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T-Cell-Independent Resistance to Infection and Generation of Immunity to *Francisella tularensis*

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The intraperitoneal 50% lethal dose (LD_{50}) for *Francisella tularensis* LVS in both normal control heterozygote BALB/c *nu*/+ mice and BALB/c *nu/nu* mice was 2×10^0. Both *nu*/+ and *nu/nu* mice given 10^7 LVS bacteria or more intradermally (i.d.) died, with a mean time to death of about 7 to 8 days. On the other hand, *nu*/+ mice given 10^6 LVS bacteria or less survived for more than 60 days and cleared systemic bacteria, while *nu/nu* mice given 10^6 LVS bacteria or less survived for more than 10 days but died between days 25 and 30. Thus, the short-term (i.e., <10-day) i.d. LD_{50} of both *nu/nu* and *nu*/+ mice was 3×10^6, but the long-term (i.e., >10-day) i.d. LD_{50} of *nu/nu* mice was less than 7×10^0. The short-term survival of i.d. infection was dependent on tumor necrosis factor and gamma interferon: treatment of *nu/nu* mice with anti-tumor necrosis factor or anti-gamma interferon at the time of i.d. infection resulted in death from infection 7 to 8 days later, whereas control infected *nu/nu* mice survived for 26 days. *nu/nu* mice infected with LVS i.d. generated LVS-specific serum antibodies, which were predominantly immunoglobulin M: titers peaked 7 days after i.d. infection but declined sharply by day 21, after which mice died. Surprisingly, *nu/nu* mice given 10^3 LVS bacteria i.d. became resistant to a lethal challenge (5,000 LD_{50}s) of LVS intraperitoneally within 2 days after i.d. infection; *nu/nu* mice similarly infected with LVS i.d. and challenged with *Salmonella typhimurium* (10 LD_{50}s) were not protected. *nu/nu* mice given *nu*/+ spleen cells intravenously as a source of mature T cells survived i.d. infection for more than 60 days and cleared bacteria. Taken together, these studies demonstrate that i.d. infection of *nu/nu* mice with LVS rapidly generates T-cell-independent, short-term, specific protective immunity against lethal challenge, but T lymphocytes are essential for long-term survival.

Francisella tularensis, the causative agent of tularemia, is a facultative intracellular bacterium found predominantly in cold-weather latitudes. Although an attenuated vaccine strain is available, its success in preventing human disease is variable (6, 39), and the mechanisms responsible for development of protective immunity to *F. tularensis* are not well understood (37). Our previous studies using an animal model demonstrated that the live vaccine strain (LVS) of *F. tularensis* is quite pathogenic for laboratory mice when introduced intraperitoneally (i.p.): a single bacterium causes a lethal infection that is similar to human disease (11, 13). The 50% lethal dose (LD_{50}) when LVS is introduced into mice intradermally (i.d.) at the base of the tail or subcutaneously (s.c.) in skin or footpads, however, is at least 4 orders of magnitude higher than the i.p. LD_{50} (11, 13). Furthermore, mice that survive sublethal i.d. or s.c. infection and clear systemic bacteria are immune to subsequent lethal i.p. or intravenous (i.v.) challenge (11, 13). Spleen cells from such i.d. infected immune mice are able to passively transfer protection to naive recipient mice (13). T-cell-depleted spleen cells were ineffective, suggesting that T cells play an important role in protective immunity. On the other hand, protective immunity develops quite rapidly in i.d. infected mice: within 3 days after initiation of a sublethal i.d. infection with LVS, mice become able to survive a lethal i.p. LVS challenge of 10,000 LD_{50}s (10). Unlike the results of passive transfer experiments, this rapid development of immunity is not consistent with the usual time requirements for the activation and function of classical T cells (10).

To directly investigate the role of T cells in *Francisella* infection, we examined the course of LVS infection in congenitally athymic *nu/nu* (nude) mice, which are severely T cell immunodeficient (33). Here we show that the initial survival of *Francisella* infection by *nu/nu* mice is T cell independent but requires endogenous tumor necrosis factor (TNF) and gamma interferon (IFN-γ) (as previously demonstrated in normal mice [22]). T cells are indeed required for eventual clearance of bacteria and long-term survival of infection. Surprisingly, substantial protective immunity that is T cell independent can be generated rapidly in *nu/nu* mice.

MATERIALS AND METHODS

Mice. Specific-pathogen-free male BALB/c *nu/nu* and BALB/c *nu*/+ mice, 4 to 5 weeks of age, were purchased from the Biological Resources Branch, Frederick Cancer Research and Development Center, National Cancer Institute, Frederick, Md., and housed in barrier facilities until used at 6 to 10 weeks of age. Before being used, all mice were quarantined for 1 week after transportation and were then age matched within an experiment. Sentinel mice were routinely screened serologically for evidence of various infections and consistently found to be negative. Spleen cells from sentinel *nu/nu* mice from 6 to 15 weeks of age were tested for evidence of peripheral T-cell development by using a panel of directly fluoresceinated anti-CD3, anti-CD4, and anti-CD8 monoclonal antibodies. No detectable levels (above background) of $CD3^+$, $CD4^+$, or $CD8^+$ spleen cells were observed at any of these ages; however, only mice 10 weeks of age or younger were used here, to minimize the

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possible contributions of the small numbers of mature T cells often observed in older *nu/nu* mice (19).

Bacteria. *F. tularensis* LVS (ATCC 29684) was obtained from the American Type Culture Collection, Rockville, Md., and cultured in either supplemented Mueller-Hinton broth in a 37°C air shaker or on modified Mueller-Hinton agar plates in a humidified 37°C incubator with 5% CO_2 (13). Stock cultures of LVS were grown to stationary phase overnight in broth from a single isolated colony, and aliquots were frozen in broth (without the addition of glycerol) at −80°C. Viable CFU after freezing were determined by a plate count of serial dilutions. *Salmonella typhimurium* W118 was a gift of Samuel Formal (Walter Reed Army Institute of Research). W118 was propagated in either L broth or on L agar plates and similarly frozen without the addition of glycerol. For the inoculation of mice, bacterial cultures were thawed immediately before use and diluted appropriately in phosphate-buffered saline (PBS); actual numbers of bacteria (CFU) inoculated were confirmed by plate counts at the time of injection.

