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Respiratory Tularemia: Comparison of Selected Routes of Vaccination in Fischer 344 Rats

JOSEPH V. JEMSKI

United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21701

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Fischer 344 rats were given the attenuated live vaccine strain of *Francisella tularensis* by small-particle aerosol, intranasal instillation, or intraperitoneal, intramuscular, or subcutaneous injection. All of the vaccinated rats developed subclinical infection by 3 days after exposure, which cleared by day 28. Temporal patterns and concentrations of the live vaccine strain organism within the hosts were dependent on the route of vaccination. Pathological alterations were limited to minimal lung lesions in aerosol-vaccinated rats and mild splenitis in intraperitoneally vaccinated rats. Agglutinins to live vaccine strain were detected in the serum of each vaccinated animal and in the bronchoalveolor wash fluids of 66% of the aerosol-vaccinated rats. Agglutinin activity of the vaccinated rats was associated predominantly with the immunoglobulin M class. Regardless of the route of vaccine administration, all vaccinated rats survived an aerosol challenge of 5.3 $\log_{10}$ cells of virulent *F. tularensis*, whereas all nonvaccinated rats died. Systemic infection did not occur in the vaccinated rats. Pulmonary infection was not prevented in the vaccinated rats after aerosol challenge, but proliferation of the virulent *F. tularensis* organisms in the lungs was significantly lower (analysis of variance, $P \leqq 0.01$) than that which occurred in the control animals. These studies demonstrate the utility of the inbred Fischer 344 rat as a model host for further investigations of *F. tularensis* infection and its associated immune response.

The stimulation of immunity in the lungs by respiratory pathogens has been considered an important factor in host resistance (20). Recent approaches to research on this issue have involved depositing an immunogen in the respiratory tract by intranasal instillation or aerosol inhalation (5, 6, 9). In my laboratory, different methods of vaccination, including inhalation of aerosols of microbial antigens, are under investigation as a means for inducing immunity against respiratory diseases, e.g., respiratory tularensis. Several different strains of rats have been used with varying results in previous studies on the pathogenesis of and resistance to tularemia (2, 10, 13, 18, 21). Preliminary dose-response evaluations in my laboratory showed the inbred Fischer 344 rat to be consistently susceptible to infection with virulent *Francisella tularensis* and that immunity could be conferred to this rat strain with the attenuated live vaccine strain (LVS) of *F. tularensis*. Initial studies, therefore, emphasized the characterization of the pathogenesis of *F. tularensis* infection in Fischer 344 rats, the ultimate goal being to elucidate the relative importance of the component arms of the immune response in providing protection against lethal respiratory tularemia.

This report presents the response of Fischer 344 rats to infection caused by the administration of the LVS of *F. tularensis* by small-particle aerosol (SPA) or by the intranasal (i.n.), intraperitoneal (i.p.), intramuscular (i.m.), or subcutaneous (s.c.) routes. Also evaluated was the protection afforded these LVS-vaccinated rats after challenging them with aerosols of virulent *F. tularensis* SCHU S4.

MATERIALS AND METHODS

Animals. Inbred male Fischer 344 rats [CDF^R (F-344)/Crl BR] were procured from Charles River Breeding Laboratories, Inc., Wilmington, Mass. Rats weighing 200 to 240 g each were housed four to six per cage and given water and commercial feed pellets (Ralston-Purina, St. Louis, Mo.) ad libitum. After challenge with virulent *F. tularensis* SCHU S4, the rats were maintained in total-containment biological safety cabinets (7).

***F. tularensis* cultures.** Both attenuated live vaccine and virulent SCHU S4 strains of *F. tularensis* were cultured in modified casein-partial hydrolysate liquid medium and stored at −60°C as previously described (3). Frozen cultures were thawed just before

use. Viable cell concentrations were estimated by plating 10-fold serial dilutions on glucose-cysteine-blood agar plates. Typical colonies were counted after incubation for 48 to 72 h at 37°C.

