INFECTION AND IMMUNITY, Feb. 1995, p. 534–538
0019-9567/95/$04.00+0
Copyright © 1995, American Society for Microbiology
Vol. 63, No. 2

Cytokine Expression in the Liver during the Early Phase of Murine Tularemia

IGOR GOLOVLIOV,[1,2] GUNNAR SANDSTRÖM,[1,2]* MATS ERICSSON,[2] ANDERS SJÖSTEDT,[1,2] AND ARNE TÄRNVIK[2]

Department of Microbiology, National Defence Research Establishment, S-901 82 Umeå,[1] and the Department of Infectious Diseases, University of Umeå, S-901 87 Umeå,[2] Sweden

Received 27 June 1994/Returned for modification 1 August 1994/Accepted 15 November 1994

Cytokine expression was determined in the livers of mice inoculated subcutaneously with *Francisella tularensis* LVS. During the first 48 h of infection, there was a logarithmic increase of bacteria in the liver, with a doubling time of 2.5 h. Within 48 h, tumor necrosis factor alpha (TNF-α), interleukin 10 (IL-10), IL-12, and gamma interferon (IFN-γ) mRNAs were expressed, and production of TNF-α and IFN-γ was demonstrated. There was no expression within 96 h of mRNA from IL-2, IL-3, or IL-4. After subcutaneous inoculation of heat-killed LVS, no expression of any of the cytokine mRNAs and no increase in the levels of TNF-α or IFN-γ occurred. The expression of TNF-α, IL-12, and IFN-γ is held to be important to evoke an early T-cell-independent host defense against *F. tularensis* as well as to drive the expansion of a protective Th1 cell response.

Intracellular bacteria and protozoa adapt to the intracellular environment of host macrophages. To successfully combat a pathogen, the macrophages need to be activated by cytokines and especially by gamma interferon (IFN-γ). Two main pathways for the activation are currently distinguished. One is the innate T-cell-independent pathway, identified mainly in experimental models of listeriosis and toxoplasmosis (4, 9, 11, 24, 27, 44). It is initiated by the release from the infected macrophages of interleukin 12 (IL-12) and tumor necrosis factor alpha (TNF-α), which trigger natural killer (NK) cells to produce IFN-γ (17, 41). This pathway is held to be important during the early phase of the infection and may help contain the infection until T cells become activated. When, later, the T-cell-dependent pathway is utilized, the macrophage activation may become more effective and eventually afford eradication of the infection. In this pathway, IL-12 is held to drive, in conjunction with IFN-γ, the differentiation of specifically sensitized $CD4^+$ T cells into the type 1 (Th1) subset (1, 8, 19), i.e., the subset producing IFN-γ.

Francisella tularensis, the causative agent of tularemia, is a facultative intracellular bacterium (15, 26). The virulence of the organism depends on its capacity to proliferate to high numbers before the development of an effective T-cell-mediated immune response. The target of this response is a variety of protein antigens (32, 36, 38). The T-cell response to the proteins is of the Th1 type, and the host defense depends on the ability of these cells to produce IFN-γ (2, 21). There is an attenuated vaccine strain, *F. tularensis* LVS, which also induces this type of response (30). Killed bacteria or bacterial antigens, on the other hand, induce no substantial T-cell response and no protection against the homologous organism (40).

As in listeriosis and toxoplasmosis, an early T-cell-independent resistance is also induced in tularemia. In a murine model of the disease, with *F. tularensis* LVS as the infecting agent, such resistance can be demonstrated and shown to be abrogated by the administration of monoclonal antibodies directed either to IFN-γ or to TNF-α (13, 14, 21). In this report, we describe the pattern of cytokine expression in the livers of *F. tularensis*-infected mice. The liver shows a rapid multiplication of LVS bacteria (3, 16, 35, 37). Similar to recent findings in studies of experimental leishmaniasis (42) and candidiasis (29), expression of IL-12 mRNA is demonstrated in the present study in immunocompetent animals during the early stage of infection with a facultative intracellular bacterium.

* Corresponding author. Phone: +46-90-106664. Fax: +46-90-106800. Electronic mail address: gsandstrom@ume.foa.se.