Inoculations. Mice were given doses of LVS as indicated either i.p., i.v. via the lateral tail vein, or i.d. at the base of the tail. Survival was monitored for 60 days. The LD_{50} of the mice was determined by inoculation of groups of five to six mice with doses of bacteria covering a 5-log range and calculated by the method of Reed and Muench (34).

Estimation of bacteria in organs. At various times after infection with 10^4 LVS bacteria i.d., mice were euthanatized by cervical dislocation. Peritoneal fluid was recovered by the injection of 5 ml of sterile PBS into the exposed peritoneal cavity and the withdrawal of fluid; generally, 3 to 4 ml of fluid was recovered. Spleens, livers, and lungs were removed aseptically and homogenized (Stomacher 80; Seward Medical, London, United Kingdom) in sterile PBS. Cells were lysed after homogenization by the addition of sodium dodecyl sulfate (final concentration, 0.05%), and the numbers of bacteria were determined by a plate count of appropriately diluted samples of each tissue. The results are expressed as the log geometric mean CFU per organ, plus or minus standard error, for groups of three mice.

Treatment of mice with anticytokine antibodies. To deplete circulating cytokines in vivo, we treated mice with a single i.p. dose (250 μg) of either purified monoclonal anti-TNF (TN3) (36), monoclonal anti-IFN-γ (H22) (35), or control polyclonal hamster immunoglobulin G (IgG) (Organon Teknika Corp., West Chester, Pa.). Immediately after inoculation of antibody, mice were given 10^4 LVS bacteria i.d., and survival was monitored for 60 days thereafter. These doses of monoclonal antibodies were sufficient to change the i.d. LD_{50} from about 5×10^4 LVS bacteria in normal untreated mice to about 1×10^1 bacteria in anti-TNF or anti-IFN-γ-treated mice (22).

Reconstitution of athymic *nu/nu* mice with normal *nu/+* splenocytes. To infuse T-cell-deficient *nu/nu* mice with a source of normal T cells, we prepared single-cell suspensions from spleens of normal immunocompetent *nu/+* heterozygote mice. Erythrocytes were lysed by treatment with ammonium chloride, and the cell suspensions were counted by using trypan blue. Viable spleen cells (10^8 per mouse) were transferred into age-matched recipient BALB/c *nu/nu* mice i.v. via the lateral tail vein. Aliquots of the single-cell suspensions were analyzed for lymphocyte distribution by flow cytometry, and cells were found to be approximately 55% IgM^+, 43% $CD3^+$, 32% $CD4^+$, and 11% $CD8^+$.

Analysis of antibody response to LVS in *nu/nu* and *nu/+* mice. Serum samples, obtained at various time points after i.d. infection with 10^4 LVS bacteria via tail bleeds, were characterized for their content of anti-LVS-specific antibodies by enzyme-linked immunosorbent assay (ELISA). Live LVS (5×10^6 bacteria per well) suspended in bicarbonate buffer (pH 9.0) was adsorbed overnight onto Immunlon 1 plates (Dynatech, Chantilly, Va.). The plates were then washed and blocked by the addition of 10% calf serum and reacted with twofold dilutions of serum samples. Antibody was detected by the addition of either enzyme-labelled goat anti-mouse immunoglobulin antibody (which reacts with mouse IgM and IgA, and all isotypes of IgG), or with enzyme-labelled isotype-specific antibodies (Southern Biotechnology, Birmingham, Ala.). The specificity and sensitivity of all reagents were optimized in separate experiments. The endpoint titer was arbitrarily defined as the lowest dilution of serum that gave an optical density value at 405 nm that was twofold greater than the value of the matched dilution of normal prebleed mouse serum and also greater than 0.050 optical density unit. Geometric mean titers were calculated from individual assays of sera from three to five mice and are presented as the antilog of the mean. Standard errors are omitted for clarity but were less than 10%.

TABLE 1. LD_{50} values after i.p. or i.d. infection with *F. tularensis* LVS[a]

Mouse strain	i.p. LD_{50}	i.d. LD_{50} at <10 days[b]	i.d. LD_{50} at >10 days[c,d]
BALB/c *nu/nu*	2×10^0	3×10^6	$<7 \times 10^0$
BALB/c *nu/+*	3×10^0	3×10^6	3×10^6

[a] Groups of five or six mice of the indicated strains were inoculated i.p. or i.d. with doses of LVS ranging from 10^0 to 10^7 bacteria; actual inoculation doses were confirmed by a plate count at the time of inoculation. Survival was monitored daily for 60 days. LD_{50} values were calculated by the method of Reed and Muench (35). These values were representative of at least three LD_{50} determinations for each combination of route and mouse strain.

[b] LD_{50} values calculated by using mortality rates through day 10 after i.d. infection.

[c] LD_{50} values calculated by using mortality rates through day 60 after i.d. infection.

[d] Five of five BALB/c *nu/nu* mice given 7×10^0 LVS bacteria i.d. died with a mean time to death, plus or minus standard error, of 38.3 ± 1.6 days. BALB/c *nu/+* mice given LVS bacteria i.d. which survived more than 7 days also survived until the experiment was terminated at 60 days.

RESULTS

Infection of BALB/c *nu/+* and *nu/nu* mice with *F. tularensis* LVS. To examine the ability of T-cell-deficient BALB/c *nu/nu* mice to survive LVS infection, we infected *nu/nu* mice and their normal heterozygote *nu/+* littermates with various doses of LVS either i.p. or i.d. The i.p. LD_{50} for both normal *nu/+* mice and immunodeficient *nu/nu* mice was quite low, approaching a single bacterium (Table 1). All *nu/+* and *nu/nu* mice infected with LVS i.p. died between 3 and 6 days after infection, regardless of dose. In contrast, the course of i.d. infection in the two strains was quite different. The i.d. LD_{50} in BALB/c *nu/+*, similar to that previously observed in other normal mice (11, 13), was 3×10^6 (Table 1). All normal mice which succumbed to i.d. infection died 6 to 9 days after infection, while survivors lived for more than 60 days. On the other hand, BALB/c *nu/nu* mice that were infected with high doses (10^7 or more) of LVS i.d. died within 7 to 9 days after infection, while those that received lower doses (10^6 or less) of LVS i.d. died between 15 and 30 days after infection. An example of the mortality pattern of *nu/+* and *nu/nu* mice infected with a lower dose, 1.3×10^5, is shown in Fig. 1.

