Specific Pulmonary Defences against \textit{Pseudomonas aeruginosa} after Local Immunization with Temperature-sensitive Mutants

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The specificity of the enhancement in lung defences after local immunization of mice with three temperature-sensitive (ts) mutants of \textit{Pseudomonas aeruginosa} was investigated. The three selected mutants display altered growth characteristics when transferred from 29 °C to mammalian body temperature. Mice immunized with the live ts mutants by aerosol exposure or multiple intranasal inoculations were challenged with aerosols containing wild-type (wt) \textit{P. aeruginosa}. Aerosol immunization with ts mutant A/10/25 significantly enhanced the lung clearance of the wt but did not enhance the clearance of either \textit{Klebsiella pneumoniae} or \textit{Staphylococcus aureus}. Aerosol immunization with ts mutants D/1/8 or E/9/9 enhanced the lung defences against the parental wt (of identical immunotype 1) but not against immunotype 4; similarly, intranasal immunization enhanced the lung defences against the parental wt but not against immunotypes 4 or 5. We conclude that local immunization with ts mutants of \textit{P. aeruginosa} enhances lung defences against the wt in a genus- and immunotype-specific fashion. It is suggested that local immunity may play a central role in immunoprophylaxis against \textit{P. aeruginosa} lung infection.

INTRODUCTION

Temperature-sensitive (ts) mutants of \textit{Pseudomonas aeruginosa} were isolated in our laboratory with the ultimate aim of constructing a live vaccine strain (Morris Hooke \textit{et al.}, 1982). These mutants replicate well at low temperatures (below 32 °C) but do not replicate, or undergo a limited number of replications, when transferred to mammalian body temperature. The safety of an actual ts vaccine strain would be ensured by the presence of multiple ts mutations, each of identical phenotype, resulting in a negligible reversion frequency (about $10^{-21}$) (Morris Hooke \textit{et al.}, 1985). Preliminary immunological evaluation has shown that immunization with ts mutants carrying one genetic lesion induces protection against intraperitoneal challenge with the wild-type (wt) (Morris Hooke \textit{et al.}, 1982, 1987). Because of our interest in developing prophylactic procedures against \textit{P. aeruginosa} pneumonia, we performed experiments using an animal model of aerosol challenge. In a previous report we showed that local immunization of C5-deficient DBA/2J mice with the ts mutant A/10/25 of \textit{P. aeruginosa} induces an increase in the pulmonary clearance of the wt (Sordelli \textit{et al.}, 1983). It was also seen that exposure to an aerosol of this mutant elicits cell responses in the lower airways (Sordelli \textit{et al.}, 1985d) and that the mutant produces factors which cause positive chemotaxis of polymorphonuclear leucocytes, findings we had observed with the wt (Sordelli \textit{et al.}, 1985b). The present study was undertaken

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Abbreviations: ts, temperature-sensitive; UBR, uncleared bacteria ratio; wt, wild-type.
to determine the specificity of the enhancement of lung defences induced by local immunization of DBA/2J mice with three different ts mutants of *P. aeruginosa*.

**METHODS**

**Bacterial strains and cultures.** The ts mutants D/1/8, A/10/25 and E/9/9 of *P. aeruginosa* were isolated after mutagenesis with nitrosoguanidine (Morris Hooke *et al.*, 1982). These three mutants were included in the study because they exhibit different growth properties after transfer to the non-permissive temperature (> 36°C): D/1/8 is a 'tight' mutant that stops growth immediately after transfer, whereas E/9/9 is a 'coaster' mutant that continues to divide for five generations after similar transfer; A/10/25 is a 'moderate coaster' that replicates two or three times after temperature shift. The parental wt, *P. aeruginosa* Fisher-Delvin-Gnasabik (F-D-G) immunotype 1 was obtained from Dr C. Heifetz (Parke-Davis, Detroit, Mich., USA) and *P. aeruginosa* immunotypes 4 and 5 were from Dr Jerald Sadoff (Walter Reed Army Institute of Research, Washington, DC, USA). *Staphylococcus aureus* ATCC 25923 and *Klebsiella pneumoniae* ATCC 27736 were obtained from the American Type Culture Collection (Rockville, Md, USA). The bacteria were propagated on tryptic soy agar (England Laboratories). Plates were incubated at 29 °C (ts mutants) or 37 °C (wt strains). In order to prepare ts *P. aeruginosa* suspensions for production of aerosols, 50 ml Trypticase Soy Broth (Difco) was inoculated with a loopful from a fresh plate culture and incubated at 29 °C with shaking (300 r.p.m.) in a G24 Environmental Incubator Shaker (New Brunswick Scientific). Cultures in mid-exponential phase, at an OD₆₀₀ equivalent to 1 × 10⁵ c.f.u. ml⁻¹, were harvested by centrifugation at 12 000 g for 10 min at 4 °C. The pellet was suspended to the appropriate cell density in cold saline (0.145 m-NaCl). Suspensions of wt *P. aeruginosa*, *S. aureus* or *K. pneumoniae* were prepared in a similar manner, but the cultures were incubated at 37 °C.