MATERIALS AND METHODS

Animals. Female 5- to 8-week-old BALB/cJ mice were purchased from Bomholtgård, Ry, Denmark. BALB/c mice have been used previously in a murine model of tularemia to demonstrate induction of T-cell-independent resistance (13, 14). The mice were housed at the National Research Establishment, Umeå, Sweden, under conventional conditions and given food and water ad libitum.

Bacteria. The vaccine strain *F. tularensis* LVS was supplied by the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Md. Bacteria were grown on modified Thayer-Martin agar (33) at 37°C to the logarithmic phase, suspended at a density of 10^{10} organisms per ml in phosphate-buffered saline (PBS) with 1% skim milk, and frozen at −70°C in 100-μl aliquots. The viability of frozen bacteria was found to be intact when tested periodically by culture on Thayer-Martin agar, and their virulence was equal to that of bacteria grown to the logarithmic phase in the defined liquid Chamberlain medium (5). For heat-killing, bacteria from the Thayer-Martin agar were suspended at a density of 10^{10} organisms per ml in PBS and treated at 65°C for 1 h. The lack of viability after heat treatment was confirmed by culture on Thayer-Martin agar, and the bacteria were stored frozen at −70°C. Before inoculation in mice, an aliquot of viable or heat-killed bacteria was thawed and the suspension was appropriately diluted in PBS.

Inoculation of bacteria and preparation of organs. Mice were inoculated subcutaneously with 2×10^5 viable *F. tularensis* LVS bacteria (approximately equivalent to 0.1 50% lethal dose), a dose known to result in rapid multiplication of bacteria in the liver, or 10^7 heat-killed LVS bacteria, a dose known to induce antibody production but no protection (unpublished data), in a total volume of 0.1 ml. At various times after inoculation, mice were killed by decapitation, the skin of each was folded back over the abdomen, and the liver was aseptically excised. A portion of the liver was homogenized in PBS for determination of viable counts. Other portions were used for estimation of cytokine levels and demonstration of cytokine mRNA. In some experiments, lymph nodes draining the site of inoculation were prepared for demonstration of cytokine mRNA.

RNA extraction and cDNA preparation. A portion of each liver was stored at −70°C in 2.5 ml of lysis buffer (4 M guanidine-isothiocyanate [Merck, Darmstadt, Germany], 25 mM Na citrate [pH 7.0], 0.5% *N*-lauroylsarcosine [Sigma Chemical Co., St. Louis, Mo.], and 100 mM 2-mercaptoethanol (Sigma]) in a 10-ml test tube. After thawing, homogenization was performed in the tube. RNA was isolated by a guanidine isothiocyanate-phenol-chloroform-isoamyl alcohol single-step method (7). Briefly, 33 μl of 3 M sodium acetate (pH 4.0), 500 μl of water-saturated phenol, and 100 μl of chloroform were mixed with 500 μl of each

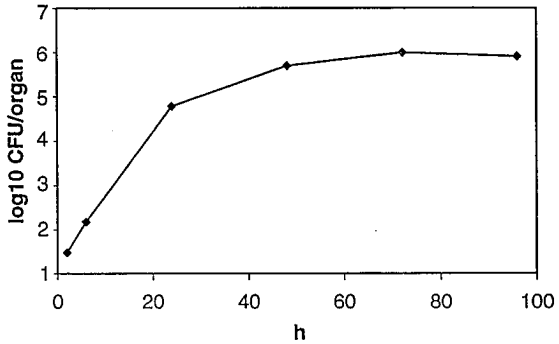

FIG. 1. Kinetics of bacterial growth of *F. tularensis* LVS in the livers of BALB/cJ mice. The animals were inoculated subcutaneously with 2×10^5 organisms and sacrificed at various times for determination of viable counts. The mean $\log_{10}$ number of CFU of two mice is indicated.