Vaccination procedures. For i.n. vaccination, a 20-μl volume of LVS suspension was instilled by micropipette (Eppendorf) into the anterior nares of rats lightly anesthetized with halothane. Preliminary studies with carbon black as an indicator system showed that the 20 μl was retained entirely in the upper respiratory tract (unpublished data). Other groups of anesthetized rats were vaccinated by i.p., i.m., or s.c. injection with 0.5 ml of LVS suspension. Aerosol vaccination was performed by exposing unanesthetized rats in a modified Henderson aerosol apparatus (17) for 10 min to SPAs of LVS generated from a Collison spray device (12). The mass median diameter of the aerosol particles was 2 μm. With this procedure, the aerosol was deposited predominantly in the lung. Control animals were sham-vaccinated by exposing them similarly to aerosols of sterile modified casein-partial hydrolysate broth. LVS aerosol concentrations were determined by collecting aerosol samples in all-glass impingers (8) containing 20 ml of sterile gelatin-saline and plating suitable dilutions on glucose-cysteine-blood agar. The presented aerosol dose for the exposed animals was calculated on the basis of the formula: (LVS concentration per liter of aerosol) × (respiratory volume of a 250-g rat per minute, i.e., 0.13 liters/min) × (duration of animal exposure in minutes) (8).

Challenge procedures. Aerosol challenge of the vaccinated and control rats with virulent *F. tularensis* SCHU S4 and determination of aerosol challenge dose were conducted in the same manner as that described for aerosol vaccinations, the only exception being that all manipulations were performed in total-containment biological safety cabinets (8).

Assay procedures. Groups of rats were killed periodically after vaccination or challenge. Lungs, spleens, and cervical and mediastinal lymph nodes were aseptically excised. Sections of these tissues were processed for histological examination, and the balance of each was homogenized in gelatin-saline diluent. Appropriate dilutions of the homogenates were streaked on duplicate glucose-cysteine-blood agar plates for determination of viable organism concentrations. The plates were incubated for 48 to 72 h at 37°C, and typical colonies were enumerated. Numbers of organisms in blood and in bronchoalveolar wash (BAW) fluids were estimated by plating 0.1 ml of undiluted samples in duplicate on glucose-cysteine-blood agar. The BAW fluids were obtained by washing the air passages of the lungs with 3 ml of sterile phosphate-buffered saline, pH 7.2, instilled via the trachea. Gentle aspiration of the saline two to three times in the lungs resulted in obtaining 1.5 to 2 ml of fluid with no gross signs of blood.

Antibody assays. Agglutinating antibody titers for sera and BAW fluids were measured by using microtiter procedures (11). The BAW fluids were concentrated to 0.5 ml by filtration through 25,000-dalton ultrafilters (Millipore Corp., Bedford, Mass.) and maintained at −20°C until assayed for antibody content. The concentrations of selected classes of immunoglobulins (IgG, IgA, IgM) of sera and BAW fluids were estimated by radial immunodiffusion procedures (14), using specific anti-rat Ig prepared in rabbits or goats (Miles Laboratories, Inc., Elkhart, Ind.).

Rat strain susceptibility evaluation. Preliminary studies were conducted to compare the susceptibility of inbred Fischer 344 rats procured from two commercial sources (Charles River Breeding Laboratories and M. A. Bioproducts, Walkersville, Md.) and that of an outbred Sprague-Dawley rat strain (Taconic Farms, Germantown, N.Y.) to aerosol challenge and to i.p. challenge with virulent *F. tularensis* SCHU S4.

