Antibodies Contribute to Effective Vaccination against Respiratory Infection by Type A *Francisella tularensis* Strains

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Pneumonic tularemia is a life-threatening disease caused by inhalation of the highly infectious intracellular bacterium *Francisella tularensis*. The most serious form of the disease associated with the type A strains can be prevented in experimental animals through vaccination with the attenuated live vaccine strain (LVS). The protection is largely cell mediated, but the contribution of antibodies remains controversial. We addressed this issue in a series of passive immunization studies in Fischer 344 (F344) rats. Subcutaneous LVS vaccination induced a robust serum antibody response dominated by IgM, IgG2a, and IgG2b antibodies. Prophylactic administration of LVS immune serum or purified immune IgG reduced the severity and duration of disease in naïve rats challenged intratracheally with a lethal dose of the virulent type A strain SCHU S4. The level of resistance increased with the volume of immune serum given, but the maximum survivable SCHU S4 challenge dose was at least 100-fold lower than that shown for LVS-vaccinated rats. Protection correlated with reduced systemic bacterial growth, less severe histopathology in the liver and spleen during the early phase of infection, and bacterial clearance by a T cell-dependent mechanism. Our results suggest that treatment with immune serum limited the sequelae associated with infection, thereby enabling a sterilizing T cell response to develop and resolve the infection. Thus, antibodies induced by LVS vaccination may contribute to the defense of F344 rats against respiratory infection by type A strains of *F. tularensis*.

Pneumonic tularemia is a highly debilitating disease caused by the Gram-negative coccobacillus *Francisella tularensis*. Strains classified under subspecies *tularensis* (type A) are the most virulent and pose the biggest challenge from a clinical perspective (28), with a mortality rate estimated to exceed 30% in untreated patients (11). Prophylactic vaccination is the best countermeasure, and there is good historical evidence that pneumonic tularemia can be prevented by vaccination with the attenuated *F. tularensis* live vaccine strain (LVS) (37). However, LVS is unlikely to be licensed for mass vaccination because the mechanism of attenuation has not been defined. Due to the potential of a major public health threat, there is an urgent need to understand the protective mechanisms associated with an effective immune response so that novel vaccines can be developed.

Protective immunity against *F. tularensis* infection is usually attributed to an effective T cell response. However, *F. tularensis* has a significant extracellular phase, which makes it accessible to humoral immune responses (18). Indeed, there is ample evidence that B cells and antibodies are necessary for mice to develop their natural resistance to primary and secondary LVS infections. Purified lipopolysaccharide (LPS) from LVS induced a population of B1-a cells within 2 to 3 days of administration that protected mice against intraperitoneal (i.p.) LVS challenge (6, 7, 14). Consistent with these results, μMT mice lacking mature B cells exhibited increased susceptibility to primary intradermal (i.d.) LVS infection and delayed bacterial clearance (15, 40). μMT mice were also more susceptible to secondary i.p. LVS infection, and this defect was corrected by reconstitution with LVS-primed B cells (15). The contribution of antibodies has been addressed repeatedly in passive immunization experiments, which showed that immune serum from humans and mice vaccinated with live or inactivated LVS protected naive mice against challenges with LVS or other low virulence strains given by a variety of routes (13, 19, 26, 29, 33, 36, 40). The dominant antibody response was directed at LPS, but antibodies against protein antigens have also been found (17, 23, 31, 41, 43). Monoclonal antibodies specific for LPS or the outer membrane protein FopA provided significant protection against LVS challenge when given either prophylactically (38) or therapeutically (30, 38). Together, these results suggest that antibodies contribute toward effective control of attenuated or low-virulence *F. tularensis* strains.

It has been much more difficult to demonstrate antibody-mediated protection against type A strains in mice (1, 20, 21, 38), even though they express many antigens recognized by LVS immune serum (13, 30). This is not surprising given the historical difficulties in generating protective immunity against type A strains in this animal model (5). However, Ray et al. recently showed that oral LVS vaccination protected mice against a pulmonary SCHU S4 challenge in an antibody-dependent manner (35). Klümper et al. also reported a similar finding using immune serum from mice cured of a lethal intranasal (i.n.) SCHU S4 infection with levofloxacin in a passive immunization model (27). Thus, the protective effects of antibodies appear not to be restricted only to low-virulence strains but may also contribute to the protection against highly virulent type A strains.

