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Nonspecific Early Protective Immunity in *Francisella* and *Listeria* Infections Can Be Dependent on Lymphocytes

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Normal mice, but not lymphocyte-deficient or B-cell-deficient mice, given a sublethal infection of *Francisella tularensis* LVS survive a secondary lethal challenge of more than 10,000 50% lethal doses given 3 days later. In this work, we show that similar early protection that is also strongly lymphocyte dependent operates in *Listeria monocytogenes* infection. Since sublethal infection with either LVS or *L. monocytogenes* protects against heterologous lethal challenge, this early protection is nonspecific.

To understand the protective mechanisms available in the mammalian immune system, we have characterized the murine protective immune response to an intracellular bacterium, *Francisella tularensis* LVS. This infection model has several convenient features; for example, mice that survive and clear sublethal intradermal (i.d.) infection are solidly immune to subsequent lethal infection with more than 10^5 50% lethal doses (LD_{50}s) given intraperitoneally (i.p.) or intravenously (i.v.) a month later (9, 10). This immunity is clearly dependent on α/β^+ T cells, but either $CD4^+$ or $CD8^+$ T cells alone can suffice (15). As with other intracellular infections such as *Listeria monocytogenes* (13), a strong innate immune response mediated by macrophages and natural killer cells and dependent on gamma interferon and tumor necrosis factor alpha permits mice to survive primary infection for as long as 30 days (7). However, an unusual feature of LVS infection in mice is the rapid generation of very strong protective immunity against lethal challenge very quickly after establishment of sublethal infection: normal mice given a sublethal dose of 10^3 LVS bacteria i.d. can survive a lethal i.p. or i.v. challenge of more than 10^6 LD_{50}s given only 3 days after the initial sublethal i.d. infection (4, 5). This mechanism is of interest because of its remarkable strength, its very quick time course, and its dependence on lymphocytes, particularly B cells. The last of these properties distinguishes this mechanism from traditional innate immunity, which is mediated largely by macrophages and natural killer cells.

Since early protection in LVS infection is dependent on lymphocytes, we have questioned whether this mechanism is specific. Previous work has studied the effect of sublethal infection with either another intracellular pathogen, *Salmonella typhimurium*, or an extracellular bacterium, pathogenic *Escherichia coli*, on protection against lethal LVS challenge 3 days later. The failure to generate heterologous protection with either bacterium suggested that protection was indeed specific, as no enhanced survival could be demonstrated (5). However, in each of these infections no early protective immunity for the homologous infection could be demonstrated; i.e., sublethal infection with *S. typhimurium* did not protect against lethal *S. typhimurium* challenge 3 days later. Thus, we could not determine whether inability to demonstrate heterologous protection against LVS challenge was due to a failure of *S. typhimurium* to stimulate an early protective response at all or due to a requirement for antigen-specific recognition and activity. Since the nature of the innate and long-term protective immunity to *L. monocytogenes* is very similar to that to LVS (3, 6, 12, 13), we have reexamined these questions in this study using *L. monocytogenes*.

F. tularensis LVS (live vaccine strain [ATCC 29684]; American Type Culture Collection, Manassas, Va.) was cultured on modified Mueller-Hinton (MH) agar plates or in modified MH broth (Difco Laboratories, Detroit, Mich.) supplemented with ferric pyrophosphate and IsoVitaleX (Becton Dickinson, Cockeysville, Md.) as previously described (5, 10). *Listeria monocytogenes* EGD (ATCC 15313) was a gift from William Schwan and was cultured in brain heart infusion broth or plates (Difco). One-milliliter aliquots of bacteria frozen in broth alone at −70°C were periodically thawed for use, and viable bacteria were quantified by plating serial dilutions on MH agar plates. The number of CFU present after thawing varied less than 5% over a 6-month period. Specific-pathogen-free, male BALB/cByJ mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and were used at 6 to 16 weeks of age. Male BALB/c.*scid* mice, as well as $Igh6^-$ (B-cell-knockout) mice and $Tcrb^-/Tcrd^-$ (double α/β and γ/δ T-cell receptor [TCR]-knockout) mice on a C57BL/6J background, were also purchased from Jackson Laboratories. Male C57BL/6NCR and BALB/c.*nu*/*nu* mice were purchased from the Biological Resources Branch, Frederick Cancer Research and Development Center, National Cancer Institute (Frederick, Md.). All mice were housed in sterile microisolator cages in a barrier environment at the Center for Biologics Evaluation and Research (CBER), fed autoclaved food and water ad lib, and routinely tested for common murine pathogens by a diagnostic service provided by the Division of Veterinary Services, CBER; all such sentinel mice tested negative over the course of these experiments. In conducting the research described in this report, the investigators adhered to a protocol approved by the Animal Care and Use Committee of the CBER. Groups of three to six mice, as indicated, were given 0.5 ml i.p. or 0.1 ml i.d. of the indicated dilution of LVS or *Listeria* EGD; actual doses of bacteria inoculated were simultaneously determined by plate count. All materials, including bacteria, were diluted in phosphate-buffered saline (PBS) (BioWhittaker, Walkersville, Md.) containing <0.01 ng of endotoxin per ml. The mean time to death (MTD) was calculated as the arithmetic mean ±