Experimental protocol. Within the three experiments reported here, groups of 50 to 70 Fischer 344 rats were vaccinated with LVS by aerosol exposure to SPA, by i.n. instillation, or by i.p., i.m., and s.c. injections. Control animals in each experiment were exposed to sterile broth aerosols in the same manner as was the LVS aerosol-vaccinated group. At 1, 3, 7, 10, 14, and 42 days after vaccination, a selected number of rats were killed by exposure to lethal doses of halothane. Sera and BAW fluids were obtained for serological assay, and tissues were cultured for LVS organisms. The remaining control and vaccinated rats were challenged with virulent *F. tularensis* aerosols 42 days after initial exposure to broth medium or vaccination. At 2, 3, and 14 days after aerosol challenge, 6 to 7 rats from each group were killed, and tissue samples were obtained, assayed for concentrations of challenge organisms, and examined for histological evidence of tularemia.

Statistical analysis. One-way analyses of variances were computed on the logarithms of organism concentrations in selected tissues from vaccinated and challenged rats and for the serum IgM levels in vaccinated rats.

RESULTS

Mortality response of rat strains to tularemia infection. The mortality of Fischer 344 inbred rats and an outbred Sprague-Dawley rat strain was compared after aerosol challenge with virulent *F. tularensis* SCHU S4 (Table 1). The effect of aerosolization on organism virulence was also determined by comparing the mortality of rats injected i.p. with the *F. tularensis* suspension before aerosolization and with *F. tularensis* organisms collected after aerosolization (Table 1). The Fischer rats from both sources

TABLE 1. *Mortality of rats exposed to F. tularensis SCHU S4*

Mode of challenge	Challenge dose (log_{10})	% Mortality (days to death) of following type of rat:[a]		
		MA-F (n = 12)	CR-F (n = 8)	Tac-SD (n = 8)
SPA	5.04	92 (3-12)	100 (5-13)	0
i.p. (pre-aerosol)	5.5	100 (2-5)	100 (2-4)	50 (4-5)
i.p. (post-aerosol)	5.11	100 (3)	88 (3-7)	63 (3-9)

[a] MA-F, Microbiological Associates-Fischer 344; CR-F, Charles River-Fischer 344; Tac-SD, Taconic Farms-Sprague-Dawley.

were highly susceptible to lethal infection with *F. tularensis*. More than 90% of these rats died 5 to 13 days after aerosol exposure to 5 $\log_{10}$ cells of *F. tularensis* and within 2 to 7 days after i.p. infection with either the pre- or postaerosolization of organisms. The Sprague-Dawley rats were fully resistant to aerosol challenge, and a combined 56% of the rats died after both types of i.p. challenge with *F. tularensis*. Based on these results, the Fischer 344 rat was selected for use in subsequent studies on LVS vaccination and protective immunity generated against respiratory tularemia.

LVS population dynamics as a function of method of vaccination. The distribution and persistence of LVS in selected tissues of rats vaccinated by different routes was determined over 28 days. The LVS assay data were recorded for lungs, spleen, and lymph nodes through day 14 only because LVS was rarely recovered thereafter (Fig. 1). None of the tissues of sham-vaccinated rats contained LVS (data not shown). In addition, data have not been presented for rats vaccinated by either i.m. or s.c. injections because, for these animals, the patterns of LVS distribution, concentration and clearance from tissues, and appearance of agglutinin titers were similar to the patterns described for the i.n. vaccinated rats.

All of the rats vaccinated by aerosol exposure yielded LVS organisms from the lungs (Fig. 1). Significantly higher concentrations of organisms (5.0 to 7.0 $\log_{10}$) were present in the lungs of these rats on each assay day, except day 1, as compared with the rats vaccinated by the i.n. or i.p. routes (analysis of variance, $P \leqq 0.01$). Surprisingly, on day 1 after exposure, LVS organisms were cultured from the lungs of 89% of the rats vaccinated by i.p. injection at concentrations not statistically different from those determined for the aerosol-vaccinated rats. LVS organisms were not isolated from the lungs of i.n. vaccinated rats until day 3, with the maximum concentration not exceeding 3.0 $\log_{10}$. For all three groups of rats, there was a progressive diminution of viable tularensis organisms in the lungs through day 14. No organisms were recovered from any rat by day 28.

