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Prophylactic and Therapeutic Use of Antibodies for Protection against Respiratory Infection with *Francisella tularensis*

Girish S. Kirimanjeswara, Jacqueline M. Golden, Chandra Shekhar Bakshi, and Dennis W. Metzger

The role of Abs in protection against respiratory infection with the intracellular bacterium *Francisella tularensis* is not clear. To investigate the ability of Abs to clear bacteria from the lungs and prevent systemic spread, immune serum was passively administered i.p. to naive mice before intranasal *F. tularensis* live vaccine strain infection. It was found that immune serum treatment provided 100% protection against lethal challenge while normal serum or Ig-depleted immune serum provided no protection. Protective efficacy was correlated with increased clearance of bacteria from the lung and required expression of FcγR on phagocytes, including macrophages and neutrophils. However, complement was not required for protection. In vitro experiments demonstrated that macrophages were more readily infected by Ab-opsonized bacteria but became highly efficient in killing upon activation by IFN-γ. Consistent with this finding, in vivo Ab-mediated protection was found to be dependent upon IFN-γ. SCID mice were not protected by passive Ab transfer, suggesting that T cells but not NK cells serve as the primary source for IFN-γ. These data suggest that a critical interaction of humoral and cellular immune responses is necessary to provide sterilizing immunity against *F. tularensis*. Of considerable interest was the finding that serum Abs were capable of conferring protection against lethal respiratory tularemia when given 24–48 h postexposure. Thus, this study provides the first evidence for the therapeutic use of Abs in *Francisella*-infected individuals. *The Journal of Immunology*, 2007, 179: 532–539.

*Francisella tularensis* is a facultative intracellular Gram-negative bacterium that causes tularemia in humans and other mammals (1). Although cutaneous tularemia is the predominant form of the disease and is rarely fatal, the less common respiratory form can cause up to a 30% mortality rate if not treated (2). The fact that as few as 10 CFU of aerosolized virulent strains of *F. tularensis* can cause disease and death among exposed humans underscores the highly infectious nature of this bacterium (3). These features have made *F. tularensis* an attractive candidate for biowarfare programs and bioterrorism.

Little is known about protective mechanisms against *F. tularensis*. Initial reports suggested that intradermal (i.d.) vaccine with the *F. tularensis* live vaccine strain (LVS) could induce protection against systemic challenge that is dependent on T cells and IFN-γ (4–7). Systemic infection models have also indicated a critical role for B cells in protective immune responses of vaccinated mice against LVS (8, 9). However, in one study (9), only primed B cells, but not unprimed B cells or immune serum, transferred protection. Unfortunately, levels of Ab titers in the recipient mice following adoptive transfer of immune B cells or serum were not measured. Indeed, other investigators have provided evidence that Abs, in addition to T cells, do play a critical role in protection against systemic LVS infection (10–13). Thus, the specific role of humoral immunity remains unclear.

Considering the high mortality rate of respiratory tularemia, there is much interest in understanding protective mechanisms against this form of disease. The LD₅₀ of LVS is 500-fold lower when given by the intranasal (i.n.) route vs the i.d. route (14, 15). Additionally, as few as 100 CFU of LVS given by the i.n. route can elicit a protective immune response against lethal i.d. challenge, whereas 10⁴–10⁵ CFU given by the i.d. route is required to provide a similar level of protection (16, 17). Interestingly, only i.n. vaccination with LVS but not i.d. vaccination, protects mice against pulmonary challenge (16). These considerations clearly call for a detailed understanding of the mechanisms responsible for protection against i.n. *F. tularensis* challenge.

In this study, we have investigated the ability of passively transferred Abs to protect mice against respiratory tularemia. Our findings suggest that humoral immune responses play a significant role in the elimination of *F. tularensis* and that a cooperative interaction between cell- and Ab-based mechanisms can provide prophylactic as well as postexposure protection against this intracellular biothreat.