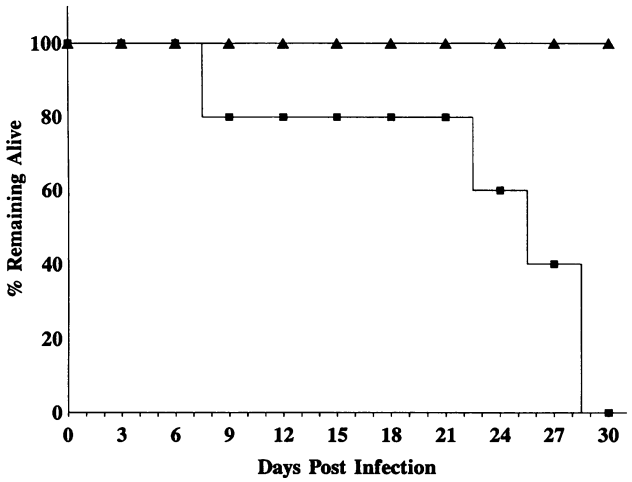

FIG. 1. Infection of BALB/c *nu*/+ (▲) and *nu/nu* (■) mice with 10^5 LVS bacteria i.d. Groups of five BALB/c *nu*/+ and BALB/c *nu/nu* mice were inoculated with 10^5 bacteria LVS i.d. at the base of the tail; actual inoculation doses were confirmed by a plate count at the time of inoculation. Survival was monitored daily for 60 days; since all *nu*/+ mice survived through 60 days, results are graphed for the first 30 days only. This experiment was representative of four experiments of similar design, all of which had comparable results.

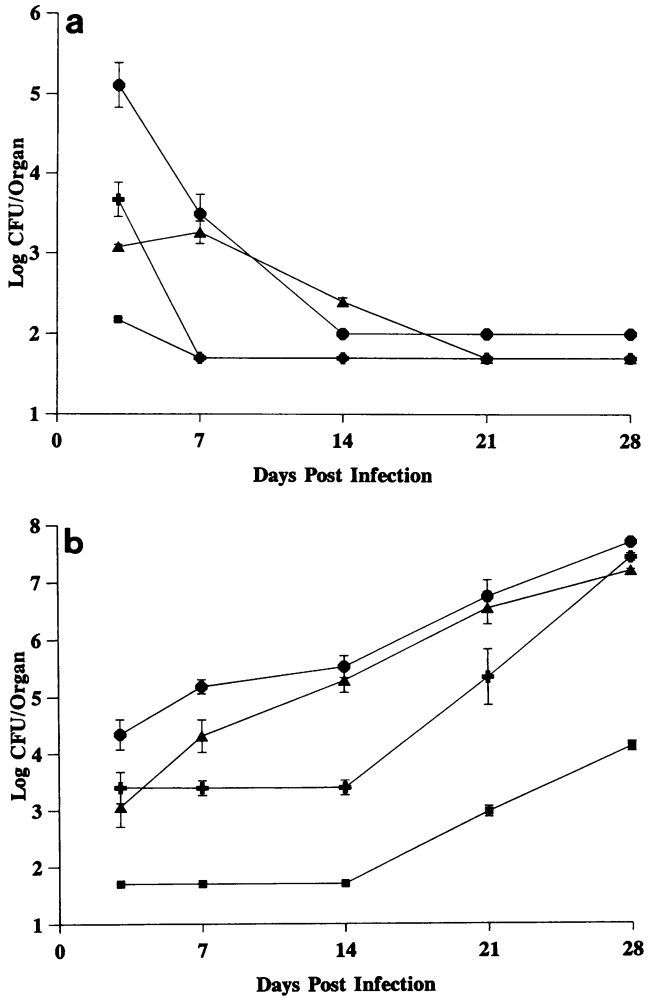

FIG. 2. Estimation of bacterial numbers in organs of BALB/c *nu*/+ (a) and *nu/nu* (b) mice after i.d. infection. Groups of BALB/c *nu*/+ and *nu/nu* mice were inoculated with 10^4 LVS bacteria i.d. at the base of the tail; actual inoculation doses were confirmed by a plate count at the time of inoculation. At days 3, 7, 14, 21, and 28 after i.d. infection, three mice from each group were sacrificed, and organs or fluid from the peritoneum was homogenized and sampled to determine the number of CFU per organ (see Materials and Methods). Symbols: ■, peritoneum; ▲, spleen; ✚, lung; ●, liver. Geometric means of the log CFU per organ ± 1 standard error are graphed. This experiment was representative of three experiments of similar design, all of which had comparable results.

While all *nu*/+ mice survived i.d. infection at this dose, one *nu/nu* mouse died on day 7, and the remaining four mice died between days 22 and 29. When the LD_{50} for i.d. infected *nu/nu* mice was calculated by considering the mortality rates through day 10 after i.d. infection, the value (3×10^6) was identical to that observed in normal *nu*/+ mice (Table 1). All i.d. infected *nu/nu* mice died by day 42, however, even at the lowest infection dose tested (7×10^0). Thus, the LD_{50} for i.d. infected *nu/nu* mice calculated by considering mortality rates at day 60 after infection was less than 7×10^0.

To examine the fate of bacteria after i.d. inoculation, we infected BALB/c *nu*/+ and *nu/nu* mice with 10^4 LVS bacteria i.d. Groups of three mice of each strain were sacrificed at various intervals after i.d. infection, and bacterial burdens in spleens, livers, lungs, and peritoneums (the major sites of infection) (13) were determined. Figure 2 illustrates the time course of systemic bacterial burdens. As observed previously in other normal mice (13), LVS bacteria spread to the major organs within 3 days after i.d. infection of *nu*/+ mice. The absolute numbers of bacteria in all organs declined thereafter, and no bacteria were detected in the organs of *nu*/+ mice by day 21 after infection (Fig. 2a). Bacteria were also detected in the major organs of *nu/nu* mice within 3 days after infection. The numbers of bacteria in the organs increased markedly from days 21 through 28 (Fig. 2b), after which all remaining mice died. Thus, T-cell-deficient mice survived infection with 10^4 LVS bacteria i.d. for several weeks but ultimately could not control systemic bacterial replication and succumbed to infection.