Spleens of the i.p. vaccinated rats contained more LVS organisms (analysis of variance, $P \leqq 0.01$) on days 1 and 3 than did those of the SPA- and i.n. vaccinated rats. From days 7 through 14, concentrations of LVS organisms in the spleen were approximately equal in all rats and ranged from 2 to 4 $\log_{10}$. As with the lungs, spleens had been cleared of LVS by 28 days.

In general, 3 to 4 $\log_{10}$ LVS organisms were recovered from a pool of cervical and mediastinal lymph nodes of each rat regardless of the route of vaccination. The lymph nodes also were cleared of LVS organisms by day 28.

Blood and BAW fluids were sampled for the presence of LVS organisms, and serum and BAW fluids were assayed for LVS agglutinin titers. A transient and low-level bacteremia occasionally was observed among the aerosol and i.n. vaccinated rats. Low levels of LVS organisms (<100 colony-forming units per ml of blood),

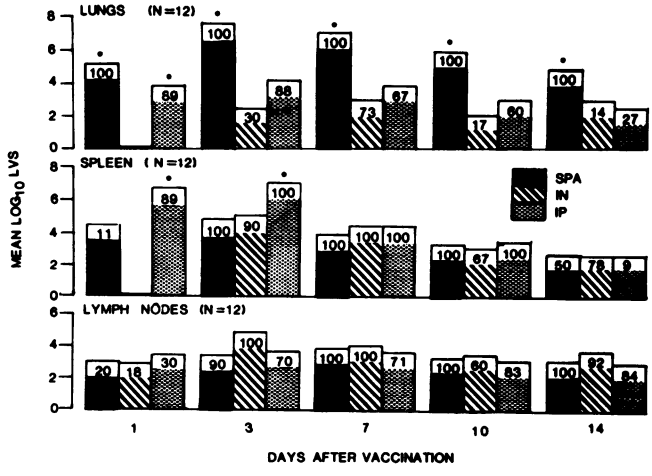

FIG. 1. *Mean number ($\log_{10}$) of LVS organisms in lungs, spleens, and lymph nodes of F-344 rats at various days after vaccination either by SP aerosols or i.n. or i.p. route (geometric mean $\log_{10}$ dose over all routes, 5.1 ± 0.59). The number at the top of each bar indicates the percentage of rats from which LVS was recovered. The asterisk above a bar indicates an LVS concentration significantly higher (analysis of variance, $P \leqq 0.01$) than another in the same group.*

however, were recovered from all of the i.p. vaccinated rats through day 3. The BAW fluids from all SPA-vaccinated rats yielded LVS organisms for 14 days at concentrations that did not exceed 3 $\log_{10}$. The LVS also was recovered from the BAW fluids from one-third of the i.p. vaccinated rats between days 3 through 10. Viable LVS bacteria were not detected in the BAW fluids of any members of the i.n. vaccinated group.

Serum and BAW fluids were assayed for LVS agglutinin titers (Table 2). For all vaccinated groups, serum titers were evident by day 7. Generally, the titers peaked by day 10 and subsided to their lowest levels (~1:200) by day 42. The highest serum titers were produced by i.p. vaccination. Low-titered BAW agglutinins were detected for about two-thirds of the SPA-vaccinated rats through day 10. Serologically positive BAW fluids were detected only on day 7 for one-third of the i.p. vaccinated rats and for one-third of the i.n. vaccinated animals only on day 10.

TABLE 2. *Serum and BAW agglutinin titers in LVS-vaccinated rats*

Vaccination route[a] and fluid	% Rats (mean titer[b]) with titer by day:			
	7	10	14	42
SPA				
Serum	100 (352)	100 (1,478)	100 (628)	100 (126)
BAW	67 (24)	67 (20)	0	0
i.n.				
Serum	73 (278)	78 (452)	100 (666)	80 (226)
BAW	0	33 (10)	0	0
i.p.				
Serum	100 (1,574)	100 (2,030)	100 (878)	100 (240)
BAW	33 (20)	0	0	0

[a] Geometric mean $\log_{10}$ vaccination dose over all routes, 5.1 ± 0.59.