homogenate. The mixture was incubated at 0°C for 15 min and centrifuged at 10,000 × *g* for 10 min at 4°C. The RNA-containing aqueous phase was precipitated with an equal volume of isopropanol (Sigma) at −20°C for at least 90 min, centrifuged at 10,000 × *g* for 10 min at 4°C, and washed once with 75% ethanol in diethylpyrocarbonate-treated double-distilled water. The precipitate was air dried and resuspended in 100 μl of diethylpyrocarbonate-treated double-distilled water. The optical density at 260 nm was used to estimate the concentration of total mRNA, yields being in the order of 100 to 200 μg per sample. For cDNA synthesis, approximately 10 μg of total RNA was incubated at 42°C for 60 min in a total volume of 30 μl in reverse transcriptase buffer (Gibco BRL, Grand Island, N.Y.) supplemented with 0.6 mM (each) dATP, dGTP, dCTP, and dTTP (Pharmacia, Uppsala, Sweden), 40 U of RNasin (Promega, Madison, Wis.), 20 U of Moloney murine leukemia virus reverse transcriptase (Gibco BRL), and 1.5 μg of random hexamers (Promega). Samples of cDNA were stored at 4°C before PCR amplification.

PCR procedure. Specific primers for murine β_2-microglobulin, IL-2, IL-3, IL-4, IL-10, the p35 subunit of IL-12 (IL-12p35), IL-12p40, IFN-γ, and TNF-α (12) were purchased from Scandinavian Gene Synthesis AB, Stockholm, Sweden. cDNA (1.0 μl) was amplified in 50 μl of *Taq* DNA polymerase buffer (500 mM KCl, 100 mM Tris-HCl [pH 9.0], 1% Triton X-100) containing 200 μM (each) dATP, dGTP, dCTP, and dTTP, 400 nM (each) 3′ and 5′ primers, and 1.2 U of *Taq* DNA polymerase (Promega). The amplification was performed with 25 (β_2-microglobulin), 30 (IFN-γ, IL-12p35, and IL-12p40) or 35 (TNF-α, IL-2, IL-3, IL-4, and IL-10) cycles at 94°C for 60 s, 60°C for 45 s, and 72°C for 2 min with a DNA thermal cycler (Perkin-Elmer Cetus Corp., Norwalk, Conn.). cDNA fragments corresponding to each cytokine were obtained from Clontech, Palo Alto, Calif., and used as positive controls. In a negative control, cDNA was omitted. A sample (10 μl) of each amplified product was subjected to electrophoresis at 100 V for 1.5 h in a 1.5% agarose gel. Gels were stained with ethidium bromide and visualized by UV illumination. As the DNA size marker, a 1-kb ladder (Gibco BRL) was used. In the analysis of IL-12p40, reproducible results were obtained only when the PCR product was subjected to a standard Southern blot procedure (22). Southern blot transfers were probed with an internal IL-12p40-specific oligonucleotide (17) and visualized by autoradiography. The probe was end labeled with ^{32}P by a standard protocol (22).

ELISA. Extraction of cytokines from the livers of inoculated mice was carried out essentially as described previously (24). Half of each liver was homogenized in 2.5 ml of RPMI 1640 (Gibco BRL) containing 1% (wt/vol) CHAPS {3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate; Sigma}. After extraction at 4°C for 1 h, the samples were centrifuged for 30 min at 6,000 × *g*, and the supernatants were stored at −70°C until used. The commercially available kits Inter-TEST-γ and Factor-TEST TNF (Genzyme Corp., Cambridge, Mass.) were utilized for determination of levels of IFN-γ and TNF-α, respectively.

RESULTS

Bacterial growth. The number of *F. tularensis* LVS bacteria in the livers of inoculated mice increased exponentially for 48 h and reached 10^6 organisms within 72 h of inoculation (Fig. 1). The doubling time was approximately 2.5 h.

Cytokine mRNA expression in the livers of mice infected with *F. tularensis* LVS. A sublethal dose of *F. tularensis* LVS (2×10^5 organisms) was inoculated subcutaneously, and the expression in the liver of mRNA from TNF-α, IFN-γ, IL-2, IL-3, IL-4, IL-10, IL-12p35, and IL-12p40 was determined at 0, 1, 2, 6, 24, 48, 72, and 96 h postinoculation. TNF-α mRNA was detected at 24 h, whereas mRNA from IFN-γ, IL-10, and IL-12p40 appeared at 48 h. As predicted (6), there was a constitutive expression of mRNA from IL-12p35 (data not shown). No expression of IL-2, IL-3, or IL-4 mRNA was detected during the 96-h interval (Fig. 2). Within 96 h of subcutaneous inoculation of 10^7 heat-killed organisms, no induction of mRNA from any of the cytokines could be demonstrated in the liver (Fig. 2).