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TABLE 1. Early protective immunity is operative in *L. monocytogenes* infection and is nonspecific[a]

Priming dose	Challenge dose	No. of deaths/total no. of mice	MTD (days)
PBS	2.5×10^5 EGD	4/5	4.0 ± 1.7
10^5 EGD	2.5×10^5 EGD	0/5	
10^5 EGD	2.5×10^6 EGD	1/5	4
10^5 EGD	2.5×10^7 EGD	5/5	3.4 ± 0.5
PBS	10^5 LVS	5/5	4.4 ± 0.9
10^4 LVS	10^5 LVS	0/5	
10^4 LVS	10^6 LVS	3/5	5.0 ± 1.7
10^4 LVS	10^7 LVS	5/5	4.4 ± 0.5
10^4 LVS	2.5×10^5 EGD	1/5	4
10^4 LVS	2.5×10^6 EGD	2/5	5.0 ± 1.4
10^4 LVS	2.5×10^7 EGD	5/5	2.6 ± 0.5
10^5 EGD	10^5 LVS	1/5	5
10^5 EGD	10^6 LVS	2/5	4.5 ± 2.1
10^5 EGD	10^7 LVS	5/5	4.0 ± 1.2

[a] BALB/cByJ mice (groups of five) were primed i.d. on day 0 and challenged i.p. on day 3 with numbers and strains of bacteria as indicated; actual priming and challenge doses were confirmed by plate count at the time of inoculation. Mice were observed for morbidity and mortality through day 60. The MTD was determined in relationship to the day of i.p. challenge. This experiment is representative of five total experiments of similar design.

standard deviation for all mice that died within a group; surviving mice were not included in this calculation. The statistical significance of differences in MTD was assessed by Student's *t* test.

Initial experiments demonstrated that both BALB/cByJ and C57BL/6 mice readily survived i.d. infection with *Listeria* EGD. The LD_{50} for i.d. challenge (i.d. LD_{50}) for BALB/cByJ mice was about 10^6 bacteria (based on two determinations using groups of five mice; in one experiment the LD_{50} was 1.1×10^6 bacteria, and in the other it was 9.4×10^5 bacteria). The i.d. LD_{50} for C57BL/6 mice was greater than 10^7 bacteria, the maximum dose that could be used to infect mice (based on two determinations using groups of five mice). The mice that survived *Listeria* EGD infection for more than 1 week also survived for over 2 months (data not shown). Mice given sublethal doses of *Listeria* EGD i.d. had no detectable bacteria in spleens, livers, and lungs 3 weeks later (data not shown) and thus were assumed to have cleared the infection. The i.p. LD_{50} for this strain of *Listeria* (EGD) was about 2.5×10^3 bacteria in BALB/cByJ mice and about 2×10^4 bacteria in C57BL/6 mice (data not shown), slightly more than the previously reported i.v. LD_{50} for this bacterial strain (12).

To determine whether the early protective immune mechanism operates in *L. monocytogenes* infection, BALB/cByJ mice were given a sublethal priming dose of 10^5 *Listeria* EGD bacteria i.d. and then lethally challenged with 2.5×10^5 *Listeria* EGD bacteria i.p., or 100 LD_{50}s, 3 days later; these mice survived both infections (Table 1). Further, 80% of mice challenged with 1,000 LD_{50}s survived, although mice given 10,000 LD_{50}s (2.5×10^7 bacteria) did not survive. As shown previously (5), mice primed with 10^4 LVS bacteria i.d. survived a challenge with more than 10^5, but not 10^7, LD_{50}s of LVS (Table 1). When BALB/cByJ mice were primed with a sublethal infection of 10^4 LVS bacteria i.d. and then challenged with heterologous *L. monocytogenes* lethal infection, survival rates were almost as good as those seen with homologous *L. monocytogenes* priming (Table 1). Conversely, mice primed with 10^5 *Listeria* EGD bacteria and challenged with various doses of LVS survived almost as well as mice primed with the homologous LVS infection (Table 1). Taken together, these data indicate that the early protective mechanism (sublethal infection followed quickly by secondary lethal infection) can readily be elicited in *L. monocytogenes* infection. As in LVS infection, early protection in *Listeria* infection is relatively strong and able to protect against a lethal challenge of about 1,000 LD_{50}s, which is a large absolute number of bacteria in *Listeria* infection. Further, heterologous protection can easily be demonstrated as well, indicating that this protective mechanism is nonspecific.

To determine the cellular basis of early protection in *L. monocytogenes* infection, various immunodeficient mice were primed with 10^5 *Listeria* EGD bacteria i.d. and challenged with a maximal lethal infection of 2.5×10^6 *Listeria* EGD bacteria (1,000 LD_{50}s) i.p. 3 days later. Normal BALB/cByJ and C57BL/6J mice readily survived priming and challenge (Table 2). However, lymphocyte-deficient *scid* mice, T-cell-deficient *nu*/*nu* mice, double TCR-knockout mice, and Igh6$^-$ (B-cell-knockout) mice were quite compromised in their ability to survive secondary lethal infection (Table 2). In combined experiments, 100% (14 of 14) of double TCR-knockout mice and 64% (9 of 14) of B-cell-knockout mice given this 1,000-LD_{50} challenge dose died in less than 10 days, indicating that early protection involves both B cells and T cells. Thus, early protection in LVS infection is similar to that in *Listeria* infection in being heavily dependent on B cells, but unlike LVS, early protection in *Listeria* infection also requires T cells. Clearly, this lymphocyte-dependent early protection is distinct from traditional innate immunity, which is a function primarily of neutrophils, macrophages, and natural killer cells (13).

This nonspecific protection is similar to observations previously described by Killar and Eisenstein (11). In those studies, mice given an avirulent vaccine strain of *S. typhimurium*, SL3235, were protected against challenge with 1,000 LD_{50}s of a virulent *S. typhimurium* challenge given 3 days, but not 1 day, after immunization. However, mice given *Salmonella* SL3235 were also protected against challenge with 100 LD_{50}s of *L. monocytogenes* given 6, but not 3 days after immunization. This nonspecific cross-protection waned by 21 to 30 days. Similarly, we have observed no reciprocal protection between LVS and *Listeria* EGD in mice given 10 LD_{50}s of bacteria as a heterologous challenge 35 days after priming (data not shown). The *Salmonella* SL3235 studies attributed protection primarily to macrophages, since adherent cells but not T cells were able

TABLE 2. Early protective immunity in *Listeria monocytogenes* infection of immunodeficient mice

Mouse strain	Priming dose	No. of deaths/total no. of mice	MTD (days)
BALB/cByJ	PBS	5/5	3.4 ± 0.9
BALB/cByJ	10^5 EGD	0/5	
BALB/c.*nu*/*nu*	10^5 EGD	5/5	9.8 ± 3.8
BALB/c.*scid*	10^5 EGD	5/5	5.2 ± 2.5
C57BL/6NCR	PBS	5/5	4.4 ± 1.1
C57BL/6NCR	10^5 EGD	0/5	
C57/Igh6$^-$	10^5 EGD	2/4	4.0 ± 0.0
C57/Tcrb$^-$/Tcrd$^-$	10^5 EGD	3/3	8.3 ± 3.8

[a] The indicated mice (groups of three to five) were primed i.d. on day 0 as indicated and challenged i.p. with 2.5×10^6 EGD bacteria on day 3; actual priming and challenge doses were confirmed by plate count at the time of inoculation. Mice were observed for morbidity and mortality through day 60. The MTD was determined in relationship to the day of i.p. challenge. This experiment is representative of three total experiments of similar design.

to adoptively transfer protection (11); nonetheless, evidence for T-cell responsiveness in proliferation assays was presented, and cross-protection was not tested in immunodeficient mice. It is possible, as originally suggested by Mackaness many years ago, that early T-cell activity is responsible for macrophage activation that leads to control of infection. Further, comparison of the present study with the earlier report (11) suggests a possible explanation for our previous inability to demonstrate early protection in *Salmonella* infection: our previous results may have been due to differences in the bacterial strains used, not to inherent lack of early protection in *Salmonella*.

The bacterial components responsible for generation of early protective immunity are currently under study. Another report demonstrating nonspecific protection in these intracellular infections, by using a different vaccine strain of *F. tularensis* and challenging 2 weeks later with the NCTC 7973 strain of *L. monocytogenes*, attempted to isolate bacterial cell surface proteins responsible for stimulating cross protection and failed to do so (2). In contrast, we have obtained evidence that both LVS chromosomal DNA and LVS lipopolysaccharide, which is neither endotoxic nor a traditional B-cell mitogen or macrophage activator (1, 14), can stimulate early protection (8). Thus, future studies will continue to investigate the cellular and molecular basis for this nonspecific, strong, early protective mechanism, and consider situations in which this rapid protection might be exploited in immunotherapies against infections.

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