INFECTION AND IMMUNITY, Oct. 1982, p. 136–140
0019-9567/82/100136-05$02.00/0

Vol. 38, No. 1

Temperature-Sensitive Mutants of *Pseudomonas aeruginosa*: Isolation and Preliminary Immunological Evaluation

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Received 3 March 1982/Accepted 2 June 1982

The immunogenicity of two temperature-sensitive (ts) mutants of *Pseudomonas aeruginosa* immunotype 1, isolated and characterized for the development of a safe, live vaccine strain, was evaluated in a mouse protection model. One mutant, A/10/25, had a limited "coasting" property (i.e., continued replication for two divisions) at the nonpermissive temperature (36°C), whereas the other mutant, E/9/9, continued replication for five generations after transfer to 36°C. Groups of 3- to 5-week-old ICR mice were immunized intraperitoneally with various doses of the two ts mutants; at various times thereafter, the mice were challenged intraperitoneally with lethal doses of the parental wild type. The more extensive coaster, E/9/9, induced 100% protection at immunizing doses lower than those required for A/10/25 to induce the same protection (1×10^8 to 2×10^8 and 6×10^8 colony-forming units, respectively). Both ts strains induced significant protection for up to 5 weeks after immunization. The results of these studies suggest that the use of *P. aeruginosa* ts mutants might provide a novel approach to the prevention of *P. aeruginosa* colonization of patients with cystic fibrosis.

Pseudomonas aeruginosa infection of patients with cystic fibrosis (CF) poses a major problem; once the lungs are colonized, the organism is almost impossible to eradicate (26). Colonization persists in the face of high titers of serum antibody to the pathogen and normally therapeutic levels of antibiotics (11). To our knowledge, no attempts have been made to immunize CF patients before they are colonized, and currently available topical or systemic vaccines are inappropriate or ineffective in terminating the infections (1, 20). Those vaccines and others under experimental investigation are composed of lipopolysaccharides, high-molecular-weight polysaccharides, outer membrane proteins, and inactivated or cross-reacting toxin-like molecules extracted from cultures of the major serotypes (4, 9, 13, 16–19, 21, 24). Together with heat-killed and Formalin-inactivated whole cells, they suffer from the diminished immunogenicity which almost invariably results from the procedures required for their preparation.

Stimulation of local defenses against respiratory and enteric pathogens by the live agent, either through natural infection or through immunization with an attenuated strain, has long been recognized as the most effective inducer of lasting immunity (3). With this in mind, we have begun the development of a safe, live, attenuated vaccine for *P. aeruginosa* and report here the preparation and preliminary immunological evaluation of two temperature-sensitive (ts) strains capable of limited but controlled replication in the host ("coasters").

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MATERIALS AND METHODS

Bacteria and culture media. The Fisher-Devlin immunotype 1 strain of *P. aeruginosa* was obtained from Carl Heifetz (Parke-Davis, Detroit, Mich.). The strain was routinely propagated on tryptic soy agar or cultured in tryptic soy broth, both from Difco Laboratories, Detroit, Mich. All mutagenesis and selection procedures were performed in G broth or on G agar (15).

Mutagenesis, enrichment, and mutant selection. The general procedure used for mutagenesis and enrichment has been described in detail previously (14). Briefly, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (in acetone) was added at 20 μg/ml to log-phase cultures of *P. aeruginosa*. After 10 min at 37°C (without aeration), the cultures were washed three times, suspended in G broth, and incubated overnight at the permissive temperature (27°C) before being subjected to two 12-h cycles of enrichment, one with carbenicillin (10 mg/ml) and one with D-cycloserine (50 mM). The addition of the antibiotics was delayed for 1 to 2 h after the cultures were shifted to the nonpermissive temperature (36°C) to enrich for ts mutants of the coasting phenotype. The surviving cells were diluted appropriately, plated, and incubated at 27°C. The colonies obtained at 27°C were replica plated and incubated at

TABLE 1. Characteristics of *P. aeruginosa* ts mutants

Mutant[a]	Phenotype[b]	Reversion rate
C/5/2	C	3×10^{-8}
A/10/25	C	1×10^{-8}
B/5/4	T	2×10^{-6}
E/9/9	C	1×10^{-6}
E/7/10	C	1×10^{-6}

[a] The cutoff temperature for all mutants was 36°C.

[b] C, Coasting: cells continue to replicate for a number of divisions after transfer to the nonpermissive temperature. T, Tight: cells cease all growth immediately after transfer to the nonpermissive temperature.

