

A Live Avirulent Mutant *Legionella pneumophila* Vaccine Induces Protective Immunity against Lethal Aerosol Challenge

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Abstract

We have examined the capacity of a live avirulent mutant form of the intracellular bacterial pathogen *Legionella pneumophila* to induce immune responses and protective immunity in guinea pigs. The mutant *L. pneumophila* is nonlethal to guinea pigs and does not revert to virulence with passage through guinea pigs. In contrast, exposure of guinea pigs to aerosols containing wild-type *L. pneumophila* induces a pneumonic illness that clinically and pathologically resembles Legionnaires' disease in humans.

Guinea pigs immunized by aerosol exposure to mutant *L. pneumophila* developed a strong humoral immune response to wild-type *L. pneumophila* antigens with reciprocal antibody titers of 32–512 (median 256) by the indirect fluorescent antibody assay, compared with titers of < 2 for control (sham immunized) guinea pigs. Mutant immunized but not control guinea pigs also developed strong cell-mediated immune responses to wild-type *L. pneumophila* antigens, as demonstrated in assays of cutaneous delayed-type hypersensitivity and in vitro splenic lymphocyte proliferation.

Mutant immunized guinea pigs developed strong protective immunity to lethal aerosol challenge with wild-type *L. pneumophila*. In four independent experiments 40–83% of mutant immunized guinea pigs survived compared with 0% of control guinea pigs. Overall, 13 of 21 (62%) mutant immunized guinea pigs survived compared with 0 of 21 (0%) control guinea pigs ($P = 0.00002$, Fisher's exact test, two-tailed). Mutant immunization induced protection comparable to wild-type immunization in these studies; 8 of 14 (57%) guinea pigs immunized by wild-type *L. pneumophila* survived.

This study demonstrates that guinea pigs immunized with a live avirulent mutant *L. pneumophila* vaccine (a) develop a strong humoral immune response to wild-type *L. pneumophila* antigens; (b) develop a strong cell-mediated immune response to wild-type *L. pneumophila* antigens; and (c) develop protective immunity to lethal aerosol challenge with wild-type *L. pneumophila*. This study demonstrates the feasibility of a vaccine against Legionnaires' disease.

Introduction

Legionella pneumophila is a facultative intracellular bacterial pathogen and the causative agent of Legionnaires' disease, a serious and often fatal form of pneumonia (1). Patients with Legionnaires' disease develop both humoral and cell-mediated immune responses to *L. pneumophila* (2–4). Cell-mediated immunity appears to play a primary role in host defense, as activated human monocytes and alveolar macrophages inhibit the intracellular multiplication of *L. pneumophila* (4–6). Humoral immunity appears to play a secondary role in host defense (2, 3).

The guinea pig is an excellent animal model for the study of Legionnaires' disease. When exposed to aerosols containing *L. pneumophila* guinea pigs develop a pneumonic illness that is clinically and pathologically similar to Legionnaires' disease in humans (7–11). A previous study from this laboratory demonstrated that guinea pigs immunized by a sublethal dose of aerosolized *L. pneumophila* develop humoral and cell-mediated immune responses and protective immunity to subsequent challenge with a lethal aerosol dose of *L. pneumophila* (12).

Another study from this laboratory described an avirulent mutant form of *L. pneumophila* that survives but does not multiply in human monocytes (13). Like the wild-type, the mutant enters monocytes by coiling phagocytosis (13, 14). Thereafter, however, the mutant follows an intracellular pathway that differs from the wild-type. Whereas the wild-type phagosome interacts sequentially with host cell smooth vesicles, mitochondria, and ribosomes, a process culminating in the formation of a ribosome-lined replicative phagosome, the mutant phagosome does not interact with these host cell organelles (13). Moreover, whereas the wild-type phagosome does not fuse with lysosomes, the mutant phagosome does fuse with lysosomes so that the mutant comes to reside in a phagolysosome (13). In all other aspects examined the mutant resembles the wild-type. Both organisms are resistant to complement in the presence or absence of anti-*L. pneumophila* antibody. Both organisms have similar colony morphology, ultrastructure, and structural and secretory protein profiles and lipopolysaccharide profiles on analysis by PAGE.