Production of TNF-α and IFN-γ after inoculation of *F. tularensis* LVS organisms. An enzyme-linked immunosorbent assay was used to estimate the levels of TNF-α and IFN-γ in homogenates of livers. After inoculation of 2×10^5 viable LVS organisms, the levels of the two cytokines increased during the interval of 24 to 48 h (Fig. 3). The presence of a slightly increased level of IFN-γ at 24 h, before mRNA was detectable by PCR (Fig. 2), could possibly represent cytokine synthesized outside the liver. In regional lymph nodes, an accumulation of IFN-γ mRNA was demonstrated within 24 h of inoculation (Fig. 4). The inoculation of killed bacteria did not result in any increase of TNF-α or IFN-γ in the liver within the 96-h period studied.

DISCUSSION

Various lines of evidence have shown TNF-α to be necessary although insufficient for the generation of an early T-cell-independent IFN-γ production in response to intracellular pathogens (4, 24, 25). More recently, the critical role of IL-12 has become apparent, mainly by in vitro experiments with spleen cells from T-cell-deficient mice (17, 41). In the present study, IL-12 mRNA was found to be expressed early in response to a facultative intracellular bacterium under in vivo conditions closely mimicking natural infection. We used immunocompetent mice, which were exposed by the dermal route of entrance as occurs in natural tularemia.

We focused on the liver, which is invaded early during the bacteremic phase of tularemia. The liver is excessively rich in resident macrophages, the primary target cells of *F. tularensis*. These cells are the probable source of the IL-12 and IL-10 mRNAs demonstrated. TNF-α may be produced not only by macrophages but also by neutrophils, which are involved in the anti-*Francisella* resistance of the liver (35). The source of IFN-γ may be NK cells, which are known to infiltrate the liver during infection with intracellular bacteria (18). The results were clear-cut insofar as no expression of mRNA from any of the cytokines was detected in the liver of noninoculated mice. In this respect, results with lymphoid organs may be less easily interpreted. In the spleens of mice infected with *L. monocytogenes*, a basal expression of several cytokines has been reported and thought to be due to a homeostatic activity independent of current exogenous stimulation (20). In accordance, we have detected TNF-α and IL-2 mRNAs in the lymph nodes and spleens of noninoculated mice (unpublished results).

The kinetics of production of TNF-α and IFN-γ in the livers of the infected mice corresponded well with the previously demonstrated early native murine host response to tularemia (13, 14). The T-cell-independent nature of the response was confirmed by the lack of expression of IL-2 mRNA in the liver during the 96-h period studied.

Whereas TNF-α, IL-12, and IFN-γ are parts of a sequence leading to the activation of infected macrophages, IL-10 is recognized as a down-regulator. Several suppressive effects on inflammation and cell-mediated immunity have been ascribed to IL-10; these include inhibition of the release of TNF-α and IL-12 from macrophages (10, 19). The role of IL-10 may, however, be highly complex, as suggested by a recently re-

