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Vaccination Against *Legionella pneumophila*: Serum Antibody Correlates with Protection Induced by Heat-Killed or Acetone-Killed Cells Against Intraperitoneal but Not Aerosol Infection in Guinea Pigs

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An aerosol model of *Legionella* infection has been established in guinea pigs. Infected animals showed growth of *Legionella* in their lungs, dissemination of organisms to the spleen, development of pneumonia and fever, and weight loss. Vaccination studies using heat-killed or acetone-killed cells were carried out, and guinea pigs were challenged intraperitoneally or by using the aerosol model of infection. Both vaccines were shown to give moderately high levels of protection against intraperitoneal challenge (28 to 145 50% lethal doses). Protection was found to be dose dependent and correlated with antibody levels as measured by enzyme-linked immunosorbent assay to an outer membrane antigen and by indirect immunofluorescence to heat-killed cells. In contrast, the same vaccination regimens that protected against intraperitoneal challenge failed to protect guinea pigs against aerosol challenge with comparable doses of *Legionella*, despite the presence of serum antibody. The results are discussed in terms of the possible requirements for immunity to aerosolized *Legionella*, including secretory immunoglobulin or cell-mediated immunity.

After the outbreak of Legionnaires disease in 1976 in Philadelphia, a previously unrecognized bacterium was isolated and designated *Legionella pneumophila* (4, 24). This organism is apparently found worldwide, as evidenced by documented outbreaks in Europe, Israel, Scandinavia, and Australia (6). Since 1976, eight additional serogroups of *L. pneumophila* and seven additional species have been recognized (2, 26). *Legionella* bacteria have been responsible for numerous focal outbreaks of pneumonia as well as isolated cases, with many of the latter appearing in immunocompromised hosts (5). There is currently considerable interest in understanding the mechanism(s) of *Legionella* pathogenesis and protective immunity to this organism, both from a theoretical point of view and from the practical aspect of potential vaccine development.

The guinea pig appears to be the animal of choice for in vivo protection experiments using legionellae, as the organism was originally isolated by intraperitoneal (i.p.) inoculation of guinea pigs with biopsy and autopsy material (7). Studies have shown that guinea pigs become infected and die after inoculation with moderate numbers of organisms by the i.p. or aerosol routes (1, 11). In studies in which guinea pigs have been vaccinated, it has been shown that heat-killed cells (HKC) given in complete Freund adjuvant, or antigenic extracts of *Legionella*, will protect against i.p. challenge (1, 11, 31). The immunoglobulin G (IgG) fraction of *Legionella*-immune goat serum has also been shown to passively protect guinea pigs against i.p. infection (32). There are, however, no published protection studies involving an aerosol route of challenge. The present study shows that HKC and acetone-killed cells (AKC) give excellent protection against *Legionella* organisms administered i.p., and that protection correlates with antibody as measured in an enzyme-linked immunosorbent assay (ELISA) and by indirect fluorescent-antibody assay. However, identical vaccination protocols failed to protect against lesser numbers of organisms given by the aerosol route.

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MATERIALS AND METHODS

Organism. *L. pneumophila*, Philadelphia 1, was obtained from the Centers for Disease Control, Atlanta, Ga. Organisms were grown on charcoal-yeast extract agar plates and lyophilized. Lyophils of each strain were passaged once on charcoal-yeast extract agar, and a saline suspension of organisms was passaged further in 7-day-old chick embryo yolk sacs. These were grown for 4 to 12 days, harvested, flash frozen, and stored at −70°C. For all studies involving vaccine preparation or challenge, vials with frozen yolk sac material were streaked on BCYEα agar (charcoal-yeast extract agar plates buffered with 1% ACES buffer [N-(2-acetamido)-2-aminoethanesulfonic acid] and 0.1% α-ketoglutaric acid) and then passaged once more on agar to remove any adherent yolk sac tissue and to ensure pure cultures.

For aerosol or i.p. infection, organisms were harvested from plates in saline. The number of *Legionella* organisms was estimated by Petroff-Hauser counts on a formalinized sample of cells. Organisms were then diluted in distilled water to the desired concentration for i.p. injection or for aerosolization. The number of viable bacteria per milliliter of challenge suspensions was always determined precisely by plating appropriate dilutions, in duplicate, on BCYEα plates.

Aerosol infection. To assess protection by various vaccines against pulmonary infection, an aerosol model of guinea pig infection was established by using a Tri-R Airborne Infection Apparatus (Tri-R Instruments, Inc., Rockville Centre, N.Y.) of the type originally designed by Middlebrook for

aerosolization of *Mycobacterium tuberculosis* (25). Guinea pigs were placed in a wire basket in the chamber of the instrument, and an aqueous suspension of *L. pneumophila* was put into the nebulizer. Aerosolization was carried out for 30 min, the chamber was flushed with fresh air for 10 min, and the surface of the fur was disinfected with UV light for 15 min. The density of *Legionella* in the chamber was estimated by drawing 1,000 cc of air through an impinger filled with distilled water and plating 0.1 ml of water on duplicate BCYEα agar plates to obtain the number of viable CFU. Using the formula of Guyton (15), the respiratory volume of each pig could be calculated based on its weight as follows: respiratory volume per minute = 2.10 × (weight of animal)$^{3/4}$, where volume is measured in milliliters and body weight is measured in grams.