In the present study we have investigated guinea pig immunologic responses to the mutant form of *L. pneumophila*, which we have found is nonlethal to guinea pigs. We shall demonstrate that guinea pigs immunized with mutant *L. pneumophila* (a) develop humoral and cell-mediated immune responses to wild-type *L. pneumophila* antigens, and (b) develop protective immunity to lethal aerosol challenge with wild-type *L. pneumophila*.

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Methods

Media. RPMI 1640 with L-glutamine was obtained from Gibco Laboratories, Grand Island, NY. Egg yolk buffer (EYB)¹ with or without 1% BSA (1) and PBS (15) were prepared as described previously.

Agar. Modified charcoal yeast extract agar was prepared as described (1).

Bacteria. Wild-type *L. pneumophila*, Philadelphia 1 strain (serogroup 1), originally obtained from the Center for Disease Control in the homogenate of a spleen of a guinea pig that had died after intraperitoneal inoculation with this strain, was passed one time only in embryonated hens' eggs, harvested, tested for viability, tested for the presence of contaminating bacteria, passed one time only on charcoal yeast extract agar, washed with EYB, flash-frozen in aliquots of 10^{11} CFU/ml, and stored at -70°C (1). Formalin-killed *L. pneumophila* (FKLP) was prepared as previously described (4). *L. pneumophila*, mutant 25D (mutant *L. pneumophila*), obtained and cloned as described (13), was aliquotted and stored in the same way as wild-type *L. pneumophila*. Before use in the aerosol inoculation system each stock preparation of bacteria was diluted in EYB to the desired concentration.

Membranes. Wild-type *L. pneumophila* membranes were prepared as described and enumerated in a Petroff-Hausser chamber (16).

Animals. Male Hartley strain guinea pigs weighing 250–300 g, purchased from Charles River Breeding Laboratories (North Wilmington, MA), were housed no more than four to a stainless steel cage and allowed free access to ascorbic acid-fortified chow and water. They were observed in the vivarium for signs of illness for 1 wk before each experiment to ensure that they were healthy. After they were exposed to aerosols of bacteria the guinea pigs were kept in filter-top cages for 1 wk and observed for signs of illness. Their rectal temperatures were taken with a telethermometer with a rectal probe (Yellow Springs Instrument Co., Yellow Springs, OH) and their weights were recorded daily.

Immunization of guinea pigs. Guinea pigs were immunized by exposure to aerosols of bacteria in the same lucite aerosol chamber described in detail in a previous study (12). Briefly, the chamber had sealable portals for introducing or removing guinea pigs, an aerosol inlet at the chamber ceiling, and an aerosol nebulizer system (Dart Industries, Ocala, FL). A vacuum pump (Gast Mfg. Co., Benton Harbor, MI) delivered positive pressure to the nebulizer and negative pressure to the chamber. Guinea pigs were immunized by exposure for 30 min to an aerosol generated from a 10-ml suspension containing a sublethal dose of 2.5×10^6 CFU of wild-type *L. pneumophila*/ml (wild-type immunized) or 5×10^9 CFU of mutant *L. pneumophila*/ml (mutant immunized). Control guinea pigs were sham immunized by exposure for 30 min to aerosols generated from 10 ml of EYB. After the pump to the nebulizer was shut off the guinea pigs were held inside the chamber for an additional 10 min before being removed and placed in filter-top cages, three to a cage. Guinea pigs shared cages only with animals exposed to the same type of aerosol. In some experiments guinea pigs were immunized twice, 3 wk apart.

Guinea pig passage. Mutant *L. pneumophila* were passaged three times through guinea pigs to assess their capacity to revert to virulence. On each passage three guinea pigs were exposed to an aerosol generated from a 10-ml suspension containing 5×10^9 CFU of mutant *L. pneumophila*/ml. Two of the guinea pigs were observed for 7 d for signs of illness including fever, weight loss, or change in feeding habits. The remaining guinea pig was killed 2 d after aerosol exposure. The right lung was sterilely removed and ground in 10 ml of EYB with a mortar and pestle, and CFU in the lung were determined by plating dilutions of the lung suspension in triplicate on charcoal yeast extract agar as described (12). CFU recovered from the lung were harvested from the agar plates, washed with EYB, counted in a Petroff-Hausser chamber, adjusted to a concentration of 5×10^9 bacterial particles/ml, and used for the subsequent passage.