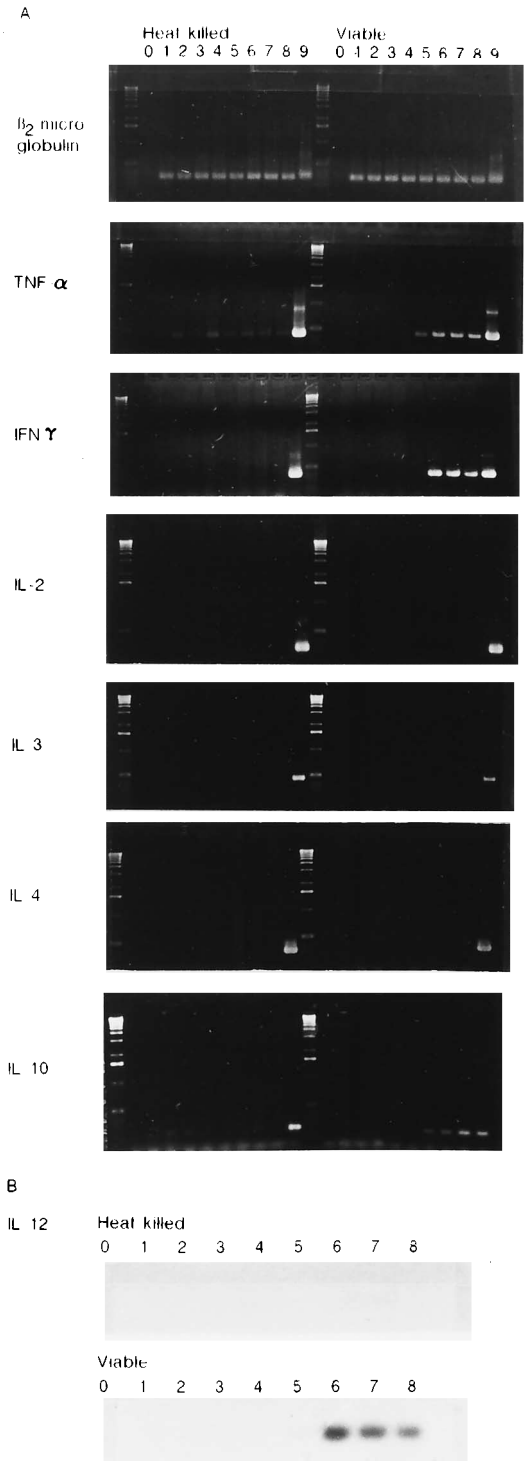

FIG. 2. PCR-assisted mRNA amplification of samples from the livers of BALB/cJ mice inoculated subcutaneously with heat-killed or live *F. tularensis* LVS organisms. Samples were prepared at various times after inoculation. Lanes: 0, negative control; 1, 0 h; 2, 1 h; 3, 2 h; 4, 6 h; 5, 24 h; 6, 48 h; 7, 72 h; 8, 96 h; 9, positive control. (A) β_2-Microglobulin, TNF-α, IFN-γ, IL-2, IL-3, IL-4, and IL-10 mRNAs. The PCR products were separated in a 1.5% agarose gel and visualized by ethidium bromide staining. (B) IL-12 mRNA. The PCR products were subjected to Southern transfer and probed with an internal IL-12p40-specific oligonucleotide.

