15) and is the same in B10.D2/oSnJ and DBA/2J mice (17). Then, only B10.D2/oSnJ × DBA/2J F_1 mice are C5-deficient hybrids (3) and, since B10.D2/oSnJ and B10.D2/nSnJ are congenic strains, both B10.D2/oSnJ × DBA/2J and B10.D2/nSnJ × DBA/2J F_1 hybrids share the same genetic background. Lung clearance of *P. aeruginosa* was investigated in those animals after deposition of 10^5 CFU of bacteria into the airways. Results are presented in Fig. 4. Significant differences ($P < 0.01$) in the number of CFU at 4 h were found between C5-deficient and C5-sufficient hybrids: $1.78 \times 10^4 \pm 0.20 \times 10^4$ CFU per lung in C5-sufficient mice and $5.57 \times 10^5 \pm 1.00 \times 10^5$ CFU per lung in C5-deficient mice. These results closely resemble the pulmonary clearance of *P. aeruginosa* seen in B10.D2 strains (Fig. 1).

DISCUSSION

It is generally accepted that phagocytosis by neutrophils is the main host defense mechanism in early lung clearance of *P. aeruginosa*. In fact, soon after aerosolization of *P. aeruginosa*, large numbers of PMN are recruited into the airways (19, 26). The mechanisms involved in this phenomenon might be associated with at least three important chemotactic factors: (i) complement-derived chemotactins (C5a and its active fragments), (ii) the macrophage-derived chemotactins for PMN described by Hunninghake et al. (12), and (iii) the chemotactic factors released by the microorganism (25, 27). Decreased numbers of PMN recruited into the lungs of C5-deficient B10.D2/oSnJ mice (compared with B10.D2/nSnJ mice) have been previously reported after intratracheal inoculation of *P. aeruginosa* by Larsen et al. (13). They suggested that the C5 molecule and its fragments are important neutrophil chemotactins during the early period (6 h) after intratracheal inoculation of *P. aeruginosa*, whereas other factors, such as macrophage and bacterial chemotactins, would possibly have their major effects during the later periods (48 h). Our results show that aerosolized *P. aeruginosa* produces an early influx of PMN into the airways of C5-deficient, as well as C5-sufficient, strains of mice. The magnitude of the PMN response, however, depends on the mouse strain investigated. Of interest, although not completely understood, is the threefold increase in the number of PMN shown by the C5-deficient DBA/2J mice when compared with their C5-sufficient counterparts 4 h after aerosolization. In addition, C5-deficient DBA/2J mice showed early accumulation of PMN not only after aerosolization of *P. aeruginosa* as presented here but also after exposure to aerosols containing *P. aeruginosa* culture supernatants (25).

Lung clearance of *P. aeruginosa* was significantly decreased in C5-deficient B10.D2/oSnJ mice compared with C5-sufficient B10.D2/nSnJ mice. The decreased number of PMN found 4 h after aerosolization in the deficient strain might account for the difference in pulmonary clearance. The importance of neutrophils in lung clearance of *P. aeruginosa* was clearly demonstrated by Rehm and coworkers (21), who reported impaired pulmonary defenses against *P. aeruginosa* in granulocytopenic mice compared with normal animals. An increased number of recruited PMN, however, does not necessarily mean that lung clearance will be improved. Excessive numbers of PMN in the

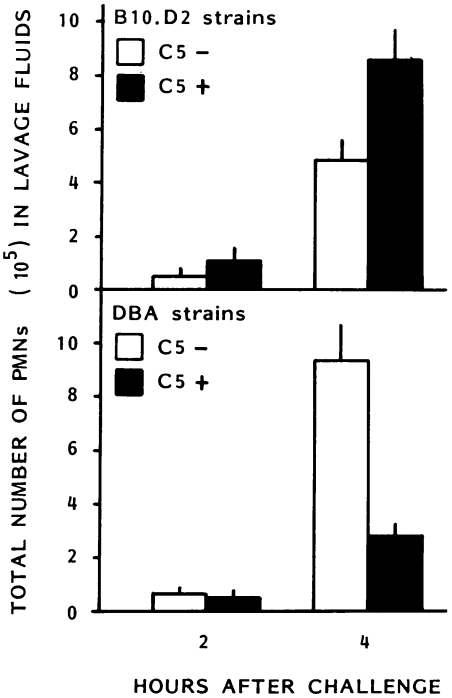

FIG. 3. Total number of PMN recovered by lung lavage 2 and 4 h after deposition of 10^5 CFU of *P. aeruginosa* per lung. Lungs were lavaged twice, and fluids were pooled for each animal. Six mice were used for each time period investigated. Panels: upper, C5-sufficient B10.D2/nSnJ and B10.D2/oSnJ inbred strains of mice; lower, C5-sufficient DBA/1J and C5-deficient DBA/2J inbred strains of mice.