The theoretical number of organisms inhaled in 30 min was determined by multiplying the respiratory volume by 30 and also by the number of organisms calculated to be in 1 cc of air. The actual number of organisms in a lung was determined by sacrificing the animal immediately after aerosolization, removing the lungs, homogenizing the tissue, adjusting the volume to 30 ml, and plating appropriate dilutions, in duplicate, on BCYEα agar plates. From the number of CFU per plate, the number of organisms per lung was calculated. There was excellent agreement between CFU per lung calculated from the impinger counts and the actual number of organisms recovered after aerosolization on necropsy. The aerosol 50% lethal dose (LD_{50}) was determined to be approximately 2 × 10^4 organisms per lung as compared with the i.p. LD_{50}, which was 10^6 CFU. The UV disinfection of the fur worked well; no *Legionella* organisms grew on BCYEα plates inoculated with swabs of guinea pig fur after aerosolization.

Animals and safety precautions. Female strain 2 guinea pigs, weighing approximately 350 g, were purchased from Murphy Breeding Laboratories, Plainfield, Ind. Pigs were housed in plastic cages with wire tops and given food and water ad libitum. After infection, they were placed in their cages in a germfree isolator with the fans reversed so that *Legionella* organisms would be contained within the isolator. The port of the isolator was disinfected with 2% peracetic acid. All manipulations of infected animals were carried out through glove ports.

Vaccine preparation. Heat-killed cells were prepared by growing bacterial lawns on BCYEα agar plates and harvesting saline suspensions in small volumes (3 ml per plate). Pooled suspensions were apportioned into tubes and treated with streaming steam at 100°C for 1 h. Cells were centrifuged, washed, and suspended in distilled water to the desired concentration as determined by Petroff-Hauser counts. Sterility was assured by plating on BCYEα agar and blood agar plates. AKC were prepared by the procedure of Landy for salmonellae (22), except that cells were harvested by filtration with Whatman no. 42 paper in a Buchner funnel, rather than by centrifugation, and the time of acetone treatment was 1 h.

Vaccination protocols. Vaccines were given i.p. in 1.0 ml of saline 2 days after preimmunization sera were obtained by cardiac puncture. Fourteen days after vaccination, animals were bled, and 2 days later (day 16) they were either boosted or challenged with *L. pneumophila*. Boosted animals were bled again 14 days after the second injection (day 30) and challenged 2 days later (day 32).

Antibody studies. Individual guinea pigs were marked by ear notching and bled by cardiac puncture before vaccination, before boosting, and before challenge as described above. Antibody to *L. pneumophila* serogroup 1 was quantitated by indirect fluorescence, using standard procedures (28), with heat-killed antigen supplied by the Centers for Disease Control. Antibody was also measured by an ELISA in which an outer membrane antigen extracted from *L. pneumophila* serogroup 1 (12) was used in a solid-phase assay. The ELISA procedure has been routinely used in our laboratory for other antigens (10). Briefly, antigen (0.1 mg/ml in phosphate-buffered saline) was allowed to adhere to minicuvettes which were washed semi-automatically three times with 0.05% Brij 35 in distilled water (Sigma Chemical Co., St. Louis, Mo.), using a Gilford EIA processor/reader (model no. 50, Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Serum at the desired dilutions was added in duplicate to the antigen-coated cuvettes and incubated for 5 h at 4°C with shaking. Serum was aspirated, cuvettes were washed five times with Brij, and peroxidase-conjugated goat anti-guinea pig serum (Litton Bionetics, Charleston, S.C.) was added. Cuvettes were incubated overnight at 4°C with shaking. Substrates, 0.01% *o*-phenylenediamine and 0.003% hydrogen peroxide, were added the next day and color developed for 1 h. Optical density was read at 450 nm. As controls, prevaccination sera were run individually and results were pooled to obtain a mean background value. The titer of sera with antibody was determined by regression analysis in which the best fit line of the log of the optical density plotted versus the log serum dilution was extrapolated to find where