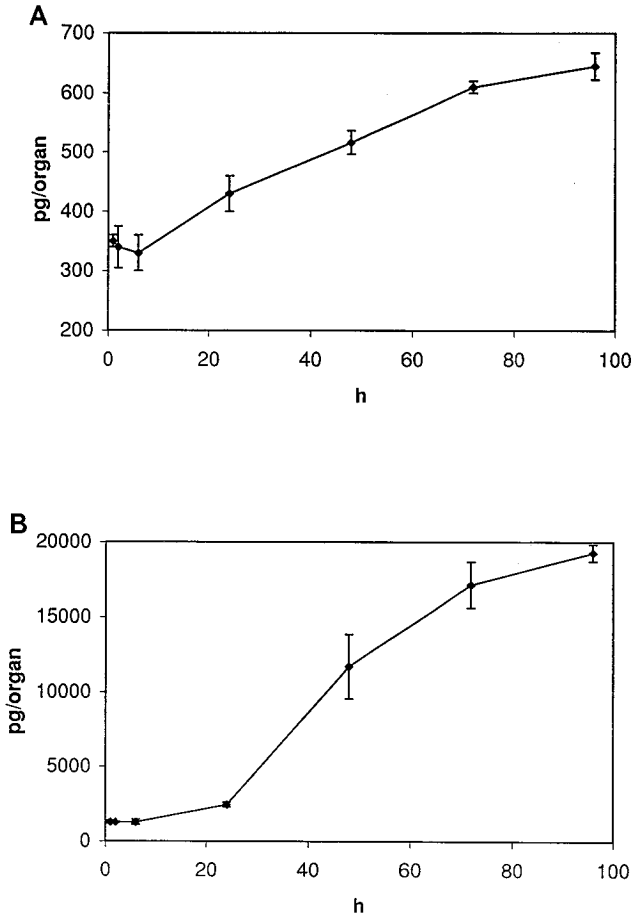

FIG. 3. Levels of TNF-α (A) and IFN-γ (B) in homogenates of livers from BALB/cJ mice inoculated subcutaneously with 2×10^5 live *F. tularensis* LVS organisms. In animals inoculated with 10^7 heat-killed LVS organisms, TNF-α and IFN-γ levels were invariably lower than 300 and 1000 ng per organ, respectively.

ported persistence of *Listeria monocytogenes* infection in mice treated with a specific anti-IL-10 monoclonal antibody (43). In the present experiments, IL-10 mRNA appeared in the liver within 48 h of subcutaneous inoculation of *F. tularensis*, indicating a possible regulatory function of this cytokine in the early phase of murine tularemia. It should be remarked, though, that the production of IL-10 was not assayed.

IL-12 has been shown recently to be critical not only as a triggering agent of the early T-cell-independent host defense but also in directing the differentiation of naive T cells into Th1 cells in response to an antigen (19). In human tularemia, most evidence indicates that the Th1 type of response predominates. When, for example, T cells from *F. tularensis*-primed individuals were stimulated in vitro with various homologous antigens, there was an invariable Th1 cell response including production of IL-2 and IFN-γ but not IL-4 (36). The early expression of IL-12 mRNA and IFN-γ demonstrated here can be assumed to counteract any Th2 cell differentiation (19). IL-4, which is believed to be important in driving a Th2 response early in immunization (34, 39), was not detected in the present study at the transcription level during the first 96 h of infection.

The cytokine expression in the liver was initiated during a phase of logarithmic bacterial growth in the organ. On the

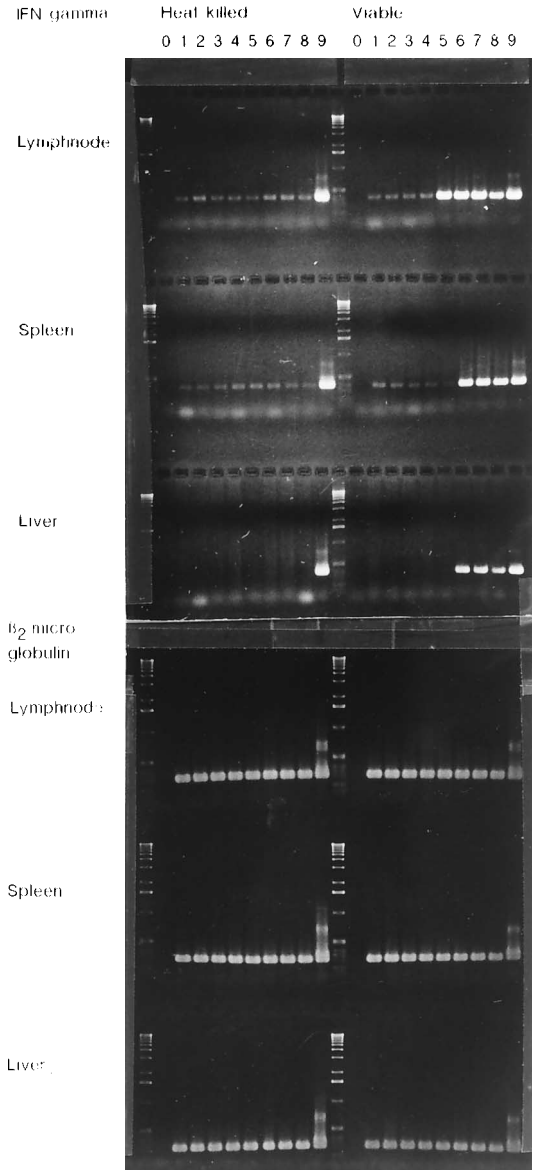

FIG. 4. PCR-assisted amplification of mRNA from IFN-γ and the control protein β_2-microglobulin in samples of lymph node, spleen, and liver from BALB/cJ mice after subcutaneous inoculation of 2×10^5 live or 10^7 heat-killed *F. tularensis* LVS organisms. Samples were prepared at various times after inoculation. Lanes: 0, negative control; 1, 0 h; 2, 1 h; 3, 2 h; 4, 6 h; 5, 24 h; 6, 48 h; 7, 72 h; 8, 96 h; 9, positive control.