[b] Reciprocal of positive animals.

Changes in concentrations of the different classes of immunoglobulins were detected only for IgM (Fig. 2). Serum IgM levels in both aerosol and i.p. vaccinated rats were significantly higher than those of the nonvaccinated control rats from days 7 through 14 (analysis of variance, $P \leq 0.05$). The serum IgM concentration for the i.n. vaccinated rats was increased significantly only at day 14. IgM also was detected in the BAW fluids of some of the SPA-vaccinated rats on days 7 and 10 and in one i.p. vaccinated rat on day 7. The increase in IgM levels in both serum and BAW fluids paralleled the increase and abatement of the LVS agglutinin titers in these fluids (Table 2).

Response of LVS-vaccinated animals to aerosol challenge. At 42 days after LVS vaccination, the sham-vaccinated control rats and the five groups of vaccinated rats were challenged with a mean aerosol dose of 5.3 $\log_{10}$ cells of virulent *F. tularensis* SCHU S4. The data shown in Fig. 3, however, are for the control-, aerosol-, i.n., and i.p. vaccinated groups of rats only. All of the control rats appeared ill 3 days after aerosol challenge. By day 7 after challenge, concentrations of challenge organisms ranged from 7 $\log_{10}$ in the lungs to about 5 $\log_{10}$ in the spleen and lymph nodes of the control rats, despite the presence of a high serum antibody titer (mean, 1:1,178). Of these rats, 94% died

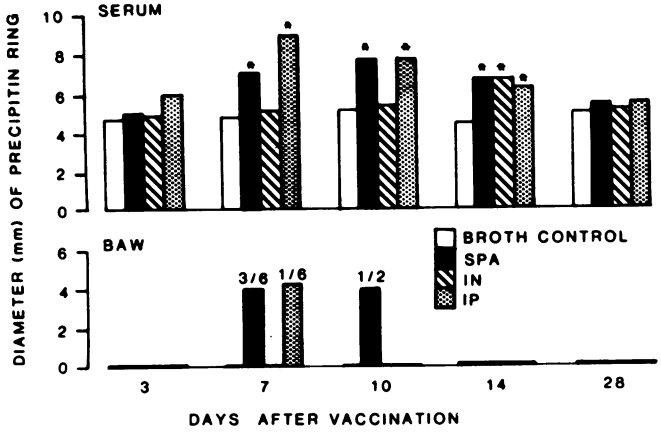

FIG. 2. *Relative levels of IgM in serum and BAW fluids as measured by radial immunodiffusion and based on diameter (millimeters) of precipitin ring. The mean diameter of the precipitin ring was calculated on the basis of three replicate samples per rat (n = 6). The asterisk above a bar for the serum samples indicates an IgM concentration significantly higher (analysis of variance, P ≦ 0.05) than that of the broth control. The numbers over the bars for the BAW samples indicate the proportion of the rats with IgM concentrations increased over baseline values.*

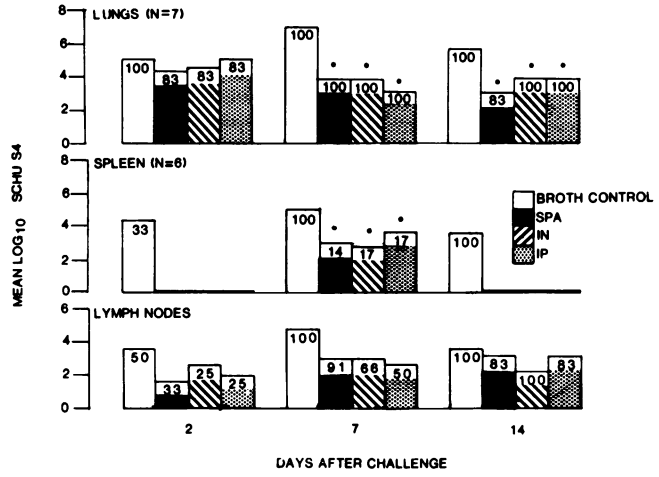

FIG. 3. *Mean $\log_{10}$ challenge organisms in lungs, spleens, and lymph nodes after exposure to an aerosol containing $10^{5.3}$ virulent F. tularensis. The number at the top of each bar indicates the percentage of rats from which tularensis organisms were recovered. The asterisk above a bar indicates an organism concentration significantly lower (analysis of variance, $P \leq 0.01$) than that of the broth control.*

between 6 and 14 days, leaving only two for assay. In the three groups of vaccinated rats, the overall mean serum agglutinin titer was 1:248 (range, 160 to 285).

Although none of these vaccinated rats died or showed signs of clinical illness for a period of 90 days after aerosol challenge, pulmonary infection was not prevented (Fig. 3). After day 2, the peak concentrations of tularensis organisms in the lungs of the vaccinated animals were 2 to 3 $\log_{10}$ lower than those in the control rats (analysis of variance, $P \leq 0.01$). Histologically, pulmonary lesions associated with *F. tularensis* infection were not detected. The vaccinated rats, therefore, apparently were protected against respiratory tularensis, even though significant numbers of virulent tularensis organisms were present in the lungs. In contrast, the typical pyogranulomatous lesions of tularemia were observed in the lungs of the control rats.

Infection was not evidenced in the spleens of rats in each of the vaccinated groups at 2 days after challenge. Furthermore, organisms were isolated from only one rat in each of these groups and only on day 7 postchallenge. The challenge organism was isolated, however, from one-third of the control rats on day 2 postchallenge and from all of the remaining control animals on days 7 and 14. Lesions typical of tularemia were observed only in the spleens of the nonvaccinated control animals. Infection of the cervical and mediastinal lymph nodes was present in all vaccinated groups of rats and in the control animals up to day 14. Concentrations of challenge organisms in the lymph nodes and the percentage of vaccinated rats with infected nodes approximated that obtained for the nonvaccinated control rats.

Bacteremia was demonstrable in all of the control rats sampled from 2 to 7 days after challenge. Concentrations of *F. tularensis* approximated 3 $\log_{10}$/ml of blood. None of the vaccinated rats yielded a positive blood culture except for one i.p. vaccinated rat, from which small numbers of organisms were recovered on day 7. The serum agglutinin titers of the vaccinated rats were no higher at 48 h after aerosol challenge than they were just before challenge. BAW fluid agglutinin titers were not detected after challenge at any time.

All of the rats vaccinated by i.m. or s.c. injections also survived the aerosol challenge with no overt signs of illness. All other response parameters of the i.m. and s.c. vaccinated rats to challenge closely resembled those observed for the i.n. vaccinated rats. The data for the i.m. and s.c. rats, therefore, were not presented in this report.

DISCUSSION

Vaccination of inbred Fischer 344 rats with the LVS of *F. tularensis* via i.p., i.m., or s.c. injection, i.n. instillation, or aerosol inhalation resulted in temporal patterns and concentrations of the LVS organism with the vaccinated host that were dependent on the route of administration. Regardless of the method of vaccination, none of the rats became febrile or exhibited overt signs of illness, even though 3 to 7 $\log_{10}$ of LVS organisms were recovered from lungs, spleens, and lymph nodes through 14 days. Histologically, the minimal lung lesions in the aerosol-vaccinated rats and the mild splenitis in the

i.p. vaccinated rats were analogous to previously described pathological alterations in Fischer 344 rats infected by i.p. inoculation with LVS (13). Thus, the subclinical infection induced by the attenuated vaccine strain of *F. tularensis* was not altered by the route of administration. A consistent bacteremia, indicative of the hematogenous transport of the LVS vaccine, occurred only in the i.p. vaccinated rats between days 1 and 3. Bacteremia was rarely observed in rats vaccinated by aerosol inhalation or by i.n. instillation, suggesting that systemic spread of the vaccine strain with these two methods of vaccination occurs early by way of the lymphatic system. The LVS organisms were cleared from all tissues of the vaccinated rats after 14 days regardless of the vaccination method.