**Mice and exposure to the aerosol.** Five-week-old male, inbred DBA/2J mice were obtained from Jackson Laboratory and kept at our facility until they were 8 weeks old, under conditions described by the 'Guide for the Care and Use of Laboratory Animals', Department of Health, Education and Welfare Publication no. 78-23 (NIH). The animals were exposed to the aerosol containing bacteria for 30 min, in a nebulization chamber built at our laboratory to the specifications of a previously described design (Henderson, 1952; Sordelli *et al.*, 1983). A 9 ml volume of a bacterial suspension (5 × 10⁵ c.f.u. ml⁻¹) was placed in the nebulizer reservoir and 6 ml was consumed in each run.

**Immunization of mice.** Two methods of inoculation were used. In order to investigate genus-specific (i.e. immunization with *P. aeruginosa*, and challenge with *P. aeruginosa*, *S. aureus* or *K. pneumoniae*) enhancement of lung defences to *P. aeruginosa*, groups of animals were immunized by exposure to an aerosol containing ts mutants of *P. aeruginosa*. Each mouse received an intrapulmonary dose of about 1 × 10⁵ c.f.u. Control groups were challenged by exposure to the aerosol with *S. aureus*, *K. pneumoniae* or saline. The challenge with wt organisms was delivered 3 weeks after immunization. To demonstrate immunotype-specific (i.e. immunization with ts immunotype 1 and challenge with immunotype 1, 4 or 5) enhancement of lung defences, mice were inoculated intranasally with 10 μl of a suspension containing ts mutant D/1/8 or E/9/9. Each mouse received 5 × 10⁵ c.f.u. and intranasal applications were repeated in the same fashion 7 and 14 d later. Control groups received 10 μl saline. Seven days after the last inoculation the animals were challenged with aerosols of *P. aeruginosa* immunotypes 1, 4 or 5.

**Pulmonary challenge.** The lung clearance of bacteria was measured by killing six mice immediately after exposure to the aerosol (t₀) and the remainder 4 h later (t₄). The lungs were excised and homogenized in 5 ml ice-cold distilled water with a Potter-Elvehjem glass grinder (A. H. Thomas). Homogenates were diluted appropriately in distilled water and cultured quantitatively on tryptic soy agar; colony counts for lung lavages were performed in animals killed 7 d after the third intranasal application of ts mutants D/1/8 or E/9/9. IgA and IgG antibodies to *P. aeruginosa* surface antigens were evaluated by a micro-ELISA technique (Sordelli *et al.*, 1985c). Gentamicin–carbenicillin-treated *P. aeruginosa*, F-D-G immunotypes 1, 4 or 5, were used as ELISA antigens; serum samples were diluted 1 in 100 and 1 in 300. The conjugates were affinity-purified goat anti-mouse γ-chain IgG conjugated with alkaline phosphatase (Cappel Laboratories) or affinity-purified goat anti-mouse α-chain IgG conjugated with alkaline phosphatase (Kirkegaard and Perry). After addition of the substrate (p-nitrophenyl phosphate, Sigma), plates were incubated for 45 min and the A₄₀₅ was recorded in an ELISA Micro-reader (Dynatech). Results were expressed as the A₄₀₅ of a given sample and compared with the A₄₀₅ generated in the assay with sera from non-immunized control animals.

**Statistical considerations.** The UBR was expressed as the arithmetic mean ± SEM, which was calculated by the
method of Wilks (1962). Individual UBR data were normalized by using the arcsin $\sqrt{\text{UBR}}$ transformation for proportions, and the transformed data were tested for significance by analysis of variance with one criterion of classification for samples of unequal size.

RESULTS

The genus-specificity of the lung defence enhancement against *P. aeruginosa* was investigated by immunizing mice by the aerosol route with the live ts mutant A/10/25. Immunized and non-immunized animals were challenged with the parental wt *P. aeruginosa* and either *S. aureus* or *K. pneumoniae* in separate experiments. Aerosol immunization with A/10/25 significantly enhanced lung defences against *P. aeruginosa* immunotype 1, but not against either *S. aureus* or *K. pneumoniae* (Fig. 1). Immunization with either *S. aureus* or *K. pneumoniae*, on the other hand, did not modify the *P. aeruginosa* UBRs (data not shown). These results indicate that the enhancement in lung defences may be genus-specific and that inflammation induced by immunization with A/10/25 aerosol neither interfered with nor enhanced the defence mechanisms against *S. aureus* and *K. pneumoniae*.