1. Abbreviations used in this paper: EYB, egg yolk buffer; FKLP, formalin-killed *L. pneumophila* (wild-type).

Serology. Anti-*L. pneumophila* antibody in the serum of guinea pigs immunized once or twice with mutant *L. pneumophila* or in the serum of control guinea pigs was assayed by the indirect fluorescent antibody technique as described for human serum by Wilkinson et al. (15). Antigen-bearing slide wells were prepared by covering each well of a 12-well microscope slide (Cel-Line Associates, Minotola, NJ) with $15 \mu\text{l}$ of a suspension containing 2×10^7 FKLP/ml. The slides were air dried, fixed in acetone for 15 min, and then air-dried again. Serum obtained by cardiac puncture from guinea pigs was serially diluted twofold in PBS up to a concentration of 1:1,024, and $20 \mu\text{l}$ of each concentration was applied to each well. The slides were incubated at 37°C in 100% humidity for 30 min, rinsed, and then soaked in PBS. $10 \mu\text{l}$ of fluorescein-conjugated IgG fraction goat anti-guinea pig IgG (Fc fragment) (Cappel Laboratories, Cochranville, PA) was placed on each well and the slides were then incubated at 37°C in 100% humidity for 30 min and rinsed with PBS. The slides were magnified 1,000 times with a fluorescence microscope (Nikon Inc., Garden City, NY). The titer of each animal's serum was expressed as the reciprocal of the dilution that produced 2+ fluorescent staining of at least 50% of the FKLP in a microscopic field.

Cutaneous delayed-type hypersensitivity. Guinea pigs were shaved over the back and flank and administered $100 \mu\text{l}$ of FKLP diluted in EYB to a concentration of 10^8 or 10^9 bacterial particles/ml or $100 \mu\text{l}$ of control buffer (EYB) intradermally at different sites in the shaved area. The diameters of areas of erythema and induration at these sites were recorded at 24, 48, and 72 h after injection.

Lymphocyte proliferation assay. Splenic lymphocytes from mutant immunized or control guinea pigs were obtained and purified, incubated with antigens (*L. pneumophila* membranes or FKLP) in triplicate microtest wells for 2 d, and then tested for capacity to incorporate [^3H]thymidine as described (12).

Studies of protective immunity. Guinea pigs were immunized once or twice by exposure to an aerosol generated from a 10-ml suspension containing 5×10^9 CFU/ml of mutant *L. pneumophila* or 2.5×10^6 CFU/ml of wild-type *L. pneumophila* (sublethal dose). Where guinea pigs were immunized twice, the second immunization was 3 wk after the first. Control guinea pigs were sham immunized once or twice. 3 wk after immunization all guinea pigs were challenged with an aerosolized dose of wild-type *L. pneumophila*. The challenge dose of wild-type *L. pneumophila* (2.5×10^8 CFU/ml), hereinafter referred to as one lethal dose (LD_{100}), was the lowest dose that consistently resulted in the death of all control guinea pigs (12). After the challenge the number of survivors in each group of animals was quantitated. Differences in survival between groups of animals were evaluated by the Fisher's exact test, two-tailed.

Results

Mutant *L. pneumophila* are nonlethal to guinea pigs. Guinea pigs were completely resistant to challenge with even very high concentrations of aerosolized mutant *L. pneumophila*. Whereas 100% of guinea pigs died after exposure to an aerosol generated from a 10-ml suspension containing 2.5×10^8 CFU of wild-type *L. pneumophila*/ml, 0% of guinea pigs died after exposure to an aerosol generated from a 10-ml suspension containing a 20-fold higher concentration (5×10^9 CFU/ml) of mutant *L. pneumophila*, the highest concentration tested.