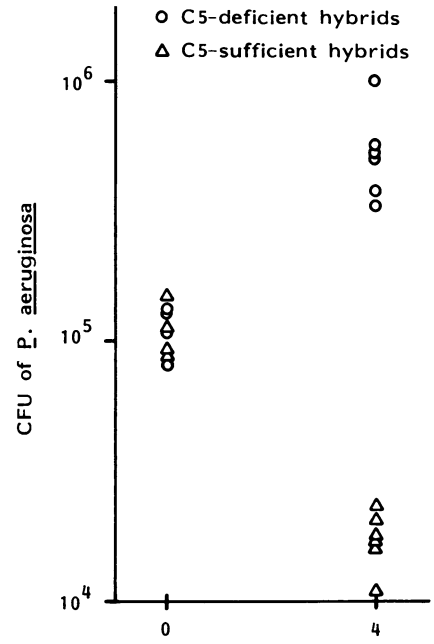

FIG. 4. Individual values of *P. aeruginosa* CFU per lung in C5-sufficient F_1 hybrid (B10.D2/nSnJ × DBA/2J) and C5-deficient F_1 hybrid (B10.D2/oSnJ × DBA/2J) mice after aerosol exposure.

lungs may induce significant tissue damage. Had this occurred in DBA/2J mice, lung clearance of *P. aeruginosa* should have been affected markedly in these animals. It would not be surprising if the C5 molecule were playing a dual role in lung defenses against *P. aeruginosa*, as has been suggested for *S. aureus* (3). In support of this hypothesis is the fact that mice treated with cobra venom factor also showed impaired lung clearance of *P. aeruginosa* (9, 10) although the number of PMN recruited into the airways was no different from that of untreated controls (10).

The role of C5 in the clearance of bacteria from murine lungs is not totally understood. Activation of the terminal components C5b through C9 of the complement system can produce critical injury to bacterial cells by the membrane attack complex. The importance of the bactericidal activity of serum in host defense against *P. aeruginosa* is still unclear (18). It was found, for example, that 91% of blood culture isolates of *P. aeruginosa* are serum resistant (28); however, the majority of saprophytic isolates of *P. aeruginosa* are also serum resistant (29). In addition to chemotaxis, C5 possesses other properties that might be equally important in the clearance of bacteria. Phagocytes have specific receptors for C5a (4, 5), and the stimulation of these receptors promotes a variety of changes in cell metabolism, including induction of lysosomal secretion, cell polarization, production of interleukin-1, and release of inflammatory mediators (2, 8, 11, 23). Thus, C5 or its active fragments could be involved in lung clearance of *P. aeruginosa* not only as a chemotactin(s) but also as a mediator(s) of phagocyte activation.

Pulmonary clearance of *P. aeruginosa* depends also on the mouse strain investigated. The two C5-deficient inbred strains of mice that we studied differ markedly in their patterns of lung clearance of *P. aeruginosa*, indicating that the ability of C5-deficient mice to eradicate *P. aeruginosa* from their lungs is influenced by the genetic background of a given strain. When C5-deficient and -sufficient pairs sharing the same genetic background were compared (B10.D2 strains and F_1 hybrids), it was found that the absence of C5 clearly results in impaired lung defenses against aerosolized *P. aeruginosa* (Fig. 1 and 4).

In conclusion, we studied the pulmonary cell responses and lung clearance of *P. aeruginosa* in two C5-deficient inbred strains of mice, and we present evidence that both phenomena are influenced by genetic background, at least in B10.D2 strains. Deficiency in the fifth component of complement adversely affects lung defenses against *P. aeruginosa* even when PMN are recruited into the airways, and it is suggested that C5 could participate in lung clearance of bacteria as a mediator of phagocyte activation besides its chemotactic role.

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