To directly assess the contribution of T cells to long-term survival of i.d. LVS infection, we prepared spleen cells (as a source of T cells) from normal BALB/c *nu*/+ mice and infused them i.v. into BALB/c *nu/nu* recipients. Three days after cell transfer, mice were inoculated with 10^5 LVS bacteria i.d. Normal immunocompetent *nu*/+ mice survived this dose of LVS (Table 2), while untreated *nu/nu* mice given 10^5 LVS bacteria i.d. died by day 30 after infection (Fig. 1). All *nu/nu* mice that received *nu*/+ spleen cells survived through day 60 after infection. Recipient *nu/nu* mice were sacrificed on day 70. Spleen cells were analyzed by flow cytometry for content of lymphocyte subpopulations, and organs were examined as described above for the presence of LVS bacteria. Spleen cells from all reconstituted *nu/nu* mice were about 24% $CD3^+$, 16% $CD4^+$, and 8% $CD8^+$; no systemic bacteria were detected in any organ. These results strongly suggested that reconstitution of T-cell-deficient mice with immunocompetent T cells permitted clearance of bacteria and long-term survival of i.d. LVS infection.

Role of TNF and IFN-γ in short-term survival of i.d. *F. tularensis* LVS infection by BALB/c *nu/nu* mice. Previous studies demonstrated that survival of i.d. LVS infection in normal mice is dependent on the presence of peripheral TNF and IFN-γ: normal mice treated with either anti-TNF or

TABLE 2. Reconstitution of athymic *nu/nu* mice with normal splenocytes[a]

Mice	Treatment i.v. (day 0)	No. of deaths/total	MTD ± SE
nu/nu	PBS	5/5	29.2 ± 5.2
	10^8 *nu/+* spleen cells	0/5	>60
nu/+	PBS	0/5	>60

[a] BALB/c *nu/nu* or BALB/c *nu/+* mice were given i.v. either 10^8 BALB/c *nu/+* spleen cells or the control diluent (PBS) as indicated. Three days later, they were inoculated with 10^5 LVS bacteria i.d. at the base of the tail; actual inoculation doses were confirmed by a plate count. Survival was monitored daily for 60 days, and mean time to death (MTD, in days) was calculated relative to the day of i.d. infection. This experiment was representative of three experiments of similar design, all of which had comparable results.

anti-IFN-γ cannot survive i.d. infection (22). To examine the role of these two cytokines in the survival of i.d. infection by athymic *nu/nu* mice, we treated control BALB/c *nu/+* mice and immunodeficient *nu/nu* mice with either control hamster IgG antibody, anti-TNF, or anti-IFN-γ and then inoculated them with 10^4 LVS bacteria i.d. Normal *nu/+* mice treated with control hamster IgG survived infection with 10^4 LVS bacteria i.d. for more than 60 days, but both anti-TNF- and anti-IFN-γ-treated *nu/+* mice died from i.d. LVS infection within about 8 days (Table 3). T-cell-deficient *nu/nu* mice treated with control hamster IgG and inoculated with 10^4 LVS bacteria i.d. died about 26 days after infection, but both anti-TNF- and anti-IFN-γ-treated mice died from i.d. LVS infection within 7 to 8 days. Thus, both TNF and IFN-γ were required for initial survival of i.d. LVS infection in *nu/nu* mice, and both cytokines could be produced in a T-cell-independent fashion.

Antibody production in BALB/c *nu/nu* mice infected i.d. with LVS. To examine the capacity of athymic *nu/nu* mice to produce LVS-specific antibodies that might contribute to initial survival of infection as well, we inoculated control BALB/c *nu/+* and BALB/c *nu/nu* mice with 10^4 LVS bacteria i.d. At various time points thereafter, serum samples were obtained and assessed for the presence of LVS-specific antibodies. Endpoint titers of LVS-specific total immunoglobulin, IgM, IgG2a, and IgG3 are shown as a function of time after i.d. infection in Fig. 3. LVS-specific IgM antibodies were detected by day 5 after i.d. infection in *nu/+* mice;

TABLE 3. Role of IFN-γ and TNF in LVS infection of *nu/nu* mice[a]

Mice	Antibody treatment	No. of deaths/total	MTD ± SE
nu/+	Hamster IgG	0/5	>60
	Anti-TNF (TN3)	5/5	8.3 ± 0.6
	Anti-IFN-γ (H22)	5/5	8.3 ± 0.6
nu/nu	Hamster IgG	5/5	26.4 ± 1.6
	Anti-TNF (TN3)	5/5	8.2 ± 1.3
	Anti-IFN-γ (H22)	5/5	7.2 ± 0.6

[a] Groups of five BALB/c *nu/+* or BALB/c *nu/nu* mice were treated i.p. with 250 μg of either polyclonal hamster IgG, monoclonal anti-TNF (TN3), or monoclonal anti-IFN-γ (H22) and then inoculated immediately thereafter with 10^4 LVS bacteria i.d.; actual inoculation doses were confirmed by a plate count at the time of inoculation. Survival was monitored daily for 60 days, and mean time to death (MTD, in days) was calculated relative to the day of i.d. infection. This experiment was representative of three experiments of similar design, all of which had comparable results.