Mutant *L. pneumophila* remain avirulent after passage through guinea pigs. Mutant *L. pneumophila* did not revert to virulence after three sequential aerosol passages through guinea pigs. During the 7-d observation period after each passage guinea pigs did not become ill. Moreover, in guinea pigs killed 2 d after passage, CFU of *L. pneumophila* in the lungs did not increase with successive passages. In contrast, in studies not presented here that used the same methodology, three

Table I. Anti-*L. pneumophila* Antibody Titers in the Sera of Mutant Immunized and Control Guinea Pigs

One immunization				Two immunizations			
Guinea pig pair	Time after immunization	Reciprocal antibody titer		Guinea pig pair	Time after first immunization	Reciprocal antibody titer	
		Mutant immunized guinea pigs	Control guinea pigs			Mutant immunized guinea pigs	Control guinea pigs
	wk				wk		
A	3	32	<2	F	6	512	<2
B	3	64	<2	G	6	256	<2
C	4	256	<2	H	6	512	<2
D	6	256	<2				
E	6	128	<2				

Guinea pigs were immunized with aerosolized mutant *L. pneumophila* or sham immunized (control guinea pigs) once or twice as indicated. Where guinea pigs were immunized twice, the second immunization was 3 wk after the first. At the time after immunization indicated, serum was obtained and the anti-*L. pneumophila* antibody titer determined by the indirect fluorescent antibody assay as described in the text. The titer was expressed as the reciprocal dilution that produced 2+ fluorescence of at least 50% of the FKLP in a microscopic field.

unrelated (noncloned) avirulent strains of *Legionellae* rapidly regained virulence upon guinea pig passage. With these strains reversion to virulence was apparent by the second passage and CFU in the lungs of guinea pigs increased dramatically (1–5 logs) with successive passages.

Guinea pigs exposed to aerosolized mutant *L. pneumophila* develop humoral immune responses to wild-type *L. pneumophila* antigens. To determine if guinea pigs exposed to aerosolized mutant *L. pneumophila* develop humoral immune responses to wild-type *L. pneumophila*, we measured the anti-

Table II. Skin Reactivity of Mutant Immunized, Wild-type Immunized, and Control Guinea Pigs in Response to Wild-type *L. pneumophila* Antigens

Experiment	Status of guinea pigs	Number of guinea pigs	Skin reaction	Extent of erythema and induration (millimeters) in response to indicated antigen concentration (bacterial particles/milliliter)					
				24 h		48 h		72 h	
				10 ⁸	10 ⁹	10 ⁸	10 ⁹	10 ⁸	10 ⁹
A	Mutant immunized	4	Erythema	8.8±2.2	9.0±1.0	8.8±1.9	12.0±1.1	4.7±0.9	9.3±0.7
			Induration	6.5±3.0	8.3±1.0	5.5±3.2	10.0±1.5	2.0±1.0	6.0±0.6
	Control	4	Erythema	8.3±1.7	8.3±1.7	4.8±2.9	8.8±3.4	0.0±0.0	5.0±2.9
			Induration	7.3±1.5	7.0±1.8	2.8±1.0	6.5±2.7	0.0±0.0	2.8±1.6
			SI (erythema)*	1.1	1.1	1.8	1.4	∞	1.9
B	Mutant immunized	3	SI (induration)	0.9	1.2	2.0	1.5	∞	2.1
			Erythema	8.0±1.3	9.3±1.5	2.3±1.5	6.2±0.4	3.3±0.9	8.7±0.7
	Control	3	Induration	0.0±0.0	4.2±0.4	0.0±0.0	4.2±0.4	0.0±0.0	3.8±0.4
			Erythema	2.8±0.4	6.0±0.6	1.5±0.9	4.3±1.2	1.5±0.5	5.8±0.6
			Induration	0.5±0.5	3.0±0.0	0.0±0.0	2.3±1.2	0.0±0.0	2.5±0.5
C	Mutant immunized	2	SI (erythema)	2.9	1.6	1.5	1.4	2.2	1.5
			SI (induration)	0.0	1.4	—	1.8	—	1.5
				10 ⁹	10 ¹⁰	10 ⁹	10 ¹⁰	10 ⁹	10 ¹⁰
			Erythema	13.8±4.3	17.5±2.5	10.3±3.3	13.0±4.0	10.5±1.5	14.8±3.3
			Induration	6.5±0.5	10.3±0.3	4.8±1.8	8.3±1.3	4.5±0.5	9.8±0.3
	Control	4	Erythema	7.8±0.8	10.7±0.8	5.8±1.5	12.5±0.9	6.6±1.1	15.6±2.1
			Induration	2.9±0.7	6.0±0.4	1.6±0.9	4.9±0.4	2.1±0.6	8.3±1.2
			SI (erythema)	1.8	1.6	1.8	1.0	1.6	0.9
	Wild-type immunized	2	SI (induration)	2.2	1.7	3.0	1.7	2.1	1.2
			Erythema	15.5±1.5	18.5±0.5	14.3±1.8	21.3±1.3	15.3±1.8	24.3±1.8
			Induration	8.5±0.5	12.5±0.5	6.8±0.8	11.5±0.5	7.0±0.0	11.5±1.5

Guinea pigs were immunized with mutant *L. pneumophila* or sham immunized (control) once (experiment A) or twice 3 wk apart (experiments B and C). In experiment C a third group of animals was immunized twice (3 wk apart) by sublethal infection with wild-type *L. pneumophila* (wild-type immunized). All animals were skin tested with FKLP as described in the text. Data given are mean±SEM. * SI, mean skin reactivity (millimeters) mutant immunized guinea pigs/mean skin reactivity (millimeters) of control guinea pigs.

body titer to FKLP in the serum of mutant immunized and control animals by the indirect fluorescent antibody assay. All mutant immunized animals developed significant antibody titers (≥ 32); the geometric mean titer for all such animals was 181. In contrast, all control guinea pigs had titers of < 2 (Table I).

Guinea pigs immunized with mutant L. pneumophila develop cutaneous delayed-type hypersensitivity to wild-type L. pneumophila antigens. To determine if mutant immunized guinea pigs develop a cutaneous delayed-type hypersensitivity response to wild-type antigens, we measured the extent of erythema and induration at skin test sites where mutant immunized and control guinea pigs were injected intradermally with various concentrations of FKLP 3 wk after immunization (Table II). Mutant immunized guinea pigs consistently had greater mean areas of erythema and induration than control guinea pigs. In 18 comparisons made between groups of immunized and control guinea pigs in three independent experi-

ments (Table II), mutant immunized guinea pigs had greater mean areas of erythema in 17 (94%) comparisons, and greater mean areas of induration in 16 (89%) comparisons. The extent of erythema and induration in immunized guinea pigs was typically about two-fold that in control guinea pigs. The erythema in control animals was distinctly lighter in color than the deep red erythema in the mutant immunized animals. Intradermal injection of the diluting buffer, EYB, did not produce erythema or induration in mutant immunized or control animals.

Splenic lymphocytes from guinea pigs immunized with mutant L. pneumophila proliferate in response to wild-type L. pneumophila antigens. To determine if mutant immunized guinea pigs expand a population of lymphocytes that recognize wild-type *L. pneumophila* antigens, we studied the proliferative responses of splenic lymphocytes from these animals to FKLP and to wild-type *L. pneumophila* membranes (Table III).

Table III. Proliferation of Splenic Lymphocytes from Mutant Immunized and Control Guinea Pigs in Response to Wild-type *L. pneumophila* Antigens

Experiment		Time after immunization	No antigen	FKLP				Membrane			
				5 × 10 ⁶ /ml		5 × 10 ⁷ /ml		5 × 10 ⁶ /ml		5 × 10 ⁷ /ml	
				SI*		SI		SI		SI	
		wk	cpm	cpm		cpm		cpm		cpm	
One immunization											
Immunized guinea pigs											
A	A1	3	142±4	1,320±52	9.3	2,199±187	15.5	452±30	3.2	1,299±131	9.1
B	B1	3	138±7	1,337±36	9.7	1,865±77	13.5	—	—	1,368±229	24.4
	B2	6	146±9	1,266±139	8.7	1,748±44	12.0	—	—	1,469±81	10.1
C	C1	3	167±88	786±133	4.7	3,394±213	20.3	616±35	3.7	4,722±327	28.3
	C2	4	97±4	239±2	2.5	454±0	4.7	193±12	2.0	523±26	5.4
	C3	6	182±7	596±39	3.3	2,432±49	13.4	519±171	2.9	3,529±1,067	19.4
Mean					6.4±1.3		13.2±2.1		3.0±0.4		16.1±3.8
Control guinea pigs											
A	A1	3	181±7	412±47	2.3	1,551±165	8.6	203±3	1.1	473±36	2.6
B	B1	3	220±11	755±37	3.4	1,317±47	6.0	—	—	2,521±126	11.5
	B2	6	110±5	179±6	1.6	308±33	2.8	—	—	535±29	4.9
C	C1	3	121±7	195±7	1.6	452±45	3.7	218±1	1.8	941±5	7.8
	C2	4	110±6	157±55	1.4	124±1	1.1	96±3	0.9	211±29	1.9
	C3	6	195±7	514±68	2.6	989±145	5.1	323±28	1.7	1,840±381	9.4
Mean					2.2±0.3		4.6±1.1		1.4±0.2		6.4±1.6
P†<					0.01		0.005		0.005		0.03
Two immunizations											
Immunized guinea pigs											
D	D1	3	221±18	1,187±27	5.4			440±15	2.0	1,845±264	8.3
	D2	3	109±4	711±65	6.5			288±32	2.6	634±32	5.8
	D3	3	113±7	1,000±33	8.8			627±32	5.5	339±29	3.0
	Mean				6.9±1.0				3.4±1.1		5.7±1.5
Control guinea pigs											
D	D1	3	267±13	634±1	2.4			325±34	1.2	550±89	2.1
	D2	3	101±5	133±26	1.3			106±1	1.0	223±15	2.2
	D3	3	109±4	109±3	2.2			127±11	1.2	188±20	1.7
	Mean				2.0±0.3				1.1±0.1		2.0±0.2
P†<					0.005				0.1		0.05