**Materials and Methods**

**Mice and bacteria**

Five- to 6-wk-old C57BL/6 and BALB/c mice were purchased from Taconic Farms. C57BL/6 C3⁻/⁻ (18) mice were a gift from Dr. R. Wetsel (University of Texas Health Sciences Center, Houston, TX) and were bred and maintained at the Albany Medical College Animal Facility. C57BL/6 FcγR⁻/⁻ mice were purchased from Taconic Farms and C57BL/6 SCID and BALB/c IFN-γ⁻/⁻ were purchased from The Jackson Laboratory and maintained at the Albany Medical College Animal Facility. All animal procedures were approved by the institutional animal care and use committee.
Animal inoculation, challenge, and adoptive transfer protocol

Mice were anesthetized by i.p. injection of 100 µl of xylazine (20 mg/ml) and ketamine (1 mg/ml). For sublethal infection, 10^7 CFU of LVS, and for lethal challenge, 10^8 CFU of LVS in 50 µl of PBS, was delivered by the i.n. route. Serum was collected by retro-orbital bleeding of anesthetized mice on days 0, 14, 28, 49, and 70 postinfection following one i.n. inoculation of live LVS (10^8 CFU). Both normal and immune sera were heated to 55°C for 30 min to inactivate complement. For adoptive transfer experiments, 250 µl of heat-inactivated normal serum or immune serum collected on day 49 postinoculation were injected by the i.p. route. In selected experiments, Ig was depleted from the antisera before transfer by incubation with anti-mouse Ig-coated beads (Sigma-Aldrich) overnight at 4°C, a procedure that caused depletion to undetectable levels. The health of all mice was monitored daily.

ELISA

F. tularensis cultures were diluted to a final concentration of 5 × 10^7 CFU/ml in carbonate buffer (0.05 M NaHCO3, 0.05 M Na2CO3, pH 9.4). Polysorp 96-well plates (Nunc) were coated with 100 µl of bacteria for 2 h at 37°C. The plates were washed with PBS containing 0.1% Tween 20, blocked with 10% FBS in PBS, and stored at 4°C until further use. Two-fold dilutions of sera were incubated in the plates for 2 h at 37°C. This was followed by washing and addition of various dilutions of biotinylated primary goat anti-mouse IgG, IgM, IgG1, IgG2a, IgG3, and IgA Abs (Caltag Laboratories). The plates were incubated for 1 h at 37°C followed by washing and addition of streptavidin-HRP (Caltag Laboratories) for 30 min. The plates were again washed and TMB peroxidase substrate solution (KPL) was added to each well. After color development, the reaction was stopped with 1.8 N H2SO4 and OD was read at 450 nm. The titers were calculated as the lowest dilution of serum that gave an OD of 0.1 above the normal serum control.

Bacterial burden analysis

On days 1, 3, 7, 14, and 21 postchallenge, mice were sacrificed by pento-barbital injection (5 mg/mouse). Lungs, livers, and spleens were harvested aseptically and homogenized in 1 ml of PBS. Various tissues of immune homogenates were plated on chocolate agar plates and incubated for 3 days at 37°C to enumerate CFU.

Histology

Lungs, livers, and spleens from mice sacrificed on day 7 postchallenge were prepared as 5-µm paraffin-embedded sections by standard methods. The sections were stained with H&E and analyzed in a blinded fashion as described previously (19).

Macrophage infection assay

The MH-S murine alveolar macrophage cell line was grown to confluency in RPMI 1640 supplemented with 10% FBS (Invitrogen Life Technologies) and 10^5 macrophages/well were incubated in a 24-well plate with or without 50 ng/ml IFN-γ (Sigma-Aldrich) for 12 h at 37°C. Cultures of LVS grown overnight in Mueller-Hinton broth were washed with PBS and adjusted to 10^6 CFU/ml in RPMI 1640. A total of 10^6 CFU of LVS (100 µl) was incubated with 50 µl of heat-inactivated normal or immune serum for 30 min on ice. The bacteria were washed with PBS and added to the macrophages for 2 h at 37°C. The macrophages were washed and incubated with 100 µg/ml gentamicin for 1 h at 37°C. The cells were then further incubated for 48 h. At various time points (0, 12, 24, and 48 h), the macrophages were lysed using 0.1% sodium deoxycholate in PBS and aliquots of various dilutions of lysates were plated on chocolate agar plates to enumerate CFU.