Serum LVS agglutinins were induced readily by all methods of vaccination, the highest titers being obtained by the i.p. route. Antibodies were detected 3 days after vaccination and reached peak levels usually by day 10. The increase in antibody production coincided with a general decrease in concentration of LVS in the tissues and with the frequency of host infection. By day 12, antibody titers leveled off at 1:126 to 1:240. At 18 months after either aerosol or i.m. vaccination, serum titers of 1:20 to 1:80 were still detectable (unpublished data). The immunoglobulin class associated with the agglutinin activity was IgM, which corroborates previous observations in humans (1); adult male volunteers given a percutaneous vaccination with 8.0 $\log_{10}$ LVS cells developed demonstrable antibody within 7 days that was predominantly IgM. The LVS agglutinins detected in the BAW fluids of the aerosol-vaccinated rats were also comprised of IgM. Previous evidence indicated that IgM was not a normal constituent of respiratory fluids (16), even though a variable number of IgM-producing cells reside within the respiratory tract parenchyma (19). Under appropriate stimuli, such as inhalation of an aerosolized antigen, it could be that such cells would synthesize IgM antibodies. It is possible also that transudation of serum IgM across respiratory mucous membranes may have occurred, as significant levels of IgM existed simultaneously in both the sera and BAW fluids of the aerosol-vaccinated rats. The data available, however, do not substantiate transudation of serum IgM into the bronchi and related spaces. Of all the rats tested after i.p. injection of i.n. instillation of LVS, only one i.p. vaccinated rat exhibited detectable IgM in BAW fluids, even though IgM was demonstrable in the sera of all of these animals. Thus, the presence of surface IgM within the lungs most likely results from direct stimulation of antibody-producing cells. Definition of the explicit source of the surface IgM and the role of these antibodies in respiratory tularemia requires further investigation.

All rats vaccinated with *F. tularensis* LVS, regardless of route (aerosol, i.n., i.p., i.m., s.c.), were protected against a lethal challenge with virulent *F. tularensis* SCHU S4. None of the vaccinated rats, each possessing low-level antibody titers, evidenced overt clinical signs, developed detectable bacteremia, or died. In contrast, all of the nonvaccinated control rats exhibited definite signs of illness and became bacteremic despite the development of high titers (mean, 1:1,178) of serum agglutinin. Of these animals, 95% died after challenge. Accordingly, it would appear that preexisting serum antibodies may have a role in preventing systemic infection in challenged rats. This contention is consistent with the findings of Proctor et al. (15). They report that peripherial neutrophils, which are rapidly mobilized during the first 18 h of infection, can limit or prevent the early dissemination of *F. tularensis* organisms by phagocytosis, but only in the presence of immune serum. In this situation, the preexisting tularensis antibodies act as specific opinions. The absence of an anamnestic reaction in the vaccinated rats after challenge also may be a result of this opsonization. A similar lack of rise in serum antibody titers in vaccinated volunteers after aerosol challenge has been reported (1). These cumulative data imply that preexisting humoral antibodies may play a significant role in the pathogenesis of early clinical tularemia in concert with cell-mediated immunity, which has been reported to be the prime immune response against lethal tularemia (4). These data also lead to the conclusion that immunity to lethal respiratory tularemia can be as effectively induced by either aerosol inhalation or i.n. instillation of LVS antigen as by the injection of LVS by the i.p., i.m., or s.c. route.

The inbred Fischer 344 rat, therefore, provides an excellent model for further investigations of *F. tularensis* infection and its associated immune responses. Comparative studies with nonimmune, immune, immunosuppressed, and immunopotentiated rats can be designed to evaluate the relative importance of the effector arms of immunity to respiratory tularemia specifically and to respiratory diseases in general.

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