In each of four independent experiments (A–D), proliferation of splenic lymphocytes from one to three pairs of mutant immunized and control animals was studied. Mutant immunized guinea pigs were exposed to mutant *L. pneumophila* once (experiments A–C) or twice, 3 wk apart (experiment D). Splenic lymphocytes (10⁷ cells/ml) were incubated in microtest wells at 37°C for 2 d without antigen or with either FKLP (5 × 10⁶ or 5 × 10⁷/ml) or wild-type *L. pneumophila* membranes (5 × 10⁶ or 5 × 10⁷/ml). The lymphocytes were then assayed for their capacity to incorporate [³H]thymidine as described in the text. For each animal the mean cpm±SEM for six wells without antigen or three wells with antigen is reported. * SI, stimulation index = (mean [³H]thymidine incorporation (cpm) of lymphocytes incubated with antigen)/(mean [³H]thymidine incorporation (cpm) of lymphocytes incubated without antigen). † P value for difference between SI of immunized and control guinea pigs by two-tailed t-test.

In four experiments involving nine pairs of mutant immunized and control guinea pigs, the mutant immunized animals consistently had significantly greater splenic lymphocyte proliferative responses to *L. pneumophila* antigens than did control animals (Table III). The immunized animals exhibited significantly stronger lymphocyte proliferative responses to both FKLP and *L. pneumophila* membrane antigens. Stimulation indices of immunized animals were approximately three-fold those of control animals in response to either of the *L. pneumophila* antigen preparations. Stimulation indices of guinea pigs immunized with mutant *L. pneumophila* twice were not significantly different from those of guinea pigs immunized once.

Guinea pigs immunized with mutant L. pneumophila are protected against lethal aerosol challenge. We performed four experiments to determine if mutant immunized guinea pigs are protected against lethal aerosol challenge with wild-type *L. pneumophila* (Table IV). Each experiment had a negative sham-immunized control group consisting of animals exposed to aerosolized diluting buffer (EYB), and three of the experiments had a positive control group consisting of animals immunized by sublethal infection with wild-type *L. pneumophila* (wild-type immunized). In the first two experiments animals were immunized once before challenge. In the second two experiments animals were immunized twice 3 wk apart before challenge. Altogether, 21 control animals, 21 mutant immunized animals, and 14 wild-type immunized animals were challenged.

Mutant immunization produced strong protective immunity. Whereas none (0%) of the control animals survived chal-

lenge in any experiment, 40–83% of mutant immunized animals survived. Similarly, 40–80% of wild-type immunized animals survived. Overall, a mean of 62% of mutant immunized animals and 57% of wild-type immunized animals survived challenge. The difference in survival between mutant immunized and control animals was highly significant ($P = 0.00002$, Fisher's exact test, two-tailed). The difference in overall survival between mutant immunized and wild-type immunized guinea pigs was not statistically significant. Somewhat more animals immunized with mutant *L. pneumophila* twice (67%) than animals immunized with mutant *L. pneumophila* once (56%) survived, but this difference was also not statistically significant.