Statistics

Survival curves were analyzed by the Kaplan-Meier log-rank test using GraphPad Prism 4 software. The Student t test was used for analyses of bacterial burden and macrophage invasion assays.

Results

Intranasal vaccination with live LVS induces a mixed Th1- and Th2-type Ab response

Groups of BALB/c and C57BL/6 mice were infected i.n. with sublethal doses (10^7 CFU) of LVS and serum anti-LVS Ab titers were determined on days 14, 28, 49, and 70 postinoculation. It was found that Ab levels increased significantly by day 28 postinoculation and peaked by day 49 in both groups of mice (Fig. 1A). By day 70, however, the Ab titers were significantly lower than those found on day 49. Consistent with these findings, the efficacy of protection against a subsequent lethal i.n. challenge with F. tularensis has been shown to significantly wane over a period of 2–3 mo (14, 16). Therefore, we used serum collected on day 49 postinoculation for further studies.

Interestingly, the anti-Francisella Ab titers of C57BL/6 mice were ~10-fold lower than those of BALB/c mice at all four time points tested but the kinetics of Ab production were similar in both strains of mice. The physiological consequence of this difference is not known. However, it is interesting to note that C57BL/6 mice are more sensitive than BALB/c mice to LVS challenge and are not as well-protected against i.n. challenge with type A strains of F. tularensis following i.n. vaccination with LVS (14, 17). The predominant Ab isotype produced against F. tularensis on day 49 postinoculation by both BALB/c and C57BL/6 mice was IgG2a (Fig. 1B). This was closely followed by IgG1 and IgG3. Significant titers of IgG2b, IgM, and IgA were also detected in both groups of mice. Taken together, these data demonstrate that a Th1-type serum Ab response is predominant after i.n. LVS infection, but significant Th2-type Ab responses are also generated in both BALB/c and C57BL/6 mice.

Anti-LVS Abs protect mice from lethal respiratory tularemia

Intranasal vaccination of mice with live LVS has been shown to protect against subsequent lethal i.n. challenge with LVS (14, 16, 17) but the mechanism responsible for this protection is unclear. To determine the ability of serum anti-LVS Abs to provide protection against respiratory tularemia, naïve BALB/c mice were treated i.p. with either heat-inactivated normal serum or heat-inactivated immune serum that was collected from BALB/c mice 49 days following sublethal LVS infection (eight mice per group).

The mice were then i.n. challenged 24 h later with a lethal dose of LVS (10^7 CFU) and survival was monitored for 3 wk. All mice that were injected with PBS or normal serum succumbed to infection by day 15 postchallenge (Fig. 2). In contrast, all mice treated...
normal serum, and the numbers reached livers and spleens of infected mice that had been inoculated with 10^4 CFU of LVS by the i.p. route. Twenty-four hours later, the mice were challenged with 10^8 CFU of LVS by the i.n. route. Survival was monitored for 3 wk. The Kaplan-Meier log-rank test was used to determine statistical significance.

with immune serum survived LVS challenge and remained healthy for 5 wk, at which time the experiment was terminated. The protection observed with immune serum was due to Ab since mice treated with Ig-depleted immune serum all succumbed to infection in a time frame that was similar to mice treated with normal serum (data not shown). These results show that serum anti-LVS Ab can confer protection against lethal i.n. challenge with LVS. Although C57BL/6 mice had lower Ab titers than BALB/c mice (Fig. 1), 100% protection was observed in both strains of mice after treatment with 250 μl of immune serum generated in either strain. Only 50% of mice survived following treatment with 150 μl of immune serum (data not shown). Furthermore, 100% mice survived when challenged with 20-fold i.n. L.D50 (2 × 10^8 CFU), while 60% of mice survived when challenged with 40-fold i.n. L.D50.