To further characterize the mechanism of antibody-medi-
ated protection, we utilized the recently characterized Fischer 344 (F344) rat model (45). Since F344 rats developed much stronger resistance to respiratory SCHU S4 challenge after LVS vaccination than previously observed in mice, we speculated that antibodies may provide better protection in this model and allow us to define their protective mechanism more thoroughly. We now show in a passive immunization model that serum antibodies from LVS-vaccinated rats conferred protection against a lethal intratracheal (i.t.) SCHU S4 challenge. Protection correlated with reduced systemic bacterial growth and less severe histopathology during the early phase of infection and bacterial clearance by a T cell-dependent mechanism. Thus, antibodies contribute to but are not sufficient for the effective control of respiratory infections by fully virulent type A strains. Our studies provide valuable insights into the protective mechanisms of antibodies that will guide future development of tularemia vaccine candidates.

MATERIALS AND METHODS

Rats. Female F344 rats and athymic nude/rnu/rnu rats were purchased from the National Cancer Institute—Frederick (Frederick, MD). The animals were housed in a specific-pathogen-free facility at the University of New Mexico Animal Resource Facility. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee and the Biosafety Committee at the University of New Mexico.

Bacteria. F. tularensis strains LVS and SCHU S4 were obtained from DynPort Vaccine Company LLC (Frederick, MD). The original stock was expanded in Chamberlain’s broth (Teknova, Hollister, CA) at 37°C for 48 h with gentle shaking, and aliquots of the culture were stored at −80°C without any preservative.

LVS vaccination and serum collection. Rats were lightly anesthetized with isoflurane (Abbott Laboratories, Chicago, IL) and vaccinated subcutaneously (s.c.) between the shoulder blades with 5 × 10^6 CFU LVS in 100 μl of phosphate-buffered saline (PBS). Four weeks after vaccination, rats were euthanized by CO2 overexposure and immune serum was collected and pooled. Normal serum was collected in a similar manner or purchased from Charles River Laboratories (Wilmington, MA). Both normal and immune sera were heated to 55°C for 30 min to inactivate complement, filter sterilized through a 0.22-μm syringe tip filter (Millipore, Billerica, MA), and stored at −80°C.

Purification of serum IgG and IgM. Serum IgG and IgM were purified using a Melon gel purification kit (Thermo Scientific Pierce Protein Research, Rockford, IL) and Capture Select IgG affinity matrix (BAC B.V., Naarden, Netherlands), respectively, following the manufacturer's instructions with a few modifications. Briefly, normal and immune sera were dialyzed against PBS for IgM purification. The suspensions were subsequently dialyzed 3 times for IgG purification. The concentration of rat IgG was determined by using a sandwich ELISA with a goat anti-rat IgG antibody and human CD2; IgG1) was from the American Type Culture Collection (Manassas, VA). A similar procedure was followed to determine the concentration of rats, purified IgG and IgM were used in some experiments; the volume given contained 100 mg/kg of body weight; Fort Dodge Animal Health, Fort Dodge, IA) on days 1, 3, 5, 7, 10, 14, and 21 postchallenge. Lungs were removed from the thorax en bloc and inflated with 10% neutral buffered formalin (NBF) for 20 min. NBF-fixed lungs were sectioned, fixed in 10% NBF for 24 h and subsequently trimmed for paraffin embedding. Paraffin-embedded tissues were sectioned at 5 μm and stained with hematoxylin and eosin for histological analysis by a board-certified veterinary pathologist. Lesions were graded in a blinded manner on a semiquantitative scale based on the severity and distribution of lesions (1, minimal; 2, mild; 3, moderate; 4, marked).

In vivo T cell depletion and flow cytometry. The hybridoma clones OX-8 (mouse anti-rat CD8; IgG1), OX-38 (mouse anti-rat CD4; IgG2a), and 55-6 (mouse anti-HIV-1 gp120; IgG2a) were obtained from the European Collection of Cell Cultures (Salisbury, United Kingdom), and TS2/18.1.1 (mouse anti-human CD2; IgG1) was from the American Type Culture Collection (Manassas, VA). Ascent fluids were generated in female ICR SCID mice, and the IgG concentrations were determined by high-performance liquid chromatography (Taconic, Albany, NY). For CD4 T cell depletion, rats were injected i.p. with 5 mg/kg of CD4 T cell-depleting antibody OX-38 or isotype control antibody 55-6 for five consecutive days and then with 1 mg/rat twice a week. For CD8 T cell depletion, 1 mg/rat of CD8 T cell-depleting antibody OX-8 or isotype control antibody TS2/18.1.1 antibodies were administered once a week. One week after the start of antibody treatment, the depletion efficiency was confirmed by flow cytometric analyses of peripheral blood mononuclear cells collected by lateral tail vein bleed. In addition, the effects of CD4 T cell depletion on the populations of NK cells, B cells, and CD8 T cells in the spleen, liver, and blood were examined. Rats were euthanized by CO2 overexposure and exanguinated by cutting the inferior vena cava. Blood collected from the chest cavity was mixed

horseradish peroxidase-conjugated goat antibodies against rat IgG (Calbiochem, San Diego, CA) and IgG1, IgG2a, IgG2b, IgG2e, IgM, and IgA (Thermo Scientific Pierce Protein Research Products, Rockford, IL) for 45 min at 37°C. Between each step, the plates were washed 5 times with 0.05% Tween 20–PBS. The plates were developed with a solution of 3,3′,5′,5′-tetramethylbenzidine (TMB), the reaction was stopped with 1.8 H2O2, and the optical density (OD) was read at 450 nm. The antibody titer was defined as the reciprocal of the highest dilution of immune serum that had a mean OD value that was at least 3 standard deviations higher than the mean OD of normal serum at the same dilution.

Passive immunization and challenge. Rats were passively immunized by i.p. injection of 250 μl of serum, unless otherwise indicated in selected experiments. Purified IgG and IgM was used in some experiments; the volume given contained the equivalent amount of LVS-specific IgG as in 250 μl of LVS-immune serum. At 24 h after passive immunization, rats were infected i.t. as described previously (45). Briefly, rats were immobilized on an inclined platform (Alpha Lab Supply, Albuquerque, NM) and intubated with a 20-gauge intravenous catheter (Terumo Medical Products, Somerset, NJ) with the help of a small animal laryngoscope with a fiber optic light source (Penn-Century, Inc., Philadelphia, PA). In some experiments 50 μl of inoculum premixed with self-illuminating quantum dots (Zymera, San Jose, CA) was delivered using a blunt-ended needle, followed by 50 μl of Coelement and a burst of ≥500 μl of air to ensure delivery of the inoculum. The infected rats were imaged in vivo using the IVIS 100 optical imaging system (Caliper Life Sciences, Hopkinton, MA). The health of the infected animals was monitored daily along with weight loss and survival. The clinical scores of infection were assigned as follows: 0, active, bright and alert, responsive to handling; 1, slight lethargy and weight loss; 2, decreased responsiveness to handling, clear premorbid signs; 3, decreased activity, ruffled coat, rapid and shallow breathing, hunched posture, eyes half closed and may have porphyrin secretion; 4, inactive and unresponsive to handling, and/or ataxic, severe weight loss, eyes completely closed with a large amount of porphyrin secretion. Animals that succumbed to infection were given a maximum score of 4.

Bacterial burden analysis. To quantify the deposited bacteria, lungs were aseptically removed 1 h after infection and homogenized in PBS using a handheld or multisample homogenizer fitted with disposable plastic homogenizing probes (Omni International, Marietta, GA). Lung homogenates were plated neat or at appropriate dilutions onto selective cytotoxic heart agar plates with 5% rabbit blood, 100 U/ml penicillin G, and 100 U/ml polymyxin B (Remel, Lexena, KS), and bacterial colonies were quantified 4 to 5 days later using Qcount (Spiral Biotech, Bethesda, MD). A similar procedure was followed to determine the bacterial burden in lungs, spleen, and liver over the course of infection. When no organism was found, a value equal to the limit of detection was used to calculate the mean bacterial burden.

Histopathological evaluation. Following i.t. challenge, rats were euthanized by i.p. injection of 150 μl of Sleepaway (>100 mg/kg of body weight; Fort Dodge Animal Health, Fort Dodge, IA) on days 1, 3, 5, 7, 10, 14, and 21 postchallenge. Lungs were removed from the thorax en bloc and inflated with 10% neutral buffered formalin (NBF) for 20 min. NBF-fixed lungs were sectioned, fixed in 10% NBF for 24 h and subsequently trimmed for paraffin embedding. Paraffin-embedded tissues were sectioned at 5 μm and stained with hematoxylin and eosin for histological analysis by a board-certified veterinary pathologist. Lesions were graded in a blinded manner on a semiquantitative scale based on the severity and distribution of lesions (1, minimal; 2, mild; 3, moderate; 4, marked).

In vivo passive immunization against respiratory tularemia. The hybridoma clones OX-8 (mouse anti-rat CD8; IgG1), OX-38 (mouse anti-rat CD4; IgG2a), and 55-6 (mouse anti-HIV-1 gp120; IgG2a) were obtained from the European Collection of Cell Cultures (Salisbury, United Kingdom), and TS2/18.1.1 (mouse anti-human CD2; IgG1) was from the American Type Culture Collection (Manassas, VA). Ascent fluids were generated in female ICR SCID mice, and the IgG concentrations were determined by high-performance liquid chromatography (Taconic, Albany, NY). For CD4 T cell depletion, rats were injected i.p. with 5 mg/kg of CD4 T cell-depleting antibody OX-38 or isotype control antibody 55-6 for five consecutive days and then with 1 mg/rat twice a week. For CD8 T cell depletion, 1 mg/rat of CD8 T cell-depleting antibody OX-8 or isotype control antibody TS2/18.1.1 antibodies were administered once a week. One week after the start of antibody treatment, the depletion efficiency was confirmed by flow cytometric analyses of peripheral blood mononuclear cells collected by lateral tail vein bleed. In addition, the effects of CD4 T cell depletion on the populations of NK cells, B cells, and CD8 T cells in the spleen, liver, and blood were examined. Rats were euthanized by CO2 overexposure and exanguinated by cutting the inferior vena cava. Blood collected from the chest cavity was mixed
FIG. 1. Subcutaneous LVS vaccination induces a robust IgM and IgG serum antibody response. F344 rats (n = 3) were vaccinated s.c. with 5 × 10⁷ CFU of LVS. Sera were collected on days 7, 15, 21, and 28 postinoculation, and the total LVS-specific IgM, IgG, and IgA antibody titers were determined for each rat by ELISA using heat-killed LVS as the capture antigen. The antibody titer was defined as the reciprocal of the highest dilution of immune serum that had a mean OD value that was greater than 3 standard deviations above the mean OD of normal serum at the same dilution. The data show the geometric mean ± standard deviation.

TABLE 2. Antibody composition in the immune serum used for passive immunization. F344 rats (n = 3 to 4) were vaccinated s.c. with 5 × 10⁷ CFU of LVS. Sera were collected 28 days postvaccination and analyzed by ELISA for the presence of LVS-specific antibodies of the indicated isotypes and subclasses by using heat-killed LVS as the capture antigen. Antibody titers were defined as described for Fig. 1. The data represent the geometric means ± standard deviations of samples combined from two independent experiments.
and surrounded by a large amount of ocular discharge. Most infected rats died within 2 weeks of infection (Fig. 3C). Although the IRS-treated rats exhibited some of the early signs of infection, weight loss occurred more gradually and rarely exceeded 10% of the initial body weight. All signs of illness resolved within 2 weeks of infection, and the rats remained outwardly healthy for the remaining 2 to 3 weeks of monitoring. LVS-vaccinated rats never lost weight or exhibited any overt signs of disease. In five independent experiments, at a SCHU S4 challenge dose of 218 to 240 CFU, 27 of 29 rats treated with IRS survived, while 29 of 30 NRS-treated rats died (Table 1). These results suggested that serum antibodies are capable of mediating protection against a lethal respiratory SCHU S4 infection.

**Purified IgG is sufficient for protection against SCHU S4 infection.** To verify that LVS-specific antibodies were responsible for the serum-mediated protection, IgG and IgM were purified from normal and immune sera. The purification process reduced the titers of contaminating antibody isotypes to <1:100 and removed most contaminating proteins, except for a prominent 75-kDa protein in the purified IgG fraction that has not been identified (Fig. 4A and B). For passive immunization, F344 rats were injected with an amount of purified IgG and/or IgM that was equivalent to that contained in 250 μl of serum. Similar to IRS, purified immune IgG provided significant protection against i.t. SCHU S4 challenge (Fig. 4C). In contrast, purified immune IgM offered no protection and the treated rats succumbed to SCHU S4 infection at the same time as rats treated with normal serum. These results indicated that LVS-specific IgG is the principal protective component in the immune serum. Since IRS and purified immune IgG provided similar levels of protection, IRS was used in all experiments described here.

**Immune serum treatment of F344 rats limits SCHU S4 growth.** Since IRS treatment provided significant protection against pulmonary SCHU S4 infection, we next investigated the effect of this treatment on SCHU S4 growth. IRS-treated rats exhibited a pattern of SCHU S4 growth and dissemination that was intermediate between the NRS-treated rats and the LVS-vaccinated rats. Bacterial expansion in the first 2 days following infection was identical between the IRS- and NRS-treated rats: in both groups, the number of lung bacteria increased to 10^7 CFU and systemic dissemination to the liver and spleen had occurred in the majority of animals (Fig. 5). The two groups started to diverge on day 3, when fewer bacteria were recovered from the IRS-treated rats. The bacterial burden in NRS-treated rats peaked on day 7 p.i., shortly before they died, with 8 x 10^8 CFU in the lungs, 4 x 10^8 CFU in the liver, and 2 x 10^7 CFU in the spleen. In contrast, the bacterial burden in the IRS-treated rats increased at a slower rate, to a peak on day 10 p.i. By day 14, the infection began to clear in the IRS-treated rats, and the bacterial load in all three tissues had dropped from their peak. These results suggest that IRS may contribute to protection of naïve F344 rats by limiting bacterial growth and facilitating development of an immune response that eventually clears the infection.

**Protection by IRS is dependent on the SCHU S4 challenge dose and the volume of IRS.** To further characterize the po-

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**TABLE 1. Summary of survival results of F344 rats challenged intratracheally with SCHU S4 after prophylactic treatment with 250 μl of serum**

<table>
<thead>
<tr>
<th>SCHU S4 challenge dose (CFU)</th>
<th>Immune rat serum</th>
<th>Normal rat serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Survival ratio (no. alive/total)</td>
<td>MTD (days)</td>
</tr>
<tr>
<td>130</td>
<td>6/6</td>
<td>1/5</td>
</tr>
<tr>
<td>218–240b</td>
<td>5/6</td>
<td>0/5</td>
</tr>
<tr>
<td>360</td>
<td>5/6</td>
<td>0/6</td>
</tr>
<tr>
<td>527</td>
<td>5/6</td>
<td>0/6</td>
</tr>
<tr>
<td>1,496</td>
<td>5/6</td>
<td>0/6</td>
</tr>
<tr>
<td>3,525</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>10,083</td>
<td>0/6</td>
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</tbody>
</table>

*Based on bacteria recovered from the lungs 1 h after infection.

b Data are from five separate experiments using a challenge dose within this range.

<MTD, mean time to death.
tency of IRS, titrations of the SCHU S4 challenge dose and the IRS volume were performed. A single treatment with 250 μl of IRS provided long-term protection to over 90% of naïve rats challenged with up to 700 CFU SCHU S4 (Table 1). The mortality rate increased when the challenge dose was increased to over 1.5×10^3 CFU, and all of the infected rats died between 10 and 13 days after challenge. There was little correlation between the challenge dose and the time to death. The cumulative results from seven independent experiments suggested that the i.t. 50% lethal dose (LD50) of SCHU S4 in F344 rats treated with 250 μl of IRS was in the range of 700 to 1,500 CFU; this was at least 100-fold less than the LD50 associated with s.c. LVS vaccination (45).

Reducing the IRS volume had a dose-dependent effect on the level of protection against a fixed challenge dose. When the IRS volume was reduced to 25 μl, the treatment prolonged the survival of rats challenged i.t. with 360 CFU by 4 to 5 days, but they eventually succumbed to infection (Fig. 6). All protective effects were eliminated when the IRS volume was further reduced to 2.5 or 0.25 μl. Increasing the IRS volume to 1 ml did not substantially delay disease onset, improve clinical signs, or accelerate resolution compared to the 250-μl treatment (data not shown). Since the effectiveness of a single IRS treatment may be limited by the availability of target organisms at or around the time of administration, rats were given repeated IRS injections in an attempt to match the increasing bacteria numbers due to proliferation over the course of infection. F344 rats were either treated with 250 μl of IRS once before SCHU S4 challenge or multiple times on days 1, 3, 6, 9, and 12 relative to challenge. The total bacterial burden in the lungs, spleen, and liver of rats was determined on days 3, 5, 7, 10, and 14 after challenge. As shown in Fig. 7, multiple antibody treatments did not alter the general pattern of SCHU S4 growth and dissemination compared to a single treatment (P > 0.05 for all three organs).

Taken together, these results showed that a single IRS treatment was sufficient to protect F344 rats against i.t. challenge of up to ~700 SCHU S4 organisms. This protection required a minimum IRS volume of 250 μl, and any amount beyond this volume threshold given in a single or multiple treatments provided little additional benefit.

Histopathology of serum-treated rats after i.t. challenge with SCHU S4. In order to determine how the IRS-treated rats survived a lethal pulmonary SCHU S4 challenge despite an extremely high bacterial burden, we next evaluated whether IRS treatment limited the histopathology in the infected tissues. Lung lesions in both IRS- and NRS-treated rats were first detected on day 3 p.i. and consisted of neutrophilic and histiocytic inflamma-

FIG. 4. Passive transfer of purified LVS-immune IgG, but not IgM, protects naïve rats against a lethal i.t. SCHU S4 challenge. IgG and IgM were purified from pooled NRS and IRS collected 28 days after s.c. LVS vaccination. The purities of enriched IgG (A) and IgM (B) preparations were analyzed with SDS-PAGE gels stained with Coomassie blue dye. IRG, immune IgG; NRG, normal IgG; IRM, immune IgM; NRM, normal IgM. The titers of LVS-specific IgM, IgG, and IgA were determined by ELISA as described for Fig. 1, and the titer of contaminating antibody isotypes in the enriched preparations was <1:100. (C) Groups of five F344 rats were treated with an amount of purified IgG or IgM that was equivalent to 250 μl of serum and challenged i.t. 1 day later with 810 CFU of SCHU S4. Survival was monitored daily.

FIG. 5. IRS-treated rats exhibit a pattern of SCHU S4 growth intermediate between NRS-treated rats and LVS-vaccinated rats. LVS-vaccinated F344 rats and naïve rats treated with 250 μl of heat-inactivated NRS or heat-inactivated IRS were challenged i.t. 1 day after serum treatment with 260 CFU of SCHU S4. Three to four rats were euthanized from each group on days 0, 1, 3, 5, 7, 10, 14, and 21 postchallenge to determine the total SCHU S4 burden in the lungs, spleen, and liver. The numbers of lung bacteria on day 0 reflect the actual lung deposition determined within 1 h of infection, and the dashed lines represent the limit of detection for each organ. Each data point represents the mean ± standard deviation.
tion within alveoli, bronchioles, and bronchi and in the perivascular spaces of adjacent blood vessels. Over the next 4 days, the lung inflammation became progressively more necrotizing in both exposure groups. Lung lesion severity was similar in the IRS- and NRS-treated rats during the first 7 days p.i., after which time the NRS-treated rats did not survive. For comparison, the lung lesions in LVS-vaccinated rats were similar in nature and severity to the IRS- and NRS-treated rats during the first 5 days p.i. but gradually decreased in severity starting at day 7 p.i.

Lesions in the liver and spleen were first detected in the IRS- and NRS-treated rats on day 3 p.i. Splenic and hepatic lesions consisted of multifocal, random neutrophilic and histiocytic inflammation on day 3 p.i. and frequently progressed to necrotizing inflammation by day 5 p.i. In both the IRS- and NRS-treated rats, maximal hepatic and splenic lesion severity scores were achieved on day 5 p.i. However, the maximal severity scores for splenic and hepatic lesions in the IRS-treated rats were lower than for the NRS-treated rats. Furthermore, the lesion severity decreased for the IRS-treated rats by day 7 p.i., but not for the NRS-treated rats. For comparison, in the vaccinated rats, lesions were sparse to nonexistent at all time points examined (Fig. 8A to F). These results demonstrate that IRS treatment reduced inflammation in the tissues to which F. tularensis is known to disseminate.

**DISCUSSION**

It is widely accepted that tularemia vaccines such as LVS must induce a potent cell-mediated immunity to be effective against highly virulent type A strains of F. tularensis. LVS vaccination also induces a strong antibody response, but its role in protection has not been thoroughly addressed. We now show that the serum antibodies provided significant protection against pulmonary infection with SCHU S4. These results suggested that a T cell-independent mechanism can temporally modify the disease process, but T cells are critical for the long-term protection associated with immune serum.

To determine the requirement for CD4 and CD8 T cells in passively immunized F344 rats, we developed a very effective *in vivo* depletion regimen using the anti-CD8 antibody OX-8 and the anti-CD4 antibody OX-38. These antibody treatment regimens reduced and maintained the peripheral blood CD4 and CD8 T cell populations to <5 and <1% of normal levels, respectively, over the course of infection. As shown in Fig. 9B, depletion of CD8 T cells completely abolished the ability of IRS to protect against SCHU S4 challenge. Depletion of CD4 T cells had only a partial effect. This may be related to a slight (<2-fold), but not statistically significant, increase in the total number of CD8 T cells in the spleen, liver, and blood; no difference in B cells (CD45R^+^) or NK cells (CD161^+^) were observed (data not shown). Indeed, the mortality rate of rats depleted of both CD4 and CD8 T cells was similar to the animals depleted of CD8 T cells alone. Thus, these results suggest that CD8 T cells are essential for IRS to protect rats against pulmonary infection with SCHU S4.
against a lethal, respiratory SCHU S4 challenge in the F344 rat passive immunization model. These results suggest that antibodies may be an important component in the overall defense against pneumonic tularemia.

We previously showed that the F344 rat is a good animal model for studying human pneumonic tularemia (45). In the present study, F344 rats were vaccinated s.c. to reproduce human vaccination by the scarification method currently used under the Special Immunization Program at USAMRIID. Similar to humans vaccinated by scarification (16, 44), the s.c. vaccinated rats developed strong IgM and IgG responses within a week of vaccination. Further studies showed that only purified serum IgG but not IgM mediated protection against i.t. SCHU S4 challenge in the rat. We have not ruled out the potential contribution of IgA to protection, especially in the lungs or other mucosal surfaces. In fact, the majority of hu-
mice (16, 44) and mice (26) developed positive serum IgA titers following scarification or i.n. vaccination, respectively. The protective capabilities of IgA have been demonstrated repeatedly in vaccine studies with IgA-deficient mice, which did not develop complete resistance against respiratory infections with LVS (2, 34) and SCHU S4 (35). A significant amount of IgA was also found in the immune mouse serum that passively transferred immunity against i.n. LVS infection (26). Thus, in addition to IgG, IgA may also play a role in protection against respiratory tularemia caused by type A strains.

It has been suggested that the morbidity and mortality associated with pneumonic tularemia are caused by the damage inflicted on the extrapulmonary tissues (8). An extension of this idea is that control of bacterial dissemination and growth outside the lungs would offer considerable survival advantage. Our results are consistent with this idea and suggest that passive immunization modified the course of acute infection by enhancing the innate immune response to disseminated bacteria. The presence of LVS-specific antibodies appeared to be most critical during the early phase of infection, because IRS failed to rescue infected rats when the treatment was delayed by 48 h (data not shown) and increasing the amount of serum or the treatment frequency did not improve the level of protection. IRS treatment had little, if any, quantitative or qualitative impact on the bacterial burden and the histopathology in the lungs. Rather, the effects of IRS on bacterial growth were more clearly observed in the infected liver and spleen. The liver is a major site of F. tularensis colonization and replication (9), and pneumonic tularemia is associated with hepatocellular damage in multiple experimental animal models, including the F344 rat (8, 32, 42, 45). Although the level of damage falls short of liver failure and may be reversible, such damage may nevertheless compromise the liver’s ability to perform essential metabolic functions and to utilize its many innate immune mechanisms to control systemic bacterial growth (22, 25). In contrast, IRS treatment reduced the bacterial burden and histopathology in the liver and, in doing so, may have preserved more liver functions in the infected rat. Similar passive immunization studies of mice with LVS point to a process that involves Fc receptor and gamma interferon (IFN-γ) (26, 36), and the liver is one of the richest sources of NK cells that are capable of producing an IFN-γ in response to F. tularensis infection (4, 12). This may also explain the ability of immune serum to delay the death of athymic nude rats and mice (36), which express higher NK cell activity than wild-type animals (10, 24). By controlling the rapid growth rate and dissemination of SCHU S4 and limiting the pathological damage associated with infection, IRS may have enabled the host to survive long enough to develop an effective T cell response that eliminated the infection. Indeed, LVS-vaccinated rats with both F. tularensis-specific antibodies and immune T cells had the lowest bacterial burden and showed the fewest histological changes following SCHU S4 infection.

T cells are required for the long-term protective effects of passive immunization. In the absence of T cells, IRS only delayed the death of athymic nude rats by several days. CD8 T cells appeared to play a more critical role than CD4 T cells, since depletion of CD8 T cells rendered rats completely susceptible to SCHU S4 infection, while only a fraction of the rats depleted of CD4 T cells succumbed. A possible explanation for the partial effect of CD4 T cell depletion is that the level of depletion may have been slightly different for each rat and the survivors had more residual cells. It is also possible that the challenge dose varied slightly for each rat and the survivors had been challenged with a lower dose.

With the growing awareness of the importance of antibodies for effective immunity against pneumonic tularemia, new vaccine designs are beginning to incorporate antibodies or elements that induce stronger antibody responses. For example, antibodies against LPS have been used to enhance antigen presentation by targeting inactivated LVS to Fc receptors on myeloid cells (34). Cholera toxin B (3) and a LVS O-polysaccharide–tetanus toxoid glycoconjugate (39) were used to induce a better antibody response to augment the cellular immunity generated with inactivated or mutant LVS. Several groups have also used protein microarrays (17, 41) and immunoproteomic approaches (23, 43) to identify immunoreactive antibodies in serum from humans and mice with previous exposure to F. tularensis. In order to further improve these novel vaccine designs and to develop antibodies into potential ancillary therapeutic agents, it will be necessary to characterize the protective response associated with any potential antibody candidate. The fact that F344 rats were consistently protected by immune serum alone suggests that the F344 rat model will be a valuable tool not only to test the protective effect of these antibodies but also to characterize their mechanism of protection.

In conclusion, our studies showed that LVS vaccination induced serum antibodies that were protective against a lethal contaminants. Ablation of contaminants was achieved by a combination of high speed centrifugation, ethanol, and proteinase K digestion. The effect of contaminants on the immune response and survival of rats was determined by the number of contaminants in the immunization preparations, as well as the time of administration after challenge.

This approach demonstrated that passive immunization is a valuable tool not only to test the protective effect of these antibodies but also to characterize their mechanism of protection. The results suggest that passive immunization is effective in protecting against pneumonic tularemia and may be a promising strategy for the development of future vaccines against this disease.
respiratory SCHU S4 infection. The protective responses defined in these studies provide valuable insights into the mechanism of antibody-mediated protection and will help guide the rational design of novel tularemia vaccines that induce not only a robust cellular immunity but also a strong humoral immunity.

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