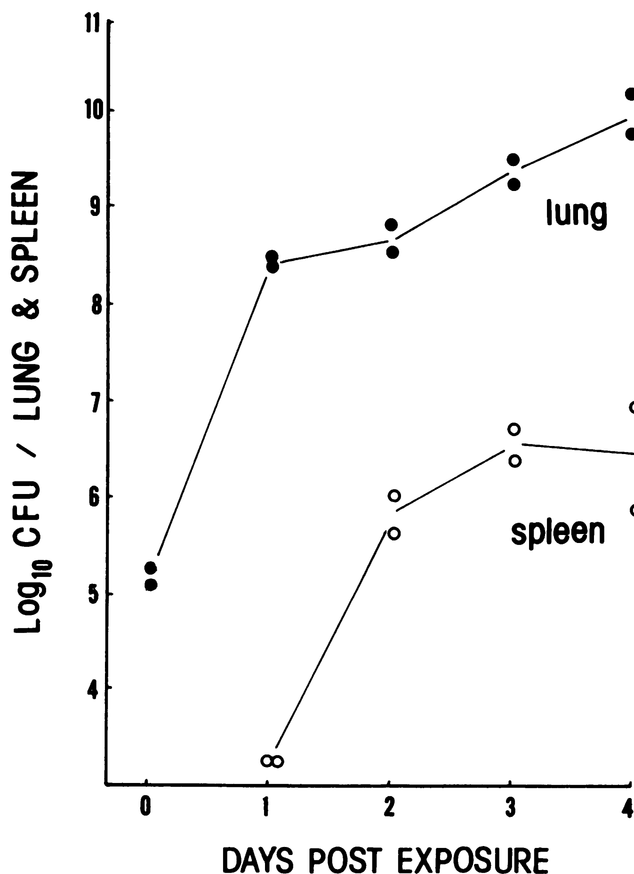

FIG. 1. Growth of *L. pneumophila* in aerosol-infected guinea pig lungs and spleen. Twelve pigs were infected, and two animals were sacrificed at each time point.