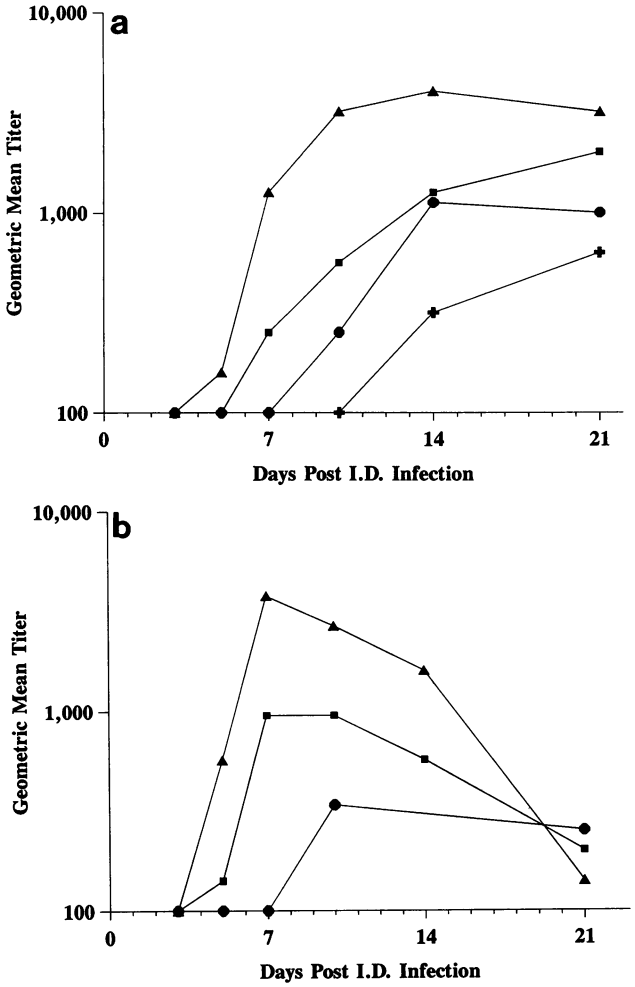

FIG. 3. Time course of anti-LVS antibody production after i.d. infection. Groups of four BALB/c *nu/+* and BALB/c *nu/nu* mice were prebled and then inoculated with 10^4 LVS bacteria i.d. at the base of the tail; actual inoculation doses were confirmed by a plate count at the time of inoculation. Serum samples from individual mice were obtained by tail bleeding on days 3, 5, 7, 10, 14, and 21 after infection, and endpoint titers of LVS-specific antibodies were determined by ELISA using LVS-coated plates (see Materials and Methods). The antilog of the geometric mean titer is graphed; standard errors were less than 10% of the mean. (a) BALB/c *nu/+* mice. Very little IgG1 or IgG2b LVS-specific antibody was detected (limit of sensitivity, about 1 to 5 ng/ml for each isotype), and therefore only results for IgM, IgG2a, and IgG3 are shown. (b) BALB/c *nu/nu* mice. Only LVS-specific antibodies of the IgM and IgG3 isotypes were detected in sera from *nu/nu* mice (limit of sensitivity, about 1 to 5 ng/ml for each isotype). Mice in this group died at days 26, 27, 32, and 38 postinfection (30.8 days ± 2.8). This experiment was representative of two experiments of similar design, both of which had comparable results. Symbols: ■, anti-immunoglobulin; ▲, anti-IgM; ✚, anti-IgG2a; ●, anti-IgG3.

IgG3 and IgG2a were detected by day 10 and day 14, respectively. Titers reached a maximum by day 21 and were maintained thereafter (Fig. 3a). Only small amounts (titers of 1:230 or less) of LVS-specific IgG1 and IgG2b antibodies were detected. Although LVS-specific IgM antibodies were likewise detected by day 5 after i.d. infection in BALB/c *nu/nu* mice, and small amounts of IgG3 were detected by day 10, titers in *nu/nu* mice reached a maximum by day 7 and

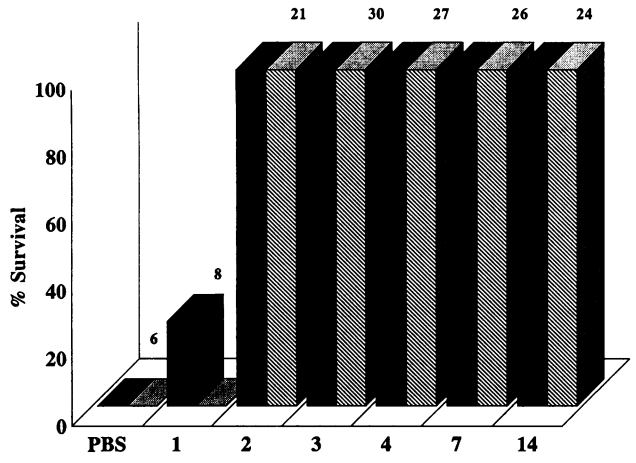

FIG. 4. Generation of resistance to i.p. LVS infection after i.d. priming in BALB/c *nu*/+ (■) and *nu/nu* (▨) mice. Groups of five BALB/c *nu*/+ and *nu/nu* mice were inoculated with 10^4 LVS bacteria i.d. on day 0 and challenged with 10^4 LVS bacteria i.p. on days 1, 2, 3, 4, 7, and 14 after i.d. infection; actual inoculation doses were confirmed by a plate count at the time of inoculation. Control mice were treated with PBS (diluent) 1 day before challenge with 10^4 LVS bacteria i.d. Survival was monitored daily for 60 days; percent survival through day 10 after each i.p. infection was plotted on the *y* axis. All BALB/c *nu*/+ mice that survived i.p. infection survived through day 60, when the experiment was terminated. Shown above each bar is the mean time to death for *nu/nu* mice, relative to the initial day of i.d. infection. This experiment was representative of three experiments of similar design, all of which had comparable results.

declined thereafter (Fig. 3b). No LVS-specific IgG1, IgG2a, or IgG2b antibodies were detected in the sera of *nu/nu* mice. By day 21, when bacterial numbers in organs were high (Fig. 2b), LVS-specific antibody titers were quite low (Fig. 3b). Thus, although *nu/nu* mice produced some T-cell-independent, LVS-specific antibodies shortly after i.d. infection, serum antibody levels were not sustained during the course of infection.

Generation of protective immunity to LVS in BALB/c *nu/nu* mice. Previous studies have demonstrated that sublethal i.d. or s.c. infection of normal mice with LVS generates systemic protective immunity to subsequent lethal LVS challenges (11, 13). Surprisingly, this protective immunity is generated quite rapidly: within 3 days after sublethal i.d. infection, normal mice are able to survive large lethal doses of LVS introduced i.p. or i.v. (10). This rapid time course is not consistent with typical requirements for activation and function of classical T cells. To test the T-cell involvement of the generation of immunity to LVS, BALB/c *nu*/+ and *nu/nu* mice were inoculated with 10^4 LVS bacteria i.d. or with control PBS. On day 1, 2, 3, 4, 7, or 14 after infection, mice were challenged with a lethal dose of 10^4 LVS bacteria (5,000 LD_{50}s; Table 1) i.p. As shown in Fig. 4, PBS-treated *nu*/+ and *nu/nu* mice both died from this i.p. lethal dose, with a mean time to death of about 6 days. By day 2 after i.d. LVS inoculation, however, 100% of both *nu*/+ and *nu/nu* mice survived lethal i.p. infection for more than 10 days. Normal *nu*/+ mice survived for more than 60 days and cleared bacteria. *nu/nu* mice eventually died from LVS infection between days 21 and 30 (relative to initial i.d. infection). Thus, the rapid generation of immunity to lethal i.p. infection was indeed T cell independent; unlike normal mice, however, all *nu/nu* mice eventually succumbed to LVS infection.