basis of experiments with a listeriolysin-deficient mutant of *L. monocytogenes*, intracellular bacterial replication may in fact be a prerequisite of cytokine induction (28). A delay in cytokine release might favor the infectivity of intracellular bacteria by allowing them to become established before the macrophages are activated. In an experimental model of bubonic plague, an avirulent mutant of *Yersinia pestis* lacking the Lcr plasmid has been found to cause a more rapid IFN-γ response than that caused by the isogenic Lcr^+ strain (23).

Our results are relevant to the unsolved question of why only live *F. tularensis* vaccines can induce effective host protection. Subcutaneous inoculation of heat-killed *F. tularensis* LVS is known to result in a pronounced antibody response but in poor protection and a poor T-cell response (40). We found no TNF-α, IL-12, or IFN-γ to be expressed in the liver early after subcutaneous inoculation of heat-killed bacteria. If killed bacteria are at all able to induce a cytokine response, the magnitude of the response would obviously be only minute in comparison with that generated intracellularly by bacterial invasion and replication in the liver and other organs.

The nature of components of intracellular bacteria initiating a protective cytokine expression is unknown. Although the lipopolysaccharide of *F. tularensis* has been found to induce TNF-α in mononuclear phagocytes in vitro (31), it is by itself not a strong candidate. In vivo, lipopolysaccharide from gram-negative bacteria has been found to induce the expression of macrophage-derived cytokines without any associated IFN-γ induction (28). Moreover, lipopolysaccharide cannot be a common denominator because there are potent intracellular pathogens such as *L. monocytogenes* which lack lipopolysaccharide. In further experiments aiming to identify crucial bacterial components, TNF-α, IL-12, and IFN-γ should all be useful markers. TNF-α and IFN-γ are readily assayed in various models of intracellular infections. IL-12 expression has been demonstrated recently in an experimental infection with the fungus *Candida albicans* (29) and the protozoan parasite *Leishmania major* (42) and can, as shown here, be monitored in experimental tularemia.

ACKNOWLEDGMENTS

We thank Lena Sundberg and Kerstin Kuoppa for excellent technical assistance.

This work was supported by grants from the Swedish Medical Research Council (project no. 9485), the Royal Swedish Academy of Sciences, the Wenner-Gren Center Foundation, the Magnus Bergvall Foundation, and the Kempe Foundations.