27 and 36°C for the detection of mutants unable to sustain growth at the nonpermissive temperature. We further tested ts isolates for their ability to continue limited replication after being shifted to the nonpermissive temperature, and suitable mutants were subjected to reversion analysis. Liquid cultures were incubated at the permissive temperature and then shifted to 34 and 36°C, and their growth was monitored both spectrophotometrically and by plating appropriate dilutions for quantitation of colony-forming units (CFU). After coasting had terminated, 0.1-ml portions of the culture were plated and incubated at the restrictive temperature for the detection of revertants.

Protection studies. Adult male and female ICR mice were purchased from Flow Laboratories, Dublin, Va., and kept under the conditions described in *Guide for the Care and Use of Laboratory Animals* (publication no. NIH 78-23, Department of Health, Education and Welfare). Bacteria for immunization or challenge were prepared by suspending in sterile saline organisms grown overnight on chocolate agar. All dilutions were performed in sterile saline. The 50% lethal dose (LD_{50}) of parental immunotype 1 was determined by the method of Reed and Muench (22). The mice were immunized intraperitoneally (i.p.) with various doses of ts mutants A/10/25 and E/9/9 and then challenged at various times thereafter with 5 to 90 LD_{50}s of the virulent wild-type parent, and mouse survival was monitored. Saline-inoculated controls usually succumbed by 24 h after challenge. The results were subjected to chi-square analysis.

RESULTS

Mutant isolation and characterization. From two separate mutagenesis-enrichment experiments, 3,072 survivors yielded 12 relatively sta-

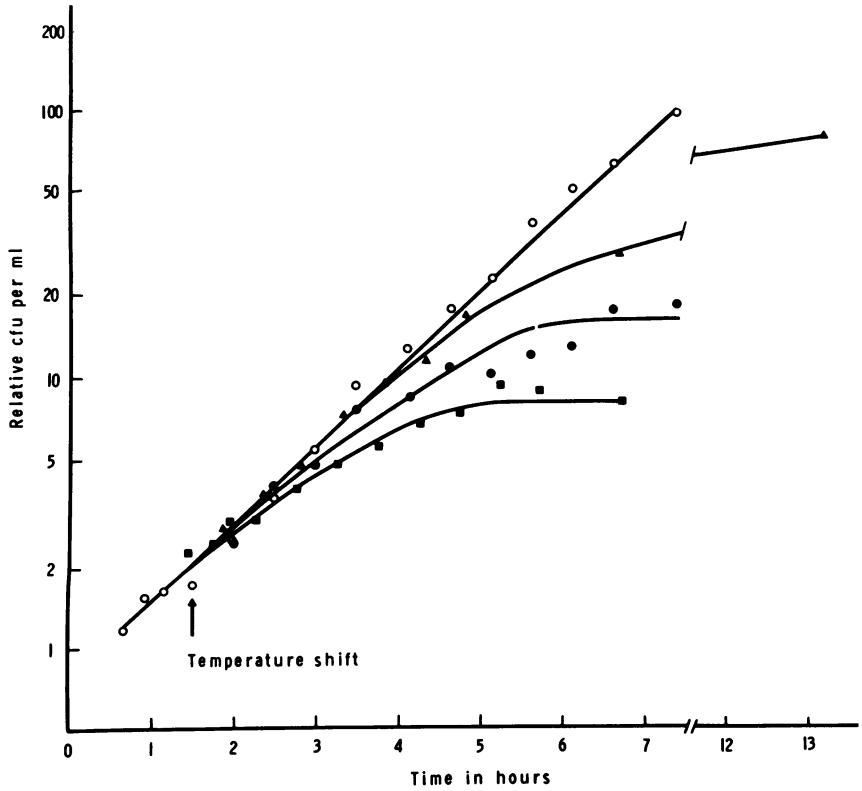

FIG. 1. Growth curves of *P. aeruginosa* ts mutants. Broth cultures of three mutants, A/10/25 (■), E/7/10 (●), and E/9/9 (▲), were grown at 29°C (○) and shifted at the time indicated by the arrow to 36°C.