TABLE 4. Specificity of resistance to lethal infection in *nu/nu* mice[a]

Mice	Sublethal priming (day 0)	Lethal challenge (day 3)	No. of deaths/total	MTD ± SE
nu/+	PBS i.d.	10^2 LVS i.p.	5/5	9.7 ± 1.0[b]
	PBS i.d.	10^2 W118 i.p.	5/5	5.8 ± 0.3[b]
	10^4 LVS i.d.	10^2 LVS i.p.	0/5	>60
	10^4 LVS i.d.	10^2 W118 i.p.	5/5	17.4 ± 1.2[c]
nu/nu	10^4 LVS i.d.	10^2 LVS i.p.	5/5	26.4 ± 1.8[c]
	10^4 LVS i.d.	10^2 W118 i.p.	5/5	13.2 ± 1.3[c]

[a] Groups of five BALB/c *nu/nu* or BALB/c *nu*/+ mice were inoculated with 10^4 LVS bacteria or the control diluent, PBS, i.d. at the base of the tail. Three days later, they were challenged i.p. with lethal doses (10^2) of either LVS or *S. typhimurium* W118 bacteria; actual inoculation doses were confirmed by a plate count at the time of inoculation. Survival was monitored daily for 60 days, and mean time to death (MTD, in days) was calculated relative to the day of i.p. challenge. This experiment was representative of two experiments of similar design, both of which had comparable results.

[b,c] Each MTD value for primed mice (*c*) is significantly different from that for unprimed mice (*b*) at the level of P of <0.002 by Student's two-tailed t test for unpaired values.

The specificity of resistance to i.p. infection after i.d. inoculation was tested by challenging i.d. LVS-inoculated mice with *S. typhimurium* W118, an unrelated bacterium. Both normal *nu*/+ mice (Table 4) and *nu/nu* mice (data not shown) succumbed to i.p. infection with 10^2 LVS bacteria (50 LD_{50}s; Table 1) and with 10^2 W118 bacteria (10 LD_{50}s) (30). As described above (Fig. 4), both BALB/c *nu*/+ and BALB/c *nu/nu* mice inoculated with 10^4 LVS bacteria i.d. on day 0 and challenged with 10^2 LVS bacteria i.p. survived for more than 10 days after i.p. challenge. *nu*/+ mice survived for more than 60 days and were considered long-term survivors, while *nu/nu* mice died from LVS infection by about day 26 (Table 4). Neither strain survived lethal challenge with W118 after i.d. LVS inoculation, although the mean time to death from *Salmonella* infection was significantly increased ($P < 0.002$; Table 4) by LVS infection, relative to uninfected mice (see reference 10). Thus, the generation of immunity in *nu/nu* (as well as *nu*/+) mice that results from i.d. infection was specific for LVS.

DISCUSSION

F. tularensis LVS is quite virulent for normal mice when administered by any systemic route (i.p., i.v., or intramuscular) (11, 13), causing a lethal disease that is similar to tularemia in humans. Not surprisingly, LVS was equally virulent when administered i.p. to athymic *nu/nu* mice, which lack functional T cells (Table 1), and i.p. LVS infection of *nu/nu* mice caused death in less than a week. Infection of *nu/nu* mice with LVS i.d., however, established a chronic infection that was progressive and eventually lethal in the face of large systemic bacterial burdens (Fig. 2). This defect could be corrected by infusion of mature T cells (Table 2), and thus these results demonstrate that resolution of i.d. infection in normal mice is dependent on mature T cells. Unlike simple resolution, however, initial generation of immunity was T cell independent: i.d. infection of *nu/nu* mice rapidly generated substantial specific protective immunity against lethal i.p. challenge (Fig. 4, Table 4). We know of no other example of this surprising observation.

Our previous studies have described an interesting disparity between the LD_{50} for systemic administration of LVS and that for skin-related introduction in normal mice: when LVS is introduced either i.d. or s.c., the murine LD_{50} is 4 to 6 orders of magnitude higher than the i.p., i.v., or intramuscular LD_{50} (11, 13). Although substantial numbers of bacteria reach the reticuloendothelial organs of i.d. infected mice, such mice are able to contain bacterial replication and clear systemic bacteria over a 2- to 3-week period (13, 22) (Fig. 2a). The ability to control i.d. LVS infection has previously been shown to depend on the rapid production of both TNF and IFN-γ (1, 22). The results presented here indicate that this cytokine production does not require mature functional T cells (Table 3). This is similar to the case with infections with *Listeria monocytogenes* (4, 9, 41) and *S. typhimurium* (26). We do not believe that these results reflect the presence of small numbers of contaminating mature T cells; all mice were used at less than 10 weeks of age, when the potential for T-cell contamination is minimal (19, 21, 23), and representative mice from each shipment were found to have no detectable $CD3^+$, $CD4^+$, or $CD8^+$ T cells in their thymocytes or spleens (see Materials and Methods). Keratinocytes, skin cells capable of cytokine production, may be a source of TNF (20); natural killer cells, which are abundant in *nu/nu* mice, are an obvious source of IFN-γ (4, 41).

Clearly, mature T cells are necessary to resolve i.d. LVS infection, with a time course consistent with the development of a conventional T-cell-mediated specific immune response. As with LVS, studies of *S. typhimurium* (17, 18, 24, 25, 29, 42) and *L. monocytogenes* (4, 7, 12, 27) have indicated that resistance to infection with these facultative intracellular bacteria can be divided into an early phase that is T cell independent and a late phase that is T cell dependent. Thus, CD-1 *nu/nu* mice infected with 10^4 *S. typhimurium* bacteria i.v. had increasing bacterial burdens in their spleens and livers and died between days 17 and 26 (29). Similarly, *nu/nu* mice infected i.v. with a dose of *Listeria* bacteria that is sublethal in *nu/+* mice exhibited a chronic infection and died about 6 weeks after infection (12, 27). Recent studies have further suggested that $CD4^+$ T cells contribute significantly to the resolution of *Salmonella* (25), *Listeria* (8), and *Mycobacterium* (31, 32) infections. Since reconstitution of *nu/nu* mice with normal T cells permits survival of i.d. LVS infection (Table 2), selective reconstitution will clarify the relative contribution of T-cell subpopulations to survival of i.d. infection.

nu/nu mice produced IgM anti-LVS antibodies in a T-cell-independent fashion (Fig. 3). These antibodies could also contribute significantly to early phase survival of LVS infection. Serum titers of anti-LVS antibodies in *nu/nu* mice peak at about 5 to 7 days after infection but fail to increase thereafter and decline precipitously between days 14 and 21 after infection. At this time range, *nu/nu* B cells may lose the capacity to sustain antibody production or circulating anti-LVS antibodies may be adsorbed by increasing bacterial burdens (Fig. 2), or both. Consumption of available antibodies, coupled with lack of further production, may be another in a cascade of events that results in the death of i.d. infected *nu/nu* mice.