REFERENCES

1. **Afonso, L. C. C., T. M. Scharton, L. Q. Vieira, M. Wysocka, G. Trinchieri, and P. Scott.** 1994. The adjuvant effect of interleukin-12 in a vaccine against *Leishmania major*. Science **263:**235–237.
2. **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.
3. **Anthony, L. S. D., and P. A. L. Kongshavn.** 1987. Experimental murine tularemia caused by *Francisella tularensis*, live vaccine strain: a model of acquired cellular resistance. Microb. Pathog. **2:**3–14.
4. **Bancroft, G. J., K. C. F. Sheehan, R. D. Schreiber, and E. R. Unanue.** 1989. Tumor necrosis factor is involved in the T cell-independent pathway of macrophage activation in *scid* mice. J. Immunol. **143:**127–130.
5. **Chamberlain, R. E.** 1965. Evaluation of live tularemia vaccine prepared in a chemically defined medium. Appl. Microbiol. **13:**232–235.
6. **Chan, S. H., B. Perussia, J. W. Gupta, M. Kobayashi, M. Pospisil, H. A. Young, S. F. Wolf, D. Young, S. C. Clark, and G. Trinchieri.** 1991. Induction of interferon γ production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with others. J. Exp. Med. **173:**869–879.
7. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
8. **Coffman, R. L., K. Varkila, P. Scott, and R. Chatelain.** 1991. Role of cytokines in the differentiation of $CD4^+$ T cell subsets in vivo. Immunol. Rev. **123:**189–207.
9. **Denkers, E. Y., R. T. Gazzinelli, D. Martin, and A. Sher.** 1993. Emergence of $NK1.1^+$ cells as effectors of IFN-γ dependent immunity to *Toxoplasma gondii* in MHC class I-deficient mice. J. Exp. Med. **178:**1465–1472.
10. **de Waal Malefyt, R., J. Abrams, B. Bennett, C. G. Figdor, and J. E. de Vries.** 1991. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J. Exp. Med. **174:** 1209–1220.
11. **Dunn, P. L., and R. J. North.** 1991. Early gamma interferon production by natural killer cells is important in defense against murine listeriosis. Infect. Immun. **59:**2892–2900.
12. **Ehlers, S., M. E. A. Mielke, T. Blankenstein, and H. Hahn.** 1992. Kinetic analysis of cytokine gene expression in the livers of naive and immune mice infected with *Listeria monocytogenes*. The immediate early phase in innate resistance and acquired immunity. J. Immunol. **149:**3016–3022.
13. **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.
14. **Elkins, K. L., T. Rhinehart-Jones, C. A. Nacy, R. K. Winegar, and A. H. Fortier.** 1993. T-cell-independent resistance to infection and generation of immunity to *Francisella tularensis*. Infect. Immun. **61:**823–829.
15. **Fortier, A. H., T. Polsinelli, S. J. Green, and C. A. Nacy.** 1992. Activation of macrophages for destruction of *Francisella tularensis*: identification of cytokines, effector cells, and effector molecules. Infect. Immun. **60:**817–825.
16. **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.
17. **Gazzinelli, R. T., S. Hieny, T. A. Wynn, S. Wolf, and A. Sher.** 1993. Interleukin 12 is required for the T-lymphocyte-independent induction of interferon γ by an intracellular parasite and induces resistance in T-cell-deficient hosts. Proc. Natl. Acad. Sci. USA **90:**6115–6119.
18. **Goossens, P. L., H. Jouin, and G. Milon.** 1991. Dynamics of lymphocytes and inflammatory cells recruited in liver during murine listeriosis. A cytofluorimetric study. J. Immunol. **147:**3514–3520.
19. **Hsieh, C.-S., S. E. Macatonia, C. S. Tripp, S. F. Wolf, A. O'Garra, and K. M. Murphy.** 1993. Development of T_H1 $CD4^+$ T cells through IL-12 produced by *Listeria*-induced macrophages. Science **260:**547–549.
20. **Iizawa, Y., J. F. Brown, and C. J. Czuprynski.** 1992. Early expression of cytokine mRNA in mice infected with *Listeria monocytogenes*. Infect. Immun. **60:**4068–4073.
21. **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.
22. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Nakajima, R., and R. R. Brubaker.** 1993. Association between virulence of Yersinia pestis and suppression of gamma interferon and tumor necrosis factor alpha. Infect. Immun. **61:**23–31.
24. **Nakane, A., T. Minagawa, and K. Kato.** 1988. Endogenous tumor necrosis factor (cachectin) is essential to host resistance against *Listeria monocytogenes* infection. Infect. Immun. **56:**2563–2569.
25. **Nakane, A., T. Minagawa, M. Kohanawa, Y. Chen, H. Sato, M. Moriyama, and N. Tsuruoka.** 1989. Interactions between endogenous gamma interferon and tumor necrosis factor in host resistance against primary and secondary *Listeria monocytogenes* infections. Infect. Immun. **57:**3331–3337.
26. **Nutter, J. E., and Q. N. Myrvik.** 1966. In vitro interactions between rabbit alveolar macrophages and *Pasteurella tularensis*. J. Bacteriol. **92:**645–651.
27. **Poston, R. M., and R. J. Kurlander.** 1991. Analysis of the time course of IFN-γ mRNA and protein production during primary murine listeriosis. The immune phase of bacterial elimination is not temporally linked to IFN production in vivo. J. Immunol. **146:**4333–4337.
28. **Poston, R. M., and R. J. Kurlander.** 1992. Cytokine expression in vivