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Enhancement of *Pseudomonas aeruginosa* Lung Clearance After Local Immunization with a Temperature-Sensitive Mutant

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We investigated the capacity of the temperature-sensitive mutant strain A/10/25 of *Pseudomonas aeruginosa* (ts-*Psa*) to induce enhancement of lung defenses against wild type *P. aeruginosa* (wt-*Psa*). Mice of the DBA/2J inbred strain were immunized by aerosolization with a single dose of 2×10^5 to 4×10^5 CFU of ts-*Psa* and were challenged 7, 14, and 21 days later with wt-*Psa*. The uncleared bacteria ratio was determined 4 h after aerosol exposure; significant enhancement in lung clearance of wt-*Psa* ($P < 0.01$) was evident as early as 7 days after immunization and detectable for at least 21 days. Aerosol immunization with *Staphylococcus aureus* did not enhance lung clearance of wt-*Psa*; however, slight but significant enhancement in *S. aureus* clearance was observed in mice immunized 7 days before with ts-*Psa*. No enhancement of *S. aureus* clearance was seen in ts-*Psa* immunized animals after 14 and 21 days. Analysis of the cell composition of lung lavage fluids revealed a transient cell response characterized by rapid increase in the absolute number of polymorphonuclear leukocytes, followed later by an increase in alveolar macrophages. The characteristics of lung lavages returned to base-line values 6 days after aerosol immunization, and a second exposure to a ts-*Psa* aerosol produced a response of similar magnitude and quality. We conclude that aerosol immunization with a temperature-sensitive mutant of *P. aeruginosa* enhances specific pulmonary defense mechanisms against the parental pathogen in mice.

Bacteria deposited by aerosol are rapidly cleared from the murine respiratory tract. Early studies reported in 1964 by Green and Kass (7) suggested that phagocytosis by alveolar macrophages was the most important clearance mechanism, but this relatively simple explanation was later revised and new hypotheses were put forward. It is currently accepted that early lung clearance of bacteria is accomplished by a variety of mechanisms acting in concert; the existence of different mechanisms for different bacterial genera has also been suggested (23). Pulmonary clearance of bacteria occurs at different rates in different experimental situations and may be influenced by (i) the mouse strain used (22), (ii) the challenging species aerosolized (8), and (iii) the number of bacteria deposited in the lungs (25). There also exists a variety of environmental, pharmacological, and chemical factors that can diminish the normal ability of mouse lungs to clear infectious agents (6). Abnormal clinical conditions, including preexisting viral infection, can further decrease lung clearance of bacteria (5, 10). One common feature of most murine lung clearance studies is that they have addressed the deleterious effects on lung defenses of different agents or experimental conditions rather than enhancement of defense mechanisms by any method. In fact, only a few studies have described lung clearance improvement. Among them, Jakab and Green (11) and, subsequently, LaForce (12) have shown that aerosol immunization enhances pulmonary clearance of gram-negative bacilli. In studies with *Proteus mirabilis* (9), Jakab also noted that aerosolization with the live microorganism was more effective in inducing enhancement than using killed bacteria or immunization by other routes. Recently, a method for producing a live vaccine for *Pseudomonas aeruginosa* was described: the combination of several coasting temperature-sensitive mutations in one strain of *P. aeruginosa*, ensuring safety (reversion rates would be negligible) and enhancing efficacy (the strain would continue to replicate for a limited number of generations at 37°C, mimicking the initial stages of a natural infection [15]). The present study was undertaken to investigate the

TABLE 1. Localization of viable ts-*Psa* after aerosol exposure

Time (h) after exposure	CFU per mouse in[a]:				
	Lung	Esophagus	Stomach	Duodenum[b]	Blood[c]
T_0	$(2.18 \pm 0.30) \times 10^5$	835 ± 147	1,378 ± 1,375	<10	<3
T_2	$(2.91 \pm 0.37) \times 10^5$	140 ± 64	1,074 ± 406	<10	<3
T_4	$(1.53 \pm 0.54) \times 10^5$	11 ± 7	<33[d]	<10	<3

[a] Expressed as mean ± standard error of the mean. Four animals were sacrificed at each time, and cultures were performed in triplicate.
[b] The limit of detection was 10 CFU; 0.3 ml of lavage fluid was plated.
[c] The limit of detection was 3 CFU per ml of blood; 0.3 ml of blood was plated.
[d] The limit of detection was 33 CFU, since 0.3 ml of lavage fluid was plated.

ability of a temperature-sensitive mutant of *P. aeruginosa* (ts-*Psa*) to induce enhancement in lung defenses against wild-type *P. aeruginosa*. The airborne route for local delivery was chosen, and some of the effects of the ts-*Psa* aerosol on the host were also addressed.

MATERIALS AND METHODS

Mice. Five-week-old male mice of the DBA/2J inbred strain were purchased from Jackson Laboratories (Bar Harbor, Maine) and kept at our laboratory until they weighed 18 to 22 g, under conditions described by the "Guide for the Care and Use of Laboratory Animals" DHEW Publication no. (NIH) 78-23.

Bacteria and culture conditions. The temperature-sensitive mutant A/10/25 (ts-*Psa*), previously isolated and characterized in our laboratory (15), was used for local immunization. This mutant grows well at 28°C and continues to divide for two generations after temperature shift to 36°C, before ceasing growth completely. The strain is propagated routinely on tryptic soy agar at 28°C. To prepare ts-*Psa* suspensions for nebulization, 10 ml of tryptic soy broth was inoculated with a loopful from an overnight plate and incubated at 28°C and 400 rpm in a G-24 water shaker bath (New Brunswick Scientific Co., New Brunswick, N.J.). Cultures were harvested in mid-log phase by centrifugation at 12,000 × *g* for 10 min at 4°C. The pellet was resuspended to 10^9 CFU/ml in cold saline. The parental wild-type, Fisher-Devlin Immunotype 1 strain of *P. aeruginosa* (wt-*Psa*) was prepared in similar fashion, except the cultures were incubated at 37°C. *Staphylococcus aureus* ATCC 25923 was also used for aerosol immunization and challenge in experiments designed to test the specificity of clearance enhancement.

Aerosol immunization and challenge. The animals were exposed to the aerosol in a nebulization chamber built in our laboratory to the specifications of a previously described design (14), for 30 min at 22 to 24°C and at −2 mm water pressure. Immunization consisted of a single deposition of 2×10^5 to 4×10^5 CFU per animal of aerosolized ts-*Psa* or *S. aureus*; controls were nebulized with saline. The animals were challenged 7, 14, and 21 days later in the same manner; each mouse received 2×10^5 to 4×10^5 CFU of wt-*Psa* or *S. aureus*. A third group of untreated DBA/2J mice was included in each run as a second control. Immediately after exposure (t_0), half of the animals were sacrificed; the remainder were sacrificed 4 h later (t_4). The lungs were excised, weighed, and homogenized in 5 ml of ice-cold distilled water with a Potter-Elvehjem glass homogenizer. The homogenates were diluted appropriately in distilled water and cultured quantitatively on tryptic soy agar; the ratio of the arithmetic means of bacterial counts at t_4/t_0 represented the uncleared bacteria ratio (UBR), an inverse expression of lung bacterial clearance. Standard errors were calculated by the method described by Wilks (26).

Organ lavage and fluid analysis. Post-mortem lung lavages were performed in several groups of animals at different times after exposure to ts-*Psa* aerosols. Six sequential lavages were carried out with the appropriate volume of sterile saline at 37°C, according to a procedure described previously (21). The cells in the lavage fluids were collected by centrifugation for 5 min at 4°C, 180 × *g* and suspended in 1 ml of Hanks balanced salt solution, and the total number of cells was counted in a hemocytometer. Smears were made by cytocentrifugation and stained with Giemsa or assayed for nonspecific esterase activity. Several animals were exposed to a ts-*Psa* challenge and were sacrificed at t_0, t_2, and t_4. The proximal portion of the esophagus and the distal part of the duodenum were clamped, and those two organs and stomach were dissected together, with incisions immediately below the pharynx and intestine, 25 mm from the pylorus. The esophagus, stomach, and duodenum were washed internally with 1, 10, and 3 ml of saline, respectively; the suspensions were diluted appropriately and plated quantitatively. Blood was obtained from the retro-orbital venous sinuses of the same mice, using heparinized capillary tubes, and was plated for culture.

RESULTS

The survival of ts-*Psa* was similar to that for wt-*Psa* during the 30-min nebulization procedure. Aerosolization of suspensions containing 10^9 CFU of either ts-*Psa* or wt-*Psa* per ml in the reservoir of the nebulizer delivered 2×10^5 to 4×10^5 CFU per mouse lung. Samples from the reservoir, taken before and after nebulization, revealed no significant changes in the number of CFU. Most (98%) of the *Pseudomonas* isolated from the animals were in the lungs, whereas 1.7% were recovered from the gastrointestinal tract; no bacteria were isolated from the blood after nebulization (Table 1). Other gram-negative bacilli and gram-positive cocci were simultaneously isolated in various numbers from the

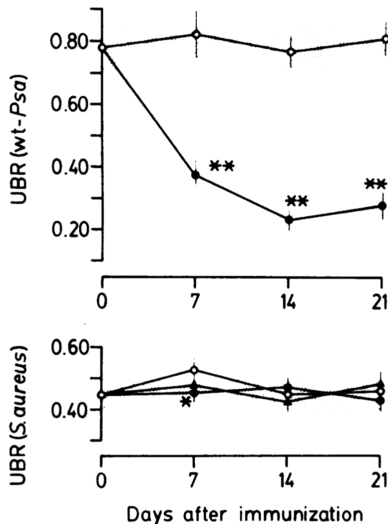

FIG. 1. Changes in UBR after immunization of DBA/2J mice with 2×10^5 to 4×10^5 CFU of ts-*Psa* (●) or 2×10^5 to 4×10^5 CFU of *S. aureus* (▲). Controls were aerosolized with saline (○). Challenges were made with wt-*Psa* (top) or *S. aureus* (bottom). Shaded areas indicate the arithmetic mean ± standard error of the mean (SEM) of the UBR from mice without previous exposure to any aerosol. Each point represents the arithmetic mean ± SEM from 6 to 14 pairs of mice. Significance levels, evaluated by Mann-Whitney *U* test, were $P < 0.05$ (*) and $P < 0.01$ (**).

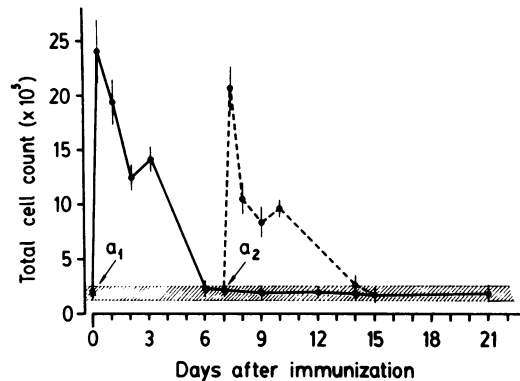

FIG. 2. Changes in the total number of pulmonary cells recovered by sequential lung lavages of DBA/2J mice after one (a_1, —) or two (a_1+a_2, ---) exposures to aerosols of ts-*Psa*. Each point represents the arithmetic mean ± SEM from 3 to 10 animals. The shaded area represents the arithmetic mean ± SEM of the total cell count from lavage fluids of nonaerosolized animals.

esophagus and stomach; no viable bacteria were recovered from the duodenum.

Mice immunized with ts-*Psa* or *S. aureus* and saline-treated and untreated control mice were challenged with wt-*Psa*, and the UBR was determined. Significant enhancement of pulmonary clearance, represented in Fig. 1 as a decrease in the UBR, was found in immunized mice when compared with controls. Enhancement was evident as early as 7 days after immunization and was detectable for at least 21 days. Pulmonary clearance of wt-*Psa* in animals immunized 7 days before with *S. aureus* did not differ significantly from controls. Pulmonary clearance of *S. aureus* was determined at 7, 14, and 21 days after the animals received an aerosol containing ts-*Psa*, *S. aureus*, or saline alone; the results are shown in Fig. 1. Immunization with ts-*Psa* induced a slight enhancement in *S. aureus* lung clearance at day 7 only ($P < 0.05$). At days 14 and 21, the UBRs from all groups were no different from those of control animals.

Lung lavages were performed at various times after aerosolization with ts-*Psa*, beginning at 4 h and for up to 21 days later. The total number of cells recoverable by lung lavage rose sharply to 2.5×10^6 (Fig. 2) as early as 4 h after aerosolization. The eightfold increase in total number, when compared with nonaerosolized controls, was due to an influx of polymorphonuclear leukocytes (PMNs); the percent of PMNs in the population increased from 1% in untreated animals to 96% 4 h after nebulization with ts-*Psa*. By 48 h after nebulization, the total number of cells had decreased to approximately 12×10^5 (four to five times resting levels), and the percentage of PMNs had decreased from 96 at the peak (t_4) to 56, indicating an influx of macrophages. Twenty-four hours later, the total number of cells appeared to rise again while the number of PMNs continued to decline. Resting levels were reached 6 to 7 days after nebulization and maintained for at least 14 more days. A second exposure of the animals to ts-*Psa* produced a respiratory cell response of similar pattern but of lesser magnitude (Fig. 2). Nevertheless, the response was associated with significant enhancement (64%) of the lung clearance of wt-*Psa*. A single aerosol of *S. aureus* did not induce any significant change in the lung free cell population for the first 24 h. The total number of cells recoverable by lung lavage did not differ from that in controls, and the population consisted of more than 92% alveolar macrophages and less than 5% PMN by 4 h after exposure to the aerosol. The cell population of lung lavage fluids 24 h after exposure did not differ from that obtained by lavage of nonaerosolized controls.

DISCUSSION

P. aeruginosa is a frequent cause of infections in patients with underlying diseases that severely impair host defense mechanisms (2, 4) or apparently provide an unusually appropriate mi-

croenvironment for bacterial growth (1). Such infections do not always respond to aggressive antibiotic therapy and often result in appreciable morbidity and mortality. In the last decade, the need for alternative therapies to eradicate *P. aeruginosa* from compromised hosts has been recognized, and special attention has focused on the cystic fibrosis respiratory tract (R. E. Wood, Abstracts of the Symposium on Basic and Clinical Research in *Pseudomonas*, p. 14, 1975. Medical College of Virginia, Virginia Commonwealth University, Richmond). Immunoprophylaxis is one approach to the problem, and several types of vaccine preparations have been tested in animal models (16, 17, 20) and humans (19). The use of vaccines to prevent *Pseudomonas* pneumonia or to ameliorate its effects is, however, still a matter of some controversy. Pennington and Kuchmy have suggested that local immunization is not required to provide adequate local *Pseudomonas* opsonins during acute pneumonia (18). Nevertheless, some patients could still benefit from local immunization. In fact, since it has been suggested that cystic fibrosis patients have an opsonic defect for *Pseudomonas* (3), presumably located at the phase of attachment of specific immunoglobulin G to alveolar macrophage Fc receptors, other effectors of humoral and cell-mediated immunity may be induced by local immunization.

We therefore decided to examine the immunogenic properties of a temperature-sensitive mutant of *P. aeruginosa*, in an attempt to characterize some of the events occurring in lungs exposed to aerosols of the organism. Aerosolization was chosen for the inoculations because it is painless and does not involve any additional manipulation of the respiratory organs, such as anesthesia or surgery for intratracheal injection. Aerosolization allows the deposition of the particulate immunogen in the lower respiratory tract, although more investigation is needed to develop a reliable and reproducible method of delivering the precise amounts of antigen which would be needed if the system were to be developed for human application. In the murine model, exposure to the aerosol has proven successful; most bacteria which impact in the upper airways may also provide additional benefits by inducing local mechanisms to prevent colonization of the nasopharynx. Deposition of a dose of 1×10^7 to 3×10^7 live ts-*Psa* (CFU/kg of body weight) in the lungs produced a mild inflammatory reaction no different from that elicited by the wt-*Psa* (this was tested in C5-deficient DBA/2J mice and also in the C5-sufficient DBA/1J inbred strain [unpublished data]). This response would mimic that occurring during the initiation of natural *Pseudomonas* infection, thus stimulating local specific defenses. The single dose of ts-*Psa* induced, 7 days later, a slight enhancement in the clearance of *S. aureus*, probably as a consequence of the last stages of the transient local inflammatory reaction. Clearance of *S. aureus* and cellular characteristics of lung lavage fluid returned to control levels by 14 days after immunization. Immunization of DBA/2J mice with *S. aureus* aerosols produced an almost insignificant change in the percentage of PMNs in lung lavage fluids; those obtained 24 h after exposure to the aerosol did not differ from controls (D. O. Sordelli, B. J. Zeligs, M. C. Cerquetti, A. Morris Hooke, and J. A. Bellanti, submitted for publication). Immunization with *S. aureus* did not result in any modification of lung clearance of wt-*Psa*, whereas immunization with ts-*Psa* produced an enhancement of wt-*Psa* clearance which lasted for at least 21 days. Enhancement did not seem to be related to the magnitude or quality of the cell response, at least when tested 7 days after immunization, but the duration of the wt-*Psa* lung clearance enhancement suggests that specific responses are involved. In fact, previous studies have demonstrated that intranasal immunization of rabbits with *Pseudomonas* lipopolysaccharide elicits a humoral response characterized by production of specific IgG, IgM, and IgA antibodies. A cellular response, assessed by release of migration inhibition factor from immune lymphocytes in respiratory cell suspensions, was also detected (24). Other studies, by LaForce and Boose, have suggested that macrophage activation, leukocyte recruitment, and local antibody are important contributing factors to heightened lung activity after aerosol immunization with *Serratia marcescens* (13). We are currently investigating the basic mechanisms responsible for the enhancement of lung clearance of *P. aeruginosa* induced by a single aerosol exposure to a temperature-sensitive mutant. In addition, we are extending the specificity studies to include challenge with other immunotypes.

In conclusion, we were able to induce enhancement of specific lung defense mechanisms against *Pseudomonas* by local, aerosol immunization of mice with ts-*Psa*, confirming that such a mutant may have a role in immunoprophylaxis of patients susceptible to pulmonary infection with this pathogen.

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