The mean weights of animals in the three groups were not significantly different ($P > 0.05$) from each other either at the start of the experiment, before administration of any immunizing doses, or on the day of challenge 3 wk after administration of the last immunizing dose. After lethal challenge guinea pigs in all three groups rapidly lost weight. 2 d after challenge, before animals died, immunized animals lost significantly more weight ($P < 0.025$) than control animals. The mean weight loss for mutant immunized guinea pigs was 47 ± 7 g, for wild-type immunized animals 44 ± 17 g, and for control animals 25 ± 7 g.

After lethal challenge, during the course of illness, control guinea pigs exhibited more frequent and severe signs of illness such as labored respirations and decreased activity than immunized animals. However, a majority of mutant immunized and wild-type immunized animals also exhibited these signs of illness at some point after challenge. Two guinea pigs from each experimental group were observed to have loose stools. Some guinea pigs had elevated temperatures over the 7-d observation period, but there was no obvious relationship between immunization status and febrile response.

Table IV. Survival Rates of Immunized and Control Guinea Pigs Administered a Lethal Aerosol Challenge of Wild-type *L. pneumophila*

Experimental status of guinea pig	Number of guinea pigs surviving per number challenged and % survival			P*
One immunization	Experiment 1	Experiment 2	Total	
Control	0/5 (0%)	0/5 (0%)	0/10 (0%)	—
Mutant immunized	3/4 (75%)	2/5 (40%)	5/9 (56%)	0.01
Wild-type immunized	2/5 (40%)	4/5 (80%)	6/10 (60%)	0.01
Two immunizations	Experiment 3	Experiment 4	Total	
Control	0/5 (0%)	0/6 (0%)	0/11 (0%)	—
Mutant immunized	5/6 (83%)	3/6 (50%)	8/12 (67%)	0.001
Wild-type immunized	2/4 (50%)	—	2/4 (50%)	0.06
Grand total				
Control			0/21 (0%)	—
Mutant immunized			13/21 (62%)	0.00002
Wild-type immunized			8/14 (57%)	0.0001

In four independent experiments guinea pigs were immunized once or twice (3 wk apart) by exposure to mutant *L. pneumophila* or to a sublethal dose of wild-type *L. pneumophila* (three experiments), or they were sham immunized (control guinea pigs). 3 wk after immunization all animals were challenged with a lethal aerosol dose of wild-type *L. pneumophila* and survival was quantitated.

* P value for difference between mutant immunized and control animals or for difference between wild-type immunized and control animals by the Fisher's exact test, two-tailed.

Discussion

We have demonstrated that immunization of guinea pigs with a live avirulent mutant form of *L. pneumophila* induces protective immunity against lethal aerosol challenge with wild-type *L. pneumophila*. This protective immunity is comparable to that induced by immunization with a sublethal dose of wild-type *L. pneumophila*.

The induction of protective immunity by immunization with mutant *L. pneumophila* is paralleled by the development of both humoral and cell-mediated immune responses. Immunized but not control animals develop high titers of antibody against wild-type *L. pneumophila* antigens, and strong cutaneous delayed-type hypersensitivity and lymphocyte proliferative responses to wild-type *L. pneumophila* antigens.

Guinea pigs immunized twice with mutant *L. pneumophila* did not develop significantly greater humoral or cell-mediated immune responses to wild-type *L. pneumophila* antigens than guinea pigs immunized once. Consistent with a role for these immune responses in host defense, guinea pigs immunized twice did not exhibit significantly greater protective immunity to lethal aerosol challenge with wild-type *L. pneumophila* than guinea pigs immunized once.

Immunization with mutant *L. pneumophila* induces strong protective immunity in guinea pigs against an extremely high challenge dose of aerosolized wild-type *L. pneumophila*. This

dose is much higher than that likely to be encountered by humans in nature. In view of this, the fact that the mutant is nonlethal to guinea pigs even at doses 20 times greater than the LD₁₀₀ of wild-type *L. pneumophila*, and the fact that the mutant remains avirulent after guinea pig passage, the mutant has potential as a prototype vaccine against Legionnaires' disease in humans.

The concept of a live avirulent bacterial vaccine against a human intracellular pathogen has ample precedent. BCG, an attenuated strain of *Mycobacterium bovis*, has been used extensively in Europe and elsewhere as a vaccine against human tuberculosis.

A safe and effective vaccine against Legionnaires' disease might be particularly valuable for persons who are in high-risk groups for this disease, e.g. cigarette smokers, persons who abuse alcohol, persons with advanced malignancy, and persons on immunosuppressive therapy, especially organ transplant recipients.

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References

1. Horwitz, M. A., and S. C. Silverstein. 1980. Legionnaires' disease bacterium (*Legionella pneumophila*) multiplies intracellularly in human monocytes. *J. Clin. Invest.* 66:441-450.
2. Horwitz, M. A., and S. C. Silverstein. 1981. Interaction of the Legionnaires' disease bacterium (*Legionella pneumophila*) with human phagocytes. I. *L. pneumophila* resists killing by polymorphonuclear leukocytes, antibody, and complement. *J. Exp. Med.* 153:386-397.
3. Horwitz, M. A., and S. C. Silverstein. 1981. Interaction of the Legionnaires' disease bacterium (*Legionella pneumophila*) with human phagocytes. II. Antibody promotes binding of *L. pneumophila* to monocytes but does not inhibit intracellular multiplication. *J. Exp. Med.* 153:398-406.
4. Horwitz, M. A. 1983. Cell-mediated immunity in Legionnaires' disease. *J. Clin. Invest.* 71:1686-1697.
5. Horwitz, M. A., and S. C. Silverstein. 1981. Activated human monocytes inhibit the intracellular multiplication of Legionnaires' disease bacteria. *J. Exp. Med.* 154:1618-1635.
6. Nash, T. W., D. M. Libby, and M. A. Horwitz. 1984. Interaction between the Legionnaires' disease bacterium (*Legionella pneumophila*) and human alveolar macrophages. Influence of antibody, lymphokines, and hydrocortisone. *J. Clin. Invest.* 74:771-782.
7. Davis, G. S., W. C. Winn, Jr., D. W. Gump, J. E. Crayhead, and H. N. Beaty. 1982. Legionnaires' pneumonia after aerosol exposure in guinea pigs and rats. *Am. Rev. Respir. Dis.* 126:1050-1057.
8. Davis, G. S., W. C. Winn, Jr., D. W. Gump, and H. N. Beaty. 1983. The kinetics of early inflammatory events during experimental pneumonia due to *Legionella pneumophila* in guinea pigs. *J. Infect. Dis.* 148:823-835.
9. Baskerville, A., R. B. Fitzgeorge, M. Broster, P. Hambleton, and P. J. Dennis. 1981. Experimental transmission of Legionnaires' disease by exposure to aerosols of *Legionella pneumophila*. *Lancet*. ii:1389-1390.
10. Katz, S. M., and S. Hashemi. 1982. Electron microscopic examination of the inflammatory response to *Legionella pneumophila* in guinea pigs. *Lab. Invest.* 46:24-32.
11. Berendt, R. F., H. W. Young, R. G. Allen, and G. L. Knutsen. 1980. Dose response of guinea pigs experimentally infected with aerosols of *Legionella pneumophila*. *J. Infect. Dis.* 141:186-192.
12. Breiman, R. F., and M. A. Horwitz. 1987. Guinea pigs sublethally infected with aerosolized *Legionella pneumophila* develop humoral and cell-mediated immune responses and are protected against lethal aerosol challenge. A model for studying host defense against lung infections caused by intracellular pathogens. *J. Exp. Med.* 164:799-811.
13. Horwitz, M. A. 1987. Characterization of avirulent mutant *Legionella pneumophila* that survive but do not multiply within human monocytes. *J. Exp. Med.* 166:1310-1328.
14. Horwitz, M. A. 1984. Phagocytosis of the Legionnaires' disease bacterium (*Legionella pneumophila*) occurs by a novel mechanism: engulfment within a pseudopod coil. *Cell*. 36:27-33.
15. Wilkinson, H. W., B. J. Fikes, and D. D. Cruce. 1979. Indirect immunofluorescence test for serodiagnosis of Legionnaires' disease: evidence for serogroup diversity of Legionnaires' disease bacterial antigens and for multiple specificity of human antibodies. *J. Clin. Microbiol.* 9:379-383.
16. Gabay, J. E., and M. A. Horwitz. 1985. Isolation and characterization of the cytoplasmic and outer membranes of the Legionnaires' disease bacterium (*Legionella pneumophila*). *J. Exp. Med.* 161:409-422.