The immunotype-specificity of the enhancement in lung defences against *P. aeruginosa* was investigated in experiments of similar design to those described above. In order to extend the validity of our previous experiments with ts mutants, we immunized mice with two other mutants of different phenotype but identical F-D-G immunotype: a ‘tight’ (D/1/8) and a ‘coaster’ (E/9/9) (see Methods), in similar doses. Because a live vaccine constructed with ts mutants of *P. aeruginosa* could not realistically be used for intrapulmonary inoculation, we investigated the effect of intranasal immunization with live D/1/8 or E/9/9 on lung defences against the wt. This route of inoculation permits more control of the dose administered and does not produce the lung inflammation seen after aerosol inoculation. Intranasal immunization with either D/1/8 or E/9/9 induced significant enhancement in lung defences against the parental wt. The results from challenge of the animals with *P. aeruginosa* immunotypes 4 or 5 showed that the enhancement was immunotype-specific (Fig. 2). Similar results were obtained when the animals were immunized by aerosol with the mutants and challenged with *P. aeruginosa* immunotype 4 (data not shown). It should be noted that although the UBRs of *P. aeruginosa* immunotypes 1, 4 and 5 from control animals are significantly different (1 vs 4, $P < 0.01$; 1 vs 5, $P < 0.01$), the

![Fig. 1. Effect of aerosol immunization with ts mutant A/10/25 of *P. aeruginosa* or saline (control) on the UBR of wt *P. aeruginosa*. The enhancement in lung defences against wt *P. aeruginosa* (seen in this figure as a decrease in UBR) after immunization with the ts mutant was significant (**, $P < 0.01$). NS, Not significant. Each bar represents the arithmetic mean ± SEM from six to eight immunized mice from each group or controls killed 4 h after aerosol challenge ($t_a$).](image-url)
Immunized with:
- Saline
- D/1/8
- E/9/9

Fig. 2. Effect of intranasal immunization with ts mutants D/1/8 or E/9/9 (both of F-D-G immunotype 1) on the UBR of P. aeruginosa F-D-G immunotype 1, 4 or 5. The enhancement in lung defences against P. aeruginosa immunotype 1 induced by both mutants was significant (**, P < 0.01). Each bar represents the arithmetic mean ± SEM from groups of six or seven mice killed at t4.

clearance rates of different Pseudomonas strains are often completely different and should not be compared per se in this study (Sordelli et al., 1985d; Southern et al., 1970).

In order to determine whether the enhancement in lung defences correlated with antibody responses induced by the immunogens, the levels of anti-P. aeruginosa IgA and IgG were measured in lung lavage fluids and sera from mice immunized intranasally with D/1/8 or E/9/9. Groups of immunized and control mice were killed 7 d after the third intranasal administration of the immunogens. No anti-P. aeruginosa immunotype 1 antibody activity was found in unconcentrated lung lavage fluids. Although intranasal immunization with either D/1/8 or E/9/9 enhanced lung defences (see above), it did not induce significant increases in the level of serum anti-P. aeruginosa IgG (Fig. 3). Intraperitoneal immunization with $4 \times 10^6$ c.f.u. of either mutant, on the other hand, induced a significant increase in the levels of IgG antibodies, when compared with those of animals injected with saline (Fig. 3). The same animals were challenged with $1 \times 10^5$ c.f.u. of P. aeruginosa immunotype 1 and the UBR determined 4 h after aerosol exposure. No significant enhancement in lung defences was observed (data not shown), indicating that circulating antibodies may not participate in the enhancement of lung defences seen after local immunization.

**DISCUSSION**

In this report we show that local immunization of the respiratory tract with ts mutants enhances lung defences against P. aeruginosa in a genus- and immunotype-specific fashion. Local immunity seems to play a more important role than circulating antibodies in the defence of the lungs against P. aeruginosa in the early stages of infection. Before the importance of these findings is discussed, certain aspects of the animal model used in this investigation merit consideration.

Bacteria presented to mice as aerosols are deposited uniformly in the lower airways, usually as single cells, rarely in clusters (Jakab, 1976). The density of bacteria deposited on respiratory epithelial surfaces of comparable area by aerosol exposure is, therefore, considerably lower than that obtained when animals are inoculated with an intratracheal or intrabronchial bolus containing identical doses of bacteria. In addition, other factors such as the animal species used and the method of inoculum preparation may also produce quite different models of P. aeruginosa infection. Most of the animal models currently utilized represent either chronic or
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Acute *P. aeruginosa* lung infection. Our model is one of *P. aeruginosa* lung clearance rather than of *P. aeruginosa* infection because the inoculum is cleared within hours and no animals die. This model is particularly useful for studying the host's defences against *P. aeruginosa* in the early stages of lung colonization, and provides a useful tool for testing immunotherapeutic protocols.

In previous studies of lung lavage fluids from mice we detected transient (5–7 d) changes in the quality and quantity of the free cell population of the lower airways after aerosol challenge with *P. aeruginosa* (Sordelli et al., 1985d), but histopathological analysis of lung sections showed that mononuclear infiltration and congestion did not resolve until 3–5 weeks later (Sordelli et al., 1985a). It seemed important, therefore, to evaluate whether lung inflammation caused by aerosol inoculation of the *P. aeruginosa* mutants enhances non-specifically the lung defences against other pathogens, and to determine the degree of non-specific enhancement of lung defences against *P. aeruginosa* induced by aerosols of *S. aureus* and *K. pneumoniae*, species that induce minimal and considerable lung inflammation respectively (Pierce et al., 1977; Toews et al., 1979). Non-specific enhancement of clearance could not be detected in either system.

Lung defences against *P. aeruginosa* immunotype 1 were enhanced not only by aerosol but also by intranasal immunization with ts mutants possessing different growth characteristics at mammalian body temperature, the 'tight' D/1/8 and the 'coaster' E/9/9, derived from the parental wt immunotype 1. *P. aeruginosa* of different immunotypes share a certain number of surface antigenic determinants (Hedstrom et al., 1984; Mutharia et al., 1982). Immunization with the two ts mutants, however, neither enhanced the lung clearance of *P. aeruginosa* immunotypes 4 and 5 nor elicited detectable levels of serum IgG to surface antigens of *P. aeruginosa* immunotype 5. These findings suggested that immunity elicited by the ts mutants was directed towards the antigens that determine the specificity of the *P. aeruginosa* immunotypes, but we have no information on the mechanism(s) responsible for that immunity.

Systemic immunization with formalin-killed *P. aeruginosa* seems to augment the lung defences against *P. aeruginosa* in an animal model of challenge by intrabronchial bolus containing the bacteria. Dunn et al. (1985) injected *P. aeruginosa* in challenge doses 29–460 times higher than those we administered to DBA/2J mice by the aerosol route. Anti-*P. aeruginosa*
serum antibody exudes into the airways of mice challenged with such high doses of \textit{P. aeruginosa}, and may have been responsible for the augmentation in lung defences against \textit{P. aeruginosa}. Interestingly, in the mouse model we used, circulating anti-\textit{P. aeruginosa} IgG does not appear to mediate the enhancement in lung defences because parenteral immunization with ts mutants elicited circulating antibodies but did not enhance the lung clearance of wt \textit{P. aeruginosa}. In other words, the enhancement in lung defences seemed to be related to local rather than systemic immunity when \textit{P. aeruginosa} was introduced into the lower airways in low doses.

The mechanism of local immunity responsible for mediating the enhancement in lung clearance of wt \textit{P. aeruginosa} aerosol is not known. Reynolds (1974) has reported that agglutinating antibodies to \textit{Pseudomonas} lipopolysaccharide were found in very low titres in concentrated (100-fold) bronchial secretions from rabbits. We were unable to detect the presence of antibodies in (unconcentrated) murine lavage fluids, but we cannot rule out the local production of immunoglobulins as one possible mechanism. Reynolds (1974) has also reported that intranasal immunization with lipopolysaccharide from \textit{P. aeruginosa} immunotype 2 induces cell-mediated immunity in the lung. The significance of cell-mediated immunity to \textit{P. aeruginosa} (in mice) was later suggested by Markham et al. (1984), who showed that T-cells provide protective immunity against the micro-organism, in the absence of antibody, and that a lymphokine mediates the killing of the bacteria. Cell-mediated immunity induced by ts mutants may, therefore, also be involved in the enhancement of lung defences against \textit{P. aeruginosa}. We speculate that these immune mechanisms may be particularly important before \textit{P. aeruginosa} colonizes the lower respiratory tract and may have relevance in immunoprophylaxis. Once these mechanisms are overwhelmed and \textit{P. aeruginosa} pneumonia is established, the immunological pattern of the lung defences of the host would change and other mechanisms, e.g. circulating antibodies, may become crucial in restricting the infection to the lungs. We hypothesize that local immunization with ts mutants of \textit{P. aeruginosa} may prevent or delay the onset of \textit{Pseudomonas} infection in the lower respiratory tract of patients with underlying disease, e.g. cystic fibrosis.

In conclusion, we have demonstrated that local immunization with ts mutants of \textit{P. aeruginosa} induces genus- and immunotype-specific enhancement of lung defences against the wild-type. Local immunity, either humoral or cell-mediated, appears to play a role in this enhancement. Further studies are needed to ascertain the precise nature of the immune mechanisms involved.

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**REFERENCES**


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