As mentioned above, the T-cell requirement for resolution of i.d. LVS infection was similar to previous observations for other intracellular bacteria such as *Salmonella* and *Listeria* spp. The mechanism responsible for the resolution of sublethal infection is separate from that operative during the generation of immunity against lethal infection. Our previous studies (13) demonstrated that both immune T cells and serum can passively transfer immunity. On the other hand, other studies using normal mice revealed a very unusual property of generation of immunity to LVS: within 3 days after sublethal i.d. LVS infection, normal mice survived a lethal i.p. challenge of over 10,000 LD_{50}s (10). This rapid time course was not consistent with the usual time requirements for activation of classical T cells. Indeed, the studies reported herein demonstrate directly that T cells are not required for the rapid generation of immunity (Fig. 4, Table 4), although T cells certainly participate in immunity when available (10).

Other studies have noted apparent nonspecific resistance to an unrelated bacterium after initial bacterial infection. For instance, normal and *nu/nu* mice infected with *S. typhimurium* and challenged with *Listeria* bacteria 7 days later exhibit about 1.5-log fewer *Listeria* bacteria in spleens than untreated mice did (25, 42). These effects on organ burdens may have a counterpart in survival, at least in normal mice, since about 50% of mice infected with avirulent *S. typhimurium* SL3235 can survive 10 to 100 LD_{50}s of *Listeria* infection (18). The only demonstrable nonspecific effect we observed after i.d. LVS priming of normal mice (10) or *nu/nu* mice (Table 4), however, was a transient increase in the mean time to death from *Salmonella* infection; both were ultimately unable to survive a *Salmonella* challenge that was only about 10 LD_{50}s.

Taken together, results with both normal mice (10) and *nu/nu* mice (present study) suggest a novel, α/β T-cell-independent mechanism responsible for the rapid generation of specific protective immunity to *F. tularensis* LVS. Since the only known cell types present in *nu/nu* mice with the potential for specific antigen recognition are γ/δ T cells, these are obvious candidates for further study. Because dendritic epithelial T cells (3, 16, 38) in *nu/nu* mice lack functional T-cell receptors (28), it seems unlikely that these cells play a major role in the rapid generation of specific immunity. Intestinal intraepithelial lymphocytes, which are γ/δ^+ (14, 15), abundant in *nu/nu* mice (5), and have a diverse repertoire of T-cell-receptor expression (2), are intriguing candidates for participation, but the relationship between intestinal intraepithelial lymphocytes and events initiated in the skin is certainly not obvious. Alternatively, new cell types with the capacity for specific antigen recognition may be revealed through further study of the LVS model.

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REFERENCES

1. **Anthony, L. S. D., E. Ghadirian, F. P. Nestel, and P. A. L. Kongshavn.** 1989. The requirement for gamma interferon in resistance of mice to experimental tularemia. Microb. Pathog. **7**:421–428.
2. **Asarnow, D. M., T. Goodman, L. Lefrancois, and J. P. Allison.** 1989. Distinct antigen receptor repertoires of two classes of murine epithelium-associated T cells. Nature (London) **341**:60–62.
3. **Asarnow, D. M., W. A. Kuziel, M. Bonyhadi, R. E. Tigelaar, P. W. Tucker, and J. P. Allison.** 1988. Limited diversity of γ/δ antigen receptor genes of Thy-1^+ dendritic epidermal cells. Cell **55**:837–847.
4. **Bancroft, G. J., R. D. Schreiber, G. C. Bosma, M. J. Bosma, and E. R. Unuanue.** 1987. A T cell-independent mechanism of macrophage activation by interferon-γ. J. Immunol. **139**:1104–1107.