**Anti-LVS Abs augment pulmonary bacterial clearance and prevent systemic spread**

Because mice treated with serum Abs were able to resist lethal doses of i.n. bacterial challenge, we next sought to determine the effect of Ab treatment on bacterial growth in the target organs. Previous reports have shown that after pulmonary infection, *F. tularensis* grows exponentially in the lungs and within 48 h, disseminates into systemic organs such as the spleen and liver (20). The bacteria have been shown to reach levels of ~10^8 CFU in these organs before the mice succumb to infection.

After treatment with either normal serum or immune serum and i.n. challenge with a lethal dose of LVS, bacterial numbers in the lungs, liver, spleen, and blood were determined on days 1, 3, 7, 14, and 21. As expected, bacterial numbers in the lungs of infected mice expanded exponentially as early as 24 h postchallenge (Fig. 3) and reached ~ 10^9 CFU on day 7, at which point the mice succumbed to the infection. The lungs of immune serum-treated mice harbored similar numbers of bacteria on day 1 postchallenge; however, by day 3, bacterial numbers were significantly lower compared with those in mice treated with normal serum. By day 7 postchallenge, bacterial numbers in the lungs of immune serum-treated mice further decreased and by day 14, no bacteria could be detected. Thus, serum anti-LVS Abs were able to limit the growth of bacteria in the lungs after 24 h and to augment bacterial clearance.

On day 3 postchallenge, ~10^5 CFU of LVS were detected in the lungs and spleens of infected mice that had been inoculated with normal serum, and the numbers reached ~10^8 by day 7. However, in mice treated with immune serum, only ~100 bacteria could be detected in the liver on day 3 and the number of bacteria in the spleen was below the level of detection. On subsequent days, only very few bacteria could be detected in the livers and spleens of immune serum-treated mice. Similarly, bacterial burden in the blood of mice treated with normal serum increased exponentially from <10 CFU/ml on day 1, to 10^3 on day 3, and 10^4 on day 7 postchallenge. However, <10 CFU could be detected in the blood of immune serum-treated mice on days 1 and 3 postchallenge and no bacteria could be detected thereafter. These results indicate that serum Ab treatment is able to prevent systemic spread of bacteria from the lungs and/or augment bacterial clearance in the liver and spleen.

Histopathological analysis of reticuloendothelial organs of infected mice treated with normal serum revealed that the lungs had extensive bronchopneumonia on day 7 postchallenge with neutrophilic and mononuclear cell infiltration into the parenchyma (Fig. 4). Extensive perivascular infiltrates and bronchial luminal exudates resulting in destruction of normal alveolar spaces were also observed. However, the lungs of immune serum-treated mice had significantly less damage with limited perivascular infiltration of mononuclear cells and scant bronchial exudate. The liver sections of normal serum-treated mice had numerous, large granulomatous lesions (~30 in each ×4 field) with central necrotic/degenerative areas. However, the livers of immune serum-treated mice had fewer granulomatous lesions (~10 in each ×4 field) that showed no central degenerative areas. The spleens of normal serum-treated mice showed extensive damage with severe necrotic lesions in both white and red pulp. However, the spleens of immune serum-treated mice had normal white pulp with extensive lymphoproliferation and fewer inflammatory infiltrates. These data indicate that immune serum-treated mice had significantly less tissue damage, findings consistent with rapid elimination of bacteria.

Ab-mediated protection is dependent on expression of FcγRs but not complement

We next investigated the mechanism responsible for Ab-mediated protection against respiratory tularemia. Because Abs are known to mediate direct complement-dependent lysis of certain Gram-negative bacteria, we first determined the role of complement in humoral protection against *F. tularensis*. Groups of naïve C3^-/-[^1]
mice, which lack the central C3 complement component (18), were treated with normal or immune serum and challenged with a lethal dose of LVS 24 h later as described above. It was found that C3<sup>−/−</sup> mice treated with normal serum succumbed to infection by day 15 postchallenge, similar to wild-type mice (Fig. 5A). Conversely, also similar to wild-type mice, all C3<sup>−/−</sup> mice treated with immune serum survived lethal LVS challenge. These data show that complement is not essential for Ab-mediated protection.

To determine the importance of FcγRs, and the potential role of opsonophagocytosis in Ab-mediated protection, C57BL/6 FcγR<sup>−/−</sup> mice, which lack effective high-affinity FcγRI and II, were treated with either normal or immune serum, and challenged i.n. 24 h later with a lethal dose of LVS. Like BALB/c mice, C57BL/6 wild-type mice treated with immune serum were resistant to subsequent lethal challenge with LVS (Fig. 5B). There was no significant difference in the kinetics of survival between wild-type and FcγR<sup>−/−</sup> mice treated with normal serum. However, FcγR<sup>−/−</sup> mice treated with immune serum were as susceptible to LVS infection as those treated with normal serum, demonstrating that FcγRs are essential for Ab-mediated protection and suggesting the importance of opsonophagocytosis as a major protective mechanism against this pathogen.

Because FcγRs are present on various phagocytic cells such as macrophages and neutrophils, we further sought to determine the cell type that is necessary for Ab-mediated protection. To address the potential role of alveolar macrophages, wild-type BALB/c mice were treated with clodronate liposomes containing clodronate. This treatment has previously been shown to deplete alveolar macrophages within 48 h (21). Another group of mice received control liposomes not containing clodronate. The mice were injected i.p. with normal or immune serum 24 h later and challenged i.n. with a lethal dose of LVS 24 h after serum treatment. It was found that mice treated with control liposomes and normal serum succumbed to infection by day 15 postchallenge, similar to that seen in wild-type mice receiving only normal serum before infection (Fig. 5C). Similarly, mice treated with control liposomes and immune serum were protected from lethal infection. In contrast, clodronate-treated mice that received normal sera were more sensitive to lethal challenge with LVS and those animals injected with immune serum were equally susceptible, with all mice succumbing to infection by day 9 postchallenge. Therefore, it appears that alveolar macrophages are critical for innate immune responses to F. tularensis and for Ab-mediated protection.

Neutrophils have previously been shown to be recruited to the lungs as early as 24 h postinfection and are essential for immunity to F. tularensis (22, 23). Because neutrophils are potent...
that IFN-γ is required for intracellular killing of opsonized bacteria by macrophages. MH-S alveolar macrophages were incubated with 50 ng/ml IFN-γ or PBS for 12 h and then exposed to LVS at an MOI of 100 at 37°C. Two hours later, gentamicin was added to kill any nonphagocytosed bacteria and at the indicated time points, the cells were lysed and bacterial counts were enumerated by plating on chocolate agar plates. The data represent the averages and ±SD from triplicate wells and are representative of three independent experiments. Statistical significance was determined by Student’s t test (*, p < 0.05; **, p < 0.001; ***, p < 0.0001).

To determine whether macrophage activation is required to trigger efficient intracellular LVS killing, MH-S cells were first incubated with IFN-γ and subsequently tested for killing of normal or immune serum-adsorbed bacteria. It was found that phagocytosis of normal serum-opsonized bacteria by activated macrophages was similar to that observed with nonactivated macrophages, showing that IFN-γ treatment did not interfere with bacterial uptake (Fig. 6). However, bacterial numbers increased only 5-fold by 48 h in activated macrophages in contrast to 100-fold increases in nonactivated macrophages. This is consistent with previous studies demonstrating that IFN-γ treatment interferes with intracellular bacterial growth (24). Uptake of immune serum-opsonized bacteria by IFN-γ-activated macrophages was similar to that observed in nonactivated macrophages. Interestingly, numbers of immune serum-opsonized bacteria rapidly decreased after incubation with activated macrophages. Within 12 h, an almost 10-fold reduction in bacterial numbers was observed and by 48 h, there was a ~1000-fold reduction. We conclude that serum Abs facilitate bacterial uptake while intracellular killing of opsonized bacteria requires IFN-γ-dependent cell activation.

In vivo Ab-mediated protection requires IFN-γ

Rhinehart-Jones et al. (12) have previously reported that nude mice succumb to i.d. challenge with LVS after passive Ab treatment, suggesting the requirement for T cells in Ab-mediated protection. Furthermore, neutralization of IFN-γ using anti-IFN-γ Abs resulted in abrogation of Ab-mediated protection against i.d. LVS challenge (12). To determine the importance of IFN-γ in Ab-mediated protection after i.n. infection, BALB/c IFN-γ−/− mice were injected with either normal or immune serum and i.n. challenged with LVS 24 h later. It was found that all IFN-γ−/− mice succumbed to infection by day 12 postchallenge regardless of whether they had received normal or immune serum (Fig. 7A). Thus, IFN-γ is critical for Ab-mediated protection against respiratory tularemia.

Previous reports have shown that IFN-γ-secreting NK and CD8 cells are recruited to the lungs as early as 24 h after i.n. LVS challenge (25). To determine the relative importance of T and NK cells in Ab-mediated protection, we next used a SCID mouse model. These mice lack B and T cells but have elevated levels of NK cells. Unlike immune serum-treated wild-type mice, SCID mice that received either normal or immune serum succumbed to infection by day 12 postchallenge (Fig. 7B). These data indicate that IFN-γ-secreting T cells are necessary for in vivo protection following Ab passive transfer and that NK cells have a limited role in this protection.
gens (26). In the case of Abs play in the control and clearance of other intracellular patho-
sisted here, there is a growing appreciation of the significant role protection against respiratory tularemia. Similar to the results pre-
tically when given within 24 – 48 h postexposure.

absence of appropriate cell-mediated immunity, it appears that Ab therapeutic agent for respiratory tularemia if administered within above organs, indicating that passive transfer of Ab confers ster-
lenic protection (9). Nevertheless, adoptive transfer of serum Abs has been shown by others (10–13) to significantly reduce bacterial numbers in the spleen and liver in systemic disease models. In addition, LPS-specific Abs have been shown to provide protection (11). The rapid dissemination of F. tularensis to various organs via the hemogenous route suggests that these bacteria have an extra-
cellular phase, whereby they are amenable to the effects of Abs. However, the role of Abs in preventing colonization and infection by the highly virulent F. tularensis subspecies tularensis, partic-
larly in the respiratory tract, is not known.

Additional experiments demonstrated the requirement for FcγRs on phagocytic cells for effective Ab-mediated bacterial clearance and in vitro analysis of opsonophagocytosis revealed that IFN-γ treatment of macrophages induced rapid killing of intracellular bacteria. Interestingly, opsonophagocytosis via FcγRs in the absence of IFN-γ failed to control bacterial replication. These results suggest that FcγR-mediated cellular activation synergy with IFN-γ for intracellular bacterial killing and are consistent with the results of in vivo experiments in which it was found that Abs were ineffective in conferring protection in the absence of IFN-γ. IFN-γ has previously been shown to interfere with bacterial escape from lysosomes, thus preventing intracellular bacterial replication (28). Surprisingly, those studies found that IFN-γ does not promote intracellular bacterial killing for at least 48 h postinvasion (28, 29). Because IFN-γ can enhance intracel-
lar reactive oxygen species and NO expression (24), and F. tu-
laresis is susceptible to these molecules, the data suggest that an additional activation signal is required to achieve optimal intracel-
lar bacterial killing.

Serum Abs failed to protect SCID mice from lethal LVS chal-
lenge suggesting that T cells, but not NK cells, are involved in Ab-mediated protection. Because, IFN-γ is required for Ab-mediated protection, it is likely that either CD4 and/or CD8 T cells contribute to the humoral response by producing IFN-γ and acti-
vating phagocytes. In fact, depletion of either CD4 or CD8 T cells or neutralization of IFN-γ at the time of challenge in LVS-vacci-
nated mice was found to result in loss of protection, even though the mice had normal serum Ab levels (16, 17). Conversely, LVS fails to induce protective immunity in B cell-deficient mice al-
though these animals have normal levels of T cells (9, 30). Taken together, it appears that both Abs and T cells are required for optimal mucosal protection against pulmonary F. tularensis infec-
tion. This underscores the close interaction of humoral and cellular immune responses in conferring optimum sterilizing immunity. In-
terestingly, recent studies have indicated that both Abs and IFN-γ are required for protection against Francisella novicida (31). How-
ever, those studies did not address the specific contribution of IFN-γ in the observed Ab-mediated protection.