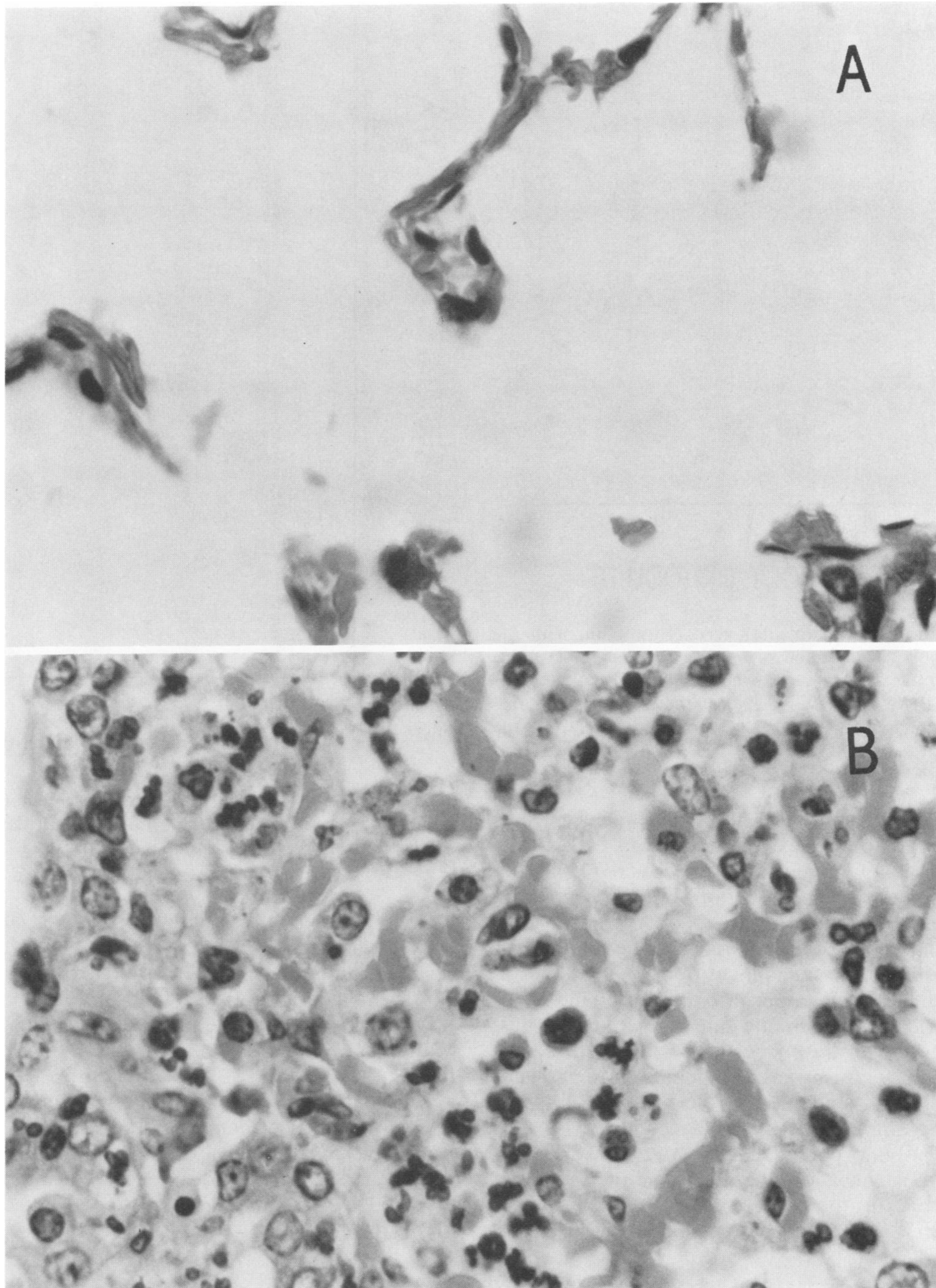

FIG. 2. Hematoxylin- and eosin-stained section of the lungs of an uninfected guinea pig (A), and a guinea pig aerosol infected 4 days previously with *L. pneumophila* (B). Magnification, ×400.

it crossed the axis formed by the background value. In every experiment, cuvettes were included with no antibody or no antigen to assure specificity of the reactions. In addition, a high-titer serum was included as a positive control to monitor day-to-day fluctuations in optical density and to allow data obtained on different days to be normalized.

Pathology. Normal and aerosol-infected guinea pigs were sacrificed, and representative sections of their lungs were fixed in 10% buffered Formalin and embedded in paraffin. Sections (3 mm) were cut and stained with hematoxylin and eosin for light microscopy.

Statistics. The levels of significance (P) for the observed frequencies were determined by the Fisher exact test for 2 × 2 tables (3).

RESULTS

Aerosol infection model. The validity of the aerosol infection model was established by infecting 12 pigs with 3.2×10^5 CFU (16 LD_{50}s), using the Tri-R apparatus. Four uninfected pigs were used as controls. Body weights and rectal temperatures of infected and control pigs were determined at time zero and daily for 4 days. Two infected pigs were

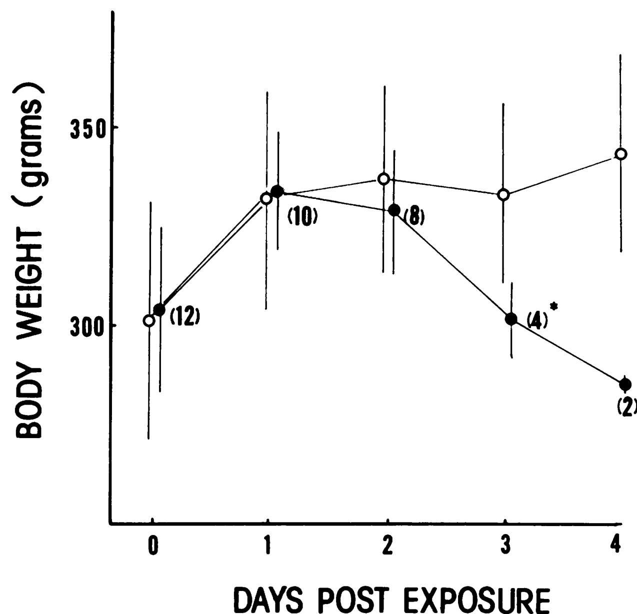

FIG. 3. Change in body weight after aerosol infection with *L. pneumophila*. Symbols: ●, infected pigs; ○, control pigs. Twelve infected and four control animals were used. Each day, two infected pigs were sacrificed to determine CFU. Numbers in parentheses are the number of pigs weighed on each day. Asterisk indicates that two pigs died of infection.

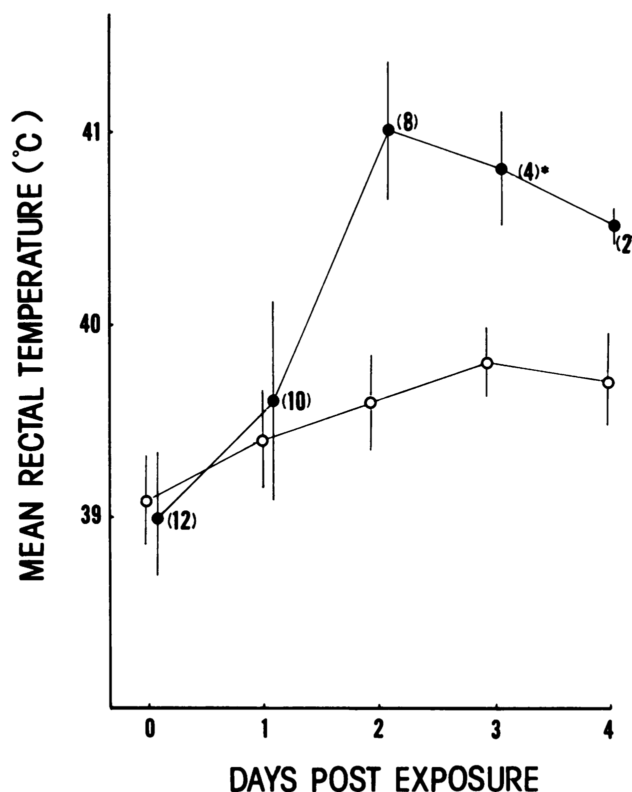

FIG. 4. Rectal temperatures of aerosol-infected guinea pigs. Symbols: ●, aerosol infected; ○, controls. Procedural details and asterisk are the same as in the legend to Fig. 3.