TABLE 2. Optimum immunizing doses of two *P. aeruginosa* ts mutants

Immunizing dose (CFU)[a]	Challenge dose (CFU)	No. of mice surviving/ no. immunized	Protection (%)
A/10/25			
6.0×10^8	3×10^9	7/7	100[b]
2.0×10^8	3×10^9	6/7	86
6.0×10^7	3×10^9	3/7	43
1.0×10^6	3×10^9	0/7	0
Control	3×10^9	0/7	0
E/9/9			
3.0×10^8	2×10^9	1/5	20
1.5×10^8	2×10^9	5/5	100[c]
3.0×10^7	2×10^9	3/5	60
1.5×10^7	2×10^9	2/5	40
3.0×10^6	2×10^9	0/5	0
Control	2×10^9	0/5	0

[a] Groups of 3- to 5-week-old ICR mice were immunized i.p. with the dose indicated and challenged 7 days later with 10 to 15 LD_{50}s of the parental wild type as described in the text.

[b] $P < 0.01$, when compared with the protection afforded by an immunizing dose of 10^6 CFU and with that afforded by no immunization (control).

[c] $P < 0.01$, when compared with the protection afforded by an immunizing dose of 3×10^6 CFU and with that afforded by no immunization (control).

ble ts mutants. Of these, two were classified as "tight" mutants (i.e., they ceased all growth immediately after transfer to the nonpermissive temperature), nine were classified as coasters (i.e., they underwent a limited number of divisions after transfer to 36°C), and one was classified as "leaky" (i.e., growth was impaired at 36°C but continued, and this isolate was eventually capable of forming single colonies). The leaky mutant was discarded. The cutoff temperatures for and the nonpermissive phenotypes and reversion frequencies of several mutants are listed in Table 1.

The growth of three mutants, A/10/25, E/7/10, and E/9/9, was studied in liquid cultures after transfer to nonpermissive temperatures. All exhibited some degree of coasting after transfer to 34 and 36°C, and all remained fully viable after extended incubation at the nonpermissive temperature (Fig. 1), properties favored by the enrichment procedure.

Protection studies. Two ts strains, A/10/25 and E/9/9, were selected for preliminary immunological evaluation in a mouse protection study. Immunization of ICR mice i.p. with 6×10^8 CFU of A/10/25 and with 2×10^8 CFU of E/9/9 induced 100% protection from challenge with the virulent parent (Table 2). A boosting dose of E/9/9 given 1 week after the initial immunization enhanced the protection induced by the lower primary doses of this mutant, but no enhancement was observed when animals were immunized and boosted with A/10/25 (Table 3). Both ts mutants induced protection from challenge 3 weeks after immunization, and all mice immunized with 5×10^8 CFU of E/9/9 survived challenge with 90 LD_{50}s 5 weeks later (Table 4).

DISCUSSION

Persistent and ineradicable colonization of the lungs with *P. aeruginosa* constitutes a major threat to survival and the quality of the lives of CF patients (26). In spite of extensive research, there is little definitive knowledge either of the bacterial mechanisms which enable *P. aeruginosa* to initiate and maintain pulmonary infection or of the precise environmental factor(s) within

TABLE 3. Effect of boosting on protection induced in mice immunized with *P. aeruginosa* ts mutants

Immunizing dose (CFU)[a]	Boosting dose (CFU)	Challenge dose (CFU)	No. of mice surviving/no. immunized	Protection (%)	Enhancement[b]
A/10/25					
6×10^8	1×10^8	3×10^9	7/7	100	No
2×10^8	1×10^8	3×10^9	6/7	86	No
6×10^7	1×10^8	3×10^9	3/7	43	No
Control	Saline	3×10^9	0/7	0	
E/9/9					
2×10^8	1×10^7	2×10^9	5/5	100	No
3×10^7	1×10^7	2×10^9	4/5	80	Yes
3×10^6	1×10^7	2×10^9	2/5	40	Yes
Control	Saline	2×10^9	0/5	0	

[a] We immunized 4- to 5-week-old ICR mice i.p. with the indicated doses, administered boosting doses 1 week later, administered challenge doses 1 week after that, and then determined survival.

[b] Enhancement was considered to have been effected if the protection induced was better than that determined in the experiments shown in Table 2.