5. **Bandeira, A., S. Itohara, M. Bonneville, O. Burlen-Defranoux, T. Mota-Santos, A. Coutinho, and S. Tonegawa.** 1991. Extrathymic origin of intestinal intraepithelial lymphocytes bearing T-cell antigen receptor γ/δ. Proc. Natl. Acad. Sci. USA **88**:43–47.
6. **Burke, D. S.** 1977. Immunization against tularemia: analysis of the effectiveness of live *Francisella tularensis* vaccine in prevention of laboratory-acquired tularemia. J. Infect. Dis. **135**:55–60.
7. **Cheers, C., and R. Waller.** 1975. Activated macrophages in congenitally athymic "nude" mice and in lethally irradiated mice. J. Immunol. **115**:844–847.
8. **Czuprynski, C. J., and J. F. Brown.** 1990. Effects of purified anti-Lyt-2 mAb treatment on murine listeriosis: comparative roles of Lyt-2$^+$ and L3T4$^+$ cells in resistance to primary and secondary infection, delayed-type hypersensitivity and adoptive transfer of resistance. Immunology **71**:107–112.
9. **Dunn, P. L., and R. J. North.** 1959. Early gamma interferon production by natural killer cells is important in defense against murine listeriosis. Infect. Immun. **59**:2892–2900.
10. **Elkins, K. L., D. A. Leiby, R. K. Winegar, C. A. Nacy, and A. H. Fortier.** 1992. Rapid generation of specific protective immunity to *Francisella tularensis*. Infect. Immun. **60**:4571–4577.
11. **Elkins, K. L., R. K. Winegar, C. A. Nacy, and A. H. Fortier.** 1992. Introduction of *Francisella tularensis* at skin sites induces resistance to infection and generation of protective immunity. Microb. Pathog., in press.
12. **Emmerling, P., H. Finger, and J. Bockemuhl.** 1975. *Listeria monocytogenes* infection in nude mice. Infect. Immun. **12**:437–439.
13. **Fortier, A. H., M. V. Slayter, R. Ziemba, M. S. Meltzer, and C. A. Nacy.** 1991. Live vaccine strain of *Francisella tularensis*: infection and immunity in mice. Infect. Immun. **59**:2922–2928.
14. **Goodman, T., and L. Lefrancois.** 1988. Expression of the γ-δ T-cell receptor on intestinal CD8$^+$ intraepithelial lymphocytes. Nature (London) **333**:855–857.
15. **Goodman, T., and L. Lefrancois.** 1989. Intraepithelial lymphocytes. Anatomical site, not T cell receptor form, dictates phenotype and function. J. Exp. Med. **170**:1569–1581.
16. **Havran, W. L., M. Poenie, R. E. Tigelaar, R. Y. Tsien, and J. P. Allison.** 1989. Phenotypic and functional analysis of γ/δ T cell receptor-positive murine dendritic epidermal clones. J. Immunol. **142**:1422–1428.
17. **Hormaeche, C. E., P. Mastroeni, A. Arena, J. Uddin, and H. S. Joysey.** 1990. T cells do not mediate the initial suppression of a salmonella infection in the RES. Immunology **70**:247–250.
18. **Killar, L. M., and T. K. Eisenstein.** 1985. Immunity to *Salmonella typhimurium* infection in C3H/HeJ and C3H/HeNCrlBR mice: studies with an aromatic-dependent live *S. typhimurium* strain as a vaccine. Infect. Immun. **47**:605–612.
19. **Kishihara, K., Y. Yoshikai, G. Matsuzaki, T. W. Mak, and K. Nomoto.** 1987. Functional alpha and beta T cell chain receptor messages can be detected in old but not in young athymic mice. Eur. J. Immunol. **17**:477–482.
20. **Kupper, T. S.** 1990. The activated keratinocyte: a model for inducible cytokine production by non-bone marrow-derived cells in cutaneous inflammatory and immune responses. J. Invest. Dermatol. **94**(Suppl. 6):146S–150S.
21. **Lawetzky, A., and T. Hunig.** 1988. Analysis of CD3 and antigen receptor expression on T cell subpopulations of aged athymic mice. Eur. J. Immunol. **17**:409–412.
22. **Leiby, D. A., A. H. Fortier, R. M. Crawford, R. D. Schreiber, and C. A. Nacy.** 1992. In vivo modulation of the murine immune response to *Francisella tularensis* LVS by administration of anticytokine antibodies. Infect. Immun. **60**:84–89.
23. **MacDonald, H. R., R. K. Lees, C. Bron, B. Sordat, and G. Miescher.** 1987. T cell antigen receptor expression in athymic (*nu/nu*) mice. Evidence for an oligoclonal beta chain repertoire. J. Exp. Med. **166**:195–209.
24. **Matsuura, M., and C. Galanos.** 1990. Induction of hypersensitivity to endotoxin and tumor necrosis factor by sublethal infection with *Salmonella typhimurium*. Infect. Immun. **58**:935–937.
25. **Nauciel, C.** 1990. Role of CD4$^+$ T cells and T-independent mechanisms in acquired resistance to *Salmonella typhimurium* infection. J. Immunol. **145**:1265–1269.
26. **Nauciel, C., and F. Espinasse-Maes.** 1992. Role of gamma interferon and tumor necrosis factor alpha in resistance to *Salmonella typhimurium* infection. Infect. Immun. **60**:450–454.
27. **Newborg, M. F., and R. J. North.** 1980. On the mechanism of T cell-independent anti-*Listeria* resistance in nude mice. J. Immunol. **124**:571–576.
28. **Nixon-Fulton, J. L., W. A. Kuziel, B. Santerse, P. R. Bergstresser, P. W. Tucker, and R. E. Tigelaar.** 1988. Thy-1$^+$ epidermal cells in nude mice are distinct from their counterparts in thymus-bearing mice. J. Immunol. **141**:1897–1903.
29. **O'Brien, A. D., and E. S. Metcalf.** 1982. Control of early *Salmonella typhimurium* growth in innately Salmonella-resistant mice does not require functional T cells. J. Immunol. **129**:1349–1351.
30. **O'Brien, A. D., D. L. Rosenstreich, E. S. Metcalf, and I. Scher.** 1980. Differential sensitivity of inbred mice to *Salmonella typhimurium*: a model for genetic regulation of innate resistance to bacterial infection, p. 101–114. *In* E. Skamene, P. Kongshaven, and M. Landy (ed.), Genetic control of natural resistance to infection and malignancy. Academic Press, Inc., New York.
31. **Orme, I. M.** 1987. The kinetics of emergence and loss of mediatory T lymphocytes acquired in response to infection with *Mycobacterium tuberculosis*. J. Immunol. **138**:293–298.
32. **Orme, I. M.** 1988. Characteristics and specificity of acquired immunologic memory to *Mycobacterium tuberculosis* infection. J. Immunol. **140**:3589–3593.
33. **Raff, M. C.** 1973. Θ-bearing lymphocytes in nude mice. Nature (London) **246**:350–351.
34. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **25**:493–497.
35. **Schreiber, R. D., L. J. Hicks, A. Celada, N. A. Buchmeier, and P. W. Gray.** 1985. Monoclonal antibodies to murine γ-interferon which differentially modulate macrophage activation and antiviral activity. J. Immunol. **134**:1609–1618.
36. **Sheehan, K. C. F., N. Ruddle, and R. D. Schreiber.** 1989. Generation and characterization of hamster monoclonal antibodies that neutralize murine tumor necrosis factors. J. Immunol. **142**:3884–3893.
37. **Tarnvik, A.** 1989. Nature of protective immunity to *Francisella tularensis*. Rev. Infect. Dis. **11**:440–451.
38. **Tigelaar, R. E., J. M. Lewis, and P. R. Bergstresser.** 1990. TCR γ/δ positive dendritic epidermal T cells as constituents of skin-associated lymphoid tissue. J. Invest. Dermatol. **94**(Suppl. 6):58S–63S.
39. **Tigertt, W. D.** 1962. Soviet viable *Pasteurella tularensis* vaccines. A review of selected articles. Bacteriol. Rev. **26**:354–373.
40. **Wentworth, P. A., and H. K. Ziegler.** 1987. Induction of macrophage Ia expression by lipopolysaccharide and *Listeria monocytogenes* in congenitally athymic nude mice. J. Immunol. **138**:3167–3173.
41. **Wherry, J. C., R. D. Schreiber, and E. R. Unanue.** 1991. Regulation of gamma interferon production by natural killer cells in scid mice: roles of tumor necrosis factor and bacterial stimuli. Infect. Immun. **59**:1709–1715.
42. **Zinkernagel, R. M.** 1976. Cell-mediated immune response to *Salmonella typhimurium* infection in mice: development of nonspecific bactericidal activity against *Listeria monocytogenes*. Infect. Immun. **13**:1069–1073.