sacrificed on each day, and their lungs and spleens were homogenized separately to assess the number of CFU of *L. pneumophila* per organ. Organisms were recovered from the lung immediately after aerosol infection, but were not detected in the spleen until 24 h later (Fig. 1) (10 organisms per organ was the minimal detectable number). The number of *Legionella* organisms in the lungs increased 3.5 logs over a 24-h period, showing rapid initial proliferation. Beyond 24 h, the rate of bacterial growth in the lung was reduced but continued to increase over the succeeding 4 days to levels of 5×10^9 per animal. Organisms were detected in the spleen after 24 h, suggesting dissemination from the lung. Examination of the lungs of control and infected pigs (Fig. 2) showed extensive polymorphonuclear infiltrates and consolidation of the alveolar spaces characteristic of an acute bacterial pneumonia in the infected pigs. Infected pigs showed a marked elevation in rectal temperature on day 2 post-aerosol infection (Fig. 4) which correlated with progressive weight loss (Fig. 3). At 4 days postinfection, surviving pigs had an average weight of 286 ± 1.0 g, compared with 344 ± 24 g for controls. Thus, infection by this method produced a progressive disease with pneumonia, in vivo multiplication of *Legionella* in the lungs, fever, and weight loss.

Vaccination and i.p. challenge. Experiments were carried out by using i.p. vaccination and i.p. challenge to establish protective immunizing doses of HKC and AKC. A single dose of 10^9 HKC was not protective against a low challenge of nine LD_{50}s, and a similar dose of AKC also did not offer 100% protection (Table 1). Either increasing the dose of

TABLE 1. Protection studies on guinea pigs given killed cells i.p. and challenged i.p.

Vaccine	Dose[a]	Booster	Challenge dose[b]		No. of survivors/ total	*P*
			No. of cells	No. of i.p. LD_{50}s		
HKC	1×10^9	No	1×10^7	9	2/5	NS[c]
	5×10^9	No	1×10^7	9	5/5	<0.01
			3×10^7	28	5/5	<0.01
AKC	1×10^9	No	1×10^7	9	3/4	NS
	1×10^9	Yes	8×10^7	72	5/5	<0.01
			2×10^8	145	5/5	<0.01
Saline			1×10^7	9.0	1/9	
			5×10^6	4.5	1/4	

[a] Injections were given i.p.
[b] Challenge was 16 days after the last vaccinating dose.
[c] NS, Not statistically significant.

TABLE 2. Comparison of protection by AKC against i.p. or aerosol challenge

Vaccine[a]	I.p. infection			Aerosol infection[b]		
	Challenge dose		No. of survivors/ total	Challenge dose		No. of survivors/ total
	No. of cells	No. of i.p. LD_{50}s		No. of cells	No. of aerosol LD_{50}s	
5×10^9 AKC	2.2×10^7	20	8/8[c]	5.5×10^5	27.5	0/6
Saline	2.2×10^7	20	2/8	5.5×10^5	27.5	0/6

[a] Injections were given i.p.
[b] Challenge was 16 days post-booster dose.
[c] $P < 0.01$.

TABLE 3. Effect of a booster dose on protection afforded guinea pigs by HKC against i.p. or aerosol challenge

Guinea pig immunized with:	I.p. infection[a]			Aerosol infection[a]		
	Challenge dose		No. of survivors/ total	Challenge dose		No. of survivors/ total
	No. of cells	No. of i.p. LD_{50}s		No. of cells	No. of aerosol LD_{50}s	
10^9 HKC (2×)	3.1×10^7	28.7	4/4	4.0×10^5	20	0/6
	1.5×10^7	13.8	4/4			
	6.2×10^6	5.7	4/4	1.3×10^5	6.5	1/6
Saline	6.2×10^6	5.7	0/4	1.3×10^5	6.5	0/4

[a] Challenge was 16 days post-booster dose.

HKC to 5×10^9 or giving two injections of 1×10 AKC was a successful regimen, resulting in 100% protection against moderately high i.p. challenge doses (28 to 145 LD_{50}s). Thus, a threshold vaccinating regimen was demonstrated for both vaccines, which was reflected in the antibody responses (see Table 4). A single dose of HKC induced little antibody as measured by ELISA or indirect fluorescent-antibody assay; a single dose of AKC was somewhat more efficacious but gave titers substantially below those induced by the more highly protective protocols of 5×10^9 HKC or two doses of 1×10^9 AKC.

Vaccination comparing i.p. and aerosol challenge. In another series of experiments, guinea pigs were vaccinated with a single dose of 5×10^9 AKC (Table 2) or with two doses of 1×10^9 HKC (Table 3), and groups were challenged i.p. or by aerosol. All of the animals that received these new vaccinating protocols and were challenged i.p. survived. Unexpectedly, only 1 of the 18 animals that were similarly vaccinated, but challenged by aerosol, survived. Thus, vaccination regimens protective against i.p. challenge failed to protect against aerosol challenge, even though the aerosol dose was lower on an LD_{50} basis than the lowest i.p. dose (9 versus 6.5 LD_{50}s). Further, since the aerosol LD_{50} was almost two logs lower than the i.p. LD_{50}, aerosol-challenged pigs exposed to 6.5 LD_{50}s (Table 2) actually received over 1.5 log fewer organisms than animals given 9 LD_{50}s i.p. Examination of antibody responses to these vaccinating regimens (Table 4) showed that the two doses of 10^9 HKC were comparable in efficacy to the previously tested protocol of two doses of 10^9 AKC. The single dose of 5×10^9 AKC gave low titers in both antibody assays, despite the solid protection observed to i.p. challenge. Assays on these sera have been performed twice, together with other sera that gave high titers, and both times low values were obtained. A possible explanation for the low ELISA titers may lie with the second antibody used in the assay, a peroxidase-conjugated anti-IgG recognizing both heavy and light chains on the guinea pig immunoglobulin molecules. It should react with IgM as well as IgG; however, if the response to one dose of AKC were mainly IgM, and if the conjugate bound to IgM less well than to IgG, it could explain why this one value is low.

Some less systematic studies were carried out on survivors of LD_{50} determinations and of vaccination experiments to determine whether previous exposure to live organisms would be protective. It was found that when guinea pigs that survived aerosol and i.p. LD_{50} experiments were rechallenged i.p. with 50 to 60 LD_{50}s, all animals survived; however, survivors of an aerosol LD_{50} all succumbed to rechallenge with 44 LD_{50}s of *Legionella* given by aerosol. Further, when guinea pigs that received 5×10^9 AKC and survived i.p. challenge (Table 2) were aerosol challenged 43 days later with two LD_{50}s, none survived. Thus, vaccination regimens with killed organisms, which provided substantial protection against i.p. challenge, failed to protect against aerosol infection at comparable or lower challenge doses. Further, even previous aerosol exposure to sublethal doses of live *Legionella* organisms failed to confer protection on guinea pigs against rechallenge by the aerosol route.

TABLE 4. Immune responses of guinea pigs vaccinated with killed *Legionella* organisms

Guinea pigs vaccinated with[a]:	Log_{10} ELISA titer (geometric mean)[b]	Mean IFA titer[c] (range)
1×10^9 HKC	3.040	32 (16–64)
5×10^9 HKC	4.347	1,152 (640–2,560)
2 doses of 1×10^9 HKC	4.806	672 (160–1,280)
1×10^9 AKC	3.564[d]	560[d] (320–640)
5×10^9 AKC	2.845[e]	512[e] (all 512)
2 doses of 1×10^9 AKC	4.859	768 (640–1,280)

[a] All vaccines were given i.p. in 1.0 ml of saline.
[b] Individual sera of five guinea pigs were tested against outer membrane antigen.
[c] Individual sera of five guinea pigs were tested against heat-killed serogroup one antigen supplied by the Centers for Disease Control.
[d] Sera of four pigs.
[e] Sera of three pigs.

DISCUSSION

The results from this study established vaccination protocols with two kinds of whole killed-cell vaccines that induced significant titers of circulating antibody to *Legionella* surface antigens as measured by ELISA and indirect fluorescent-antibody assay, and which gave solid protection against i.p. infection. The same vaccination protocols did not protect against aerosol infection, despite high levels of serum antibody. We do not know whether failure to protect against aerosol challenge is due to (i) a requirement for secretory antibody; (ii) a requirement in the lung for antibody to an antigen not expressed on HKC or AKC; (iii) a requirement for cellular immunity in the lung; or (iv) a hypersensitivity reaction occurring in the lungs of immunized pigs which obscures resistance.

To assess whether vaccination regimens with killed cells induce secretory antibody, determinations must be made on lung lavage fluids. Unlike studies on serum antibody, these experiments would require sacrifice of the animal. The lack of protection against aerosol challenge was unanticipated, and experiments to assess antibody in the lung have yet to be carried out in a systematic fashion.

In regard to the second hypothesis, there is not enough information available to evaluate the likelihood that it is correct. In humans, a rise in antibodies to HKC correlates with clinical disease, but it is presently unknown whether these antibodies are protective. Toxins of *Legionella* have been described (13, 16), and a possibility to consider is that antitoxin antibodies are necessary for resistance in the lung. The third hypothesis is perhaps the most appealing, as evidence supports the concept that *Legionella* organisms are facultative intracellular pathogens. They have been shown to grow in vitro in alveolar cells of the cynolmolgus monkey (20), in guinea pig peritoneal macrophages (21), and in human peripheral blood monocytes (17). Further, *Legionella* organisms have been shown to invade and grow intracellularly in tissue-cultured cells which are not phagocytes (9, 27, 33). Electron micrographs of lung tissue from infected humans and guinea pigs, and from human monocytes, are consistent with an interpretation of growth of the bacteria in ribosome-studded vesicles of macrophages (17, 19, 29, 30). In general, control over growth of other obligate or facultative intracellular pathogens is believed to occur by induction of cell-mediated immunity. In this pathway, macrophage activation is mediated by lymphokines released from sensitized T cells which have contacted the antigens of the organism to which the T cells have been previously exposed. The activated macrophage then acquires enhanced microbicidal or tumoricidal capacity or both (8, 23). Only live microorganisms or killed cells presented in complete Freund adjuvant are believed to induce cell-mediated immunity.

The fourth hypothesis has arisen from observations made in several experiments in which multiply immunized animals were aerosol challenged. They seemed to die faster and in greater respiratory distress than controls. Although our data on this point are preliminary, we are exploring the possibility that hypersensitivity in the lungs is abrogating protection. Precedent for such a situation is documented in the case of children receiving a killed vaccine of respiratory syncytial virus. They experienced greater incidence of disease, with more severe pneumonia, than did controls (14, 18).

The results presented in this paper cast doubt on the validity of using an i.p. route of challenge in experimental *Legionella* infections, and raise theoretical questions as to the relative importance of various mechanisms of host resistance to *Legionella*, as well as practical considerations to be assessed in vaccine development.

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LITERATURE CITED

1. **Berendt, R. F., H. W. Young, R. G. Allen, and G. L. Knudsen.** 1980. Dose-response of guinea pigs experimentally infected with aerosols of *Legionella pneumophila*. J. Infect. Dis. **141**:186–192.
2. **Bissett, M. L., J. O. Lee, and D. S. Lindquist.** 1983. New serogroup of *Legionella pneumophila*, serogroup 8. J. Clin. Microbiol. **17**:887–891.
3. **Bliss, C. J.** 1967. Statistics in biology, vol. 3, p. 63–65. McGraw-Hill Book Co., New York.
4. **Brenner, D. J., A. G. Steigerwalt, and J. E. McDade.** 1979. Classification of the Legionnaires' disease bacterium: *Legionella pneumophila*, genus novum, species nova, of the family Legionellaceae, familia nova. Ann. Intern. Med. **90**:656–658.
5. **Broome, C. V., and D. W. Fraser.** 1979. Epidemiologic aspects of legionellosis. Epidemiol. Rev. **1**:1–6.
6. **Centers for Disease Control.** 1978. Legionnaires' Disease—Australia. Morbid. Mortal. Weekly Rep. **27**:523.
7. **Chandler, F. W., J. E. McDade, M. D. Hicklin, J. A. Blackmon, B. M. Thomason, and E. P. Ewing.** 1979. Pathologic findings in guinea pigs inoculated intraperitoneally with the Legionnaire's disease bacterium. Ann. Intern. Med. **90**:671–675.
8. **Collins, F. M.** 1979. Cellular antimicrobial immunity. Crit. Rev. Microbiol. **7**:27–91.
9. **Daisy, J. A., C. E. Benson, J. McKitrick, and H. M. Friedman.** 1981. Intracellular replication of *Legionella pneumophila*. J. Infect. Dis. **143**:460–464.
10. **Eisenstein, T. K., B. J. De Cueninck, D. Resavy, G. D. Shockman, R. B. Carey, and R. M. Swenson.** 1983. Quantitative determination in human sera of vaccine-induced antibody to type-specific polysaccharides of group B streptococci using an enzyme-linked immunosorbent assay. J. Infect. Dis. **147**: 847–856.
11. **Elliott, J. A., W. Johnson, and C. M. Helms.** 1981. Ultrastructural localization and protective activity of a high-molecular-weight antigen isolated from *Legionella pneumophila*. Infect. Immun. **31**:822–824.
12. **Flesher, A. R., H. J. Jennings, C. Lugowski, and D. L. Kasper.** 1982. Isolation of a serogroup 1-specific antigen from *Legionella pneumophila*. J. Infect. Dis. **145**:224–233.
13. **Friedman, R. L., B. H. Iglewski, and R. D. Miller.** 1980. Identification of a cytotoxin produced by *Legionella pneumophila*. Infect. Immun. **29**:271–274.
14. **Fulginiti, V. A., J. J. Eller, O. F. Sieber, J. W. Joyner, M. Minamitani, and G. Meiklejohn.** 1969. Respiratory virus immunization. I. A field trial of two inactivated respiratory virus vaccines; an aqueous trivalent parainfluenza virus vaccine and an alum-precipitated respiratory syncytial virus vaccine. Am. J. Epidemiol. **89**:435–448.
15. **Guyton, A. C.** 1947. Measurement of respiratory volumes. Am. J. Physiol. **150**:70–77.
16. **Hedlund, K. W., and R. W. Larson.** 1982. The identification of a *Legionella pneumophila* toxin with *in vivo* lethality. Semin. Infect. Dis. **4**:450–455.
17. **Horwitz, M. A., and S. C. Silverstein.** 1980. Legionnaires' disease bacterium (*Legionella pneumophila*) multiplies intracellularly in human monocytes. J. Clin. Invest. **66**:441–450.
18. **Kapikian, A. Z., R. H. Mitchell, R. M. Chanock, R. A. Shvedoff, and C. E. Stewart.** 1969. An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. Am. J. Epidemiol. **89**:405–421.
19. **Katz, S. M., and S. Hashemi.** 1982. Electron microscopic examination of the inflammatory response to *Legionella pneumophila* in guinea pigs. Lab. Invest. **45**:24–32.
20. **Kishimoto, R. A., M. D. Kastello, J. D. White, F. G. Shirey, V. G. McGann, E. W. Larson, and K. W. Hedlund.** 1979. In vitro interaction between normal cynomolgus monkey alveolar macrophages and Legionnaires disease bacteria. Infect. Immun.