TABLE 4. Length of protection induced by immunization with *P. aeruginosa* ts mutants

Immunizing dose (CFU)[a]	Challenge dose (CFU)	No. of mice surviving/no. immunized			
		7 days	14 days	21 days	35 days
A/10/25					
6×10^8	3×10^9	7/7[b]	7/7[b]	7/7[b]	ND[c]
2×10^8	3×10^9	6/7	5/7	2/7	ND
6×10^7	3×10^9	3/7	3/7	0/7	ND
Control	3×10^9	0/7	0/7	0/7	ND
E/9/9					
5×10^8	1×10^9–9×10^9	4/6	5/6[b]	6/6[b]	6/6[b]
Control	1×10^9–9×10^9	0/6	0/6	0/5	0/4

[a] Groups of 4- to 5-week-old ICR mice were immunized with the dose indicated and challenged at various times thereafter with 5 to 90 LD_{50}s of the parental wild type, as described in the text.

[b] $P < 0.01$, when compared with the protection afforded by immunizing doses of less than 2×10^8 CFU (A/10/25) and afforded by no immunization (for A/10/25 and E/9/9).

[c] ND, Not done.

the lungs which allows the organism to persist in the face of aggressive antibiotic therapy and high titers of serum antibody. The ability of mucoid strains to sequester themselves in copious quantities of alginate, the elaboration of proteases which may inactivate opsonizing antibody and complement, and the elaboration of toxins which may have deleterious effects on phagocytic cells have been cited as possible microbial factors involved in persistent colonization (5, 7, 13, 23). Hypersecretion and electrolyte imbalance in mucoviscoid lungs, defects in immune defenses, or CF factors which specifically impair the clearance of *P. aeruginosa* have either been postulated for or demonstrated in CF patients (2, 25, 26). Parenteral immunization with currently available vaccines has failed to ameliorate the situation (20).

Recent studies of a model of acute *Pseudomonas* pneumonia have shown that intratracheal immunization with the heptavalent lipopolysaccharide vaccine Pseudogen or the cell wall-extracted material in PEV-01 protects guinea pig lungs from acute damage and enhances the pulmonary clearance of virulent organisms (17, 19). A high-molecular-weight polysaccharide vaccine purified from immunotype 1 was ineffective in the same model. Similar immunization with the lipopolysaccharide vaccine enhances the clearance of *P. aeruginosa* encased in agar beads and deposited in guinea pig lungs but has no effect when administered after chronic infection is established (18).

The vaccine potential of ts bacterial mutants has been investigated, and the efficacy of such strains in inducing protection from virulent challenge has been established (6, 8, 10). The inherent problem with all these strains is reversion to virulence at a highly significant rate (ca. 10^{-7}), rendering them unsafe for human use. However, ts strains offer several specific advantages over other strains attenuated by either genetic or physicochemical means. First, ts mutants with lesions in genes coding for essential products cannot grow at the restrictive temperature in any nutritional environment because there is no way to correct the effect of the mutation. Second, surface antigens remain intact and immunogenicity is uncompromised. Third, the method used for isolating ts mutants can be manipulated to yield coasting strains which replicate to a limited degree in the vaccinee, thus mimicking the initial stages of natural infection and prolonging the stimulation of the immune system.

The problem of genetic instability can be overcome by the combination in one strain of two or more mutations of identical phenotype, thus reducing the reversion frequency to negligible levels (ca. 10^{-21}). The difficulty of identifying recombinants whose phenotype is identical to that of the parental strains can be alleviated by exploiting linkage of the *ts* mutations to positively selectable chromosomal markers. The feasibility of this approach to vaccine development has been demonstrated in a model with *Haemophilus influenzae* (A. Morris Hooke, J. A. Bellanti, and M. P. Oeschger. Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, E61, p. 60).

Encouraged by the success with *H. influenzae*, we began the application of the same method to *P. aeruginosa*. After the isolation of several promising ts mutants, we subjected two of them to preliminary immunological evaluation. Both moderate and extreme coasters were capable of inducing protection from challenge with the virulent parental wild type. As expected, the optimum immunizing dose for the extreme coaster (E/9/9) was much lower (more than threefold) than that required for A/10/25 to in-

duce 100% protection. It should be noted that doses of E/9/9 higher than 2×10^8 CFU failed to induce good protection in challenged mice until 2 to 3 weeks after immunization (Tables 2 and 4). The reasons for this are not entirely clear but may be related to the extreme coasting ability of the strain, which may contribute to a Schwartzman-like reaction. The length of the protection induced by the mutants suggests that specific mechanisms are involved. Immunization with E/9/9 protected the animals from challenge with 90 LD_{50}s 35 days later (Table 4). The specificity of this protection is currently being investigated.

ACKNOWLEDGMENTS

We thank Diane Hargrave for expert secretarial assistance. The Cystic Fibrosis Foundation provided generous support.

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