Because IFN-γ is critical for Ab-mediated protection, it is in-
triguing that adoptive transfer of serum Abs alone was able to protect mice from lethal i.n. LVS challenge. One explanation for this efficacy is that upon LVS challenge, bacteria induce a proin-
flammatory response that includes IFN-γ, which in turn, facilitates serum Ab-mediated bacterial clearance. In fact, previous results (Refs. 20 and 22 and S. Olmos and D. W. Metzger, unpublished observations) have shown that moderate amounts of IFN-γ are detected in the lungs within 24–48 h following i.n. LVS inocula-
tion. In support of this finding, a significant number of IFN-γ+ CD8 and NK cells have also been shown to be recruited to the lungs within 24–48 h of infection (25). Histological analysis has likewise revealed a recruitment of macrophages and neutrophils to

**FIGURE 8.** Postexposure protection against respiratory tularemia by adoptive transfer of serum Abs. Groups of 5- to 6-wk-old BALB/c (eight mice per group) were challenged with 10^4 CFU of LVS by the i.n. route. A total of 250 μl of immune serum (IS) was injected i.p. 24 h (+24 h), 48 h (+48 h), or 72 h (+72 h) postchallenge and then additional immune serum was given every 3 days thereafter. Survival was monitored for 21 days. Statistical significance was determined by Kaplan-Meier log-rank test.

**Postexposure protection against respiratory tularemia by Ab treatment**

The above results demonstrated that serum Abs can be used pro-
phylactically to protect against i.n. LVS infection. To determine whether serum Abs could be efficacious if administered postchal-
lenge, groups of naive BALB/c mice were i.n. challenged with a lethal dose of LVS and then injected with immune serum 24, 48, or 72 h later. In each case, passive Ab therapy was continued every 3 days following the initial inoculation. All mice that received immune serum 24 h postchallenge survived lethal bacterial chal-
lenge (Fig. 8). The group that received immune serum 48 h after challenge showed a modest but statistically significant increase in median time to death (9.5 days after treatment with normal serum vs 13.5 days after immune serum treatment) and 25% of the ani-
mals survived to at least day 21, when the experiment was termi-
nated. However, immune serum delivered 72 h after challenge was not protective. In mice that received immune serum 24 h postin-
fection, no bacteria could be detected in the lungs, livers, or spleens on day 21. In surviving mice that received immune serum 48 h postinfection, <10^2 bacteria could be recovered from the above organs, indicating that passive transfer of Ab confers ster-
ilizing immunity. These results show that Abs can be an effective therapeutic agent for respiratory tularemia if administered within 24–48 h postexposure.

**Discussion**

The results of the current study clearly demonstrate that serum Abs can contribute to protective immunity against respiratory tularemia by preventing the spread of bacteria from the lungs to liver and spleen, and facilitating rapid bacterial clearance in the lungs. Pro-
tection was found to be dependent on FcγRs and phagocytes such as macrophages and neutrophils, suggesting that opsonophagocy-
tosis is a critical LVS clearance mechanism. Furthermore, cell-
mediated immunity appears to act synergistically with humoral immunity in providing protection, as demonstrated by the fact that only IFN-γ-activated macrophages could rapidly kill the bacteria and Abs provided no protection in IFN-γ−/− mice. Indeed, in the absence of appropriate cell-mediated immunity, it appears that Ab may actually facilitate bacterial infection of macrophages. Finally, and of considerable interest, Ab treatment could be used therapeu-
tically when given within 24–48 h postexposure.

Our results clearly show that passive transfer of Ab can provide protection against respiratory tularemia. Similar to the results pre-
sented here, there is a growing appreciation of the significant role Abs play in the control and clearance of other intracellular patho-
gens (26). In the case of F. tularensis, although heterologous an-
tisera were extensively used to treat human tularemia with signif-
icant success before antibiotics were discovered (27), more recently, B cells but not Abs were reported to be critical for sys-
temic protection (9). Nevertheless, adoptive transfer of serum Abs has been shown by others (10–13) to significantly reduce bacterial numbers in the spleen and liver in systemic disease models. In addition, LPS-specific Abs have been shown to provide protection (11). The rapid dissemination of F. tularensis to various organs via the hemogenous route suggests that these bacteria have an extra-
cellular phase, whereby they are amenable to the effects of Abs. However, the role of Abs in preventing colonization and infection by the highly virulent F. tularensis subspecies tularensis, partic-
ularly in the respiratory tract, is not known.

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the lungs following LVS infection (20). It is conceivable that serum Abs also reach the site of infection due to increased vascular permeability associated with local inflammation. Thus, transudation of serum Abs can lead to opsonization of the bacteria and promotion of phagocytosis and intracellular killing. It is of interest to note that adoptively transferred serum Abs had little or no effect on bacterial numbers during the first 24 h of infection either in vivo or in vitro, supporting the idea that induced IFN-γ expression is needed for efficient killing of opsonized bacteria.

Additional experiments showed that adoptive transfer of LVS-specific immune serum failed to significantly protect mice against type A F. tularensis SchuS4 although an increase in median time to death was observed (data not shown). This observation is not surprising because IFN-γ, a cytokine that we have shown to be critical for Ab-mediated protection, could not be detected during first 72 h following challenge with SchuS4. It was recently reported that SchuS4 severely suppresses proinflammatory cytokines such as TNF-α and IL-12 during the first 3 days of infection (32). Additionally, we have failed to observe any histological changes indicating recruitment of inflammatory cells into the lungs of mice during the first 72 h following SchuS4 infection (C. S. Bakshi and D. W. Metzger, unpublished data). In contrast, when pulmonary IFN-γ can be detected, by day 4, bacterial numbers in the lungs and systemic organs are at such a high level (10⁷–10⁸) (32) that Abs would likely be ineffective in reversing the course of infection. The possibility that serum Abs could play a major role in protection against SchuS4 with concerted help from T cell-mediated immunity, such as has been reported with LVS (16, 17), is the focus of current investigation.

Adoptively transferred serum Abs still conferred protection even when given 24–48 h postchallenge, at a time when the bacteria had not yet escaped from the lungs and the lung environment contained only moderate amounts of IFN-γ and inflammatory cells. After 48 h, however, bacterial numbers reach ~10⁶ CFU in the lungs and are associated with a great amount of inflammation and escape of bacteria to the systemic organs. Hence, adoptively transferred serum Abs have limited efficacy if given later than 48 h after infection. It is worth mentioning that in vaccinated mice, mucosal Abs such as IgA in addition to Ag-specific T cells may also contribute to overall protection (19). We are currently investigating the specific role of mucosal Abs in providing a first line defense against pulmonary tularemia.

Our results may have relevance to vaccination with both attenuated and subunit tularemia vaccines. LVS has been shown to induce protective immunity against virulent type A strains and the i.n. vaccination regimen has been found to be superior to more common i.d. vaccination routes (14, 16, 17). CD4 and CD8 T cells have been implicated in LVS-induced protection against pulmonary tularemia but the protection does not appear to be sterilizing or long-lasting. Our results suggest the need for concerted actions of both Abs and cell-mediated immunity against F. tularensis at mucosal surfaces.

Respiratory tularemia, either in the form of a natural epidemic or caused by an intentional act of terror, can be a serious life-threatening disease. Control of the disease after a large-scale exposure to F. tularensis is complicated by the absence of a licensed vaccine and the prospect of antibiotic-resistant bacteria, a likelihood during an intentional act of bioterrorism. An alternative approach to conventional antibiotic treatment is to transfer specific Abs to individuals after exposure to bacteria. This method has several advantages over conventional methods of treatment: it is rapid, specific, can be used for therapeutic as well as prophylactic purposes even among immunocompromised individuals, and the chances of developing resistant bacteria is minimal. Additionally, it can enhance host immune responses to the bacteria following exposure, thus acting as a surrogate vaccine. The results of our study, in addition to demonstrating the contribution of serum Abs to protective immunity and the requirements for optimal Ab-mediated protection during pulmonary infection with F. tularensis, have demonstrated that serum Abs can be used for both prophylactic and therapeutic purposes.

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Disclosures

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References