25:761–763.

21. **Kishimoto, R. A., J. D. White, F. G. Shirey, V. G. McGann, R. F. Berendt, E. W. Larson, and K. W. Hedlund.** 1981. In vitro response of guinea pig peritoneal macrophages to *Legionella pneumophila*. Infect. Immun. **31**:1209–1213.
22. **Landy, H.** 1953. Enhancement of the immunogenicity of typhoid vaccines by retention of the Vi antigen. Am. J. Hyg. **58**:148–164.
23. **Mackaness, G. B.** 1971. Resistance to intracellular infection. J. Infect. Dis. **123**:439–445.
24. **McDade, J. E., C. C. Shepard, D. Fraser, T. R. Tsai, M. A. Redus, and W. R. Dowdle, and the Laboratory Investigation Team.** 1977. Legionnaire's disease: isolation of a bacterium and demonstration of its role in other respiratory disease. N. Engl. J. Med. **297**:1197–1203.
25. **Middlebrook, G.** 1952. An apparatus for airborne infection. Proc. Soc. Exp. Biol. Med. **80**:105–110.
26. **Orrison, L. H., W. B. Cherry, R. L. Tyndall, C. B. Fliermans, S. B. Gough, M. A. Lambert, L. K. McDougal, W. F. Bibb, and D. J. Brenner.** 1983. *Legionella oakridgensis*: unusual new species isolated from cooling tower water. Appl. Environ. Microbiol. **45**:536–545.
27. **Rinaldo, C. R., Jr., A. W. Pasculle, R. L. Myerowitz, F. M. Gress, and J. N. Dowling.** 1981. Growth of the Pittsburgh pneumonia agent in animal cell cultures. Infect. Immun. **33**:939–943.
28. **Wilkinson, H. W., D. D. Cruce, B. J. Fikes, L. P. Yealy, and C. E. Farshy.** 1979. Indirect immunofluorescence test for Legionnaires' disease, p. 112–116. *In* G. L. Jones and G. A. Hebert (ed.), Laboratory manual: "Legionnaires' " the disease, the bacterium and methodology. Centers for Disease Control, Atlanta, Ga.
29. **Winn, W. C., Jr., G. S. Davis, D. W. Gump, J. E. Craighead, and H. N. Beaty.** 1982. Legionnaires' pneumonia after intratracheal inoculation of guinea pigs and rats. Lab. Invest. **47**:568–578.
30. **Winn, W. C., Jr., and M. L. Myerowitz.** 1981. The pathology of the *Legionella pneumophila*. Hum. Pathol. **12**:401–422.
31. **Wong, K. H., W. O. Schalla, M. C. Wong, P. R. B. McMaster, J. C. Feeley, and R. J. Arko.** 1982. Biologic activities of antigens from *Legionella pneumophila*, p. 434–443. *In* J. B. Robbins, J. C. Hill, and J. C. Sadoff (ed.), Bacterial vaccines. Thieme-Stratton, New York.
32. **Wong, K. W., W. O. Schalla, R. J. Arko, J. C. Bullard, and J. C. Feeley.** 1979. Immunochemical, serologic and immunologic properties of major antigens isolated from the Legionnaire's disease bacterium. Ann. Intern. Med. **90**:634–638.
33. **Wong, M. C., E. P. Ewing, Jr., C. S. Callaway, and W. L. Peacock, Jr.** 1980. Intracellular multiplication of *Legionella pneumophila* in cultured human embryonic lung fibroblasts. Infect. Immun. **28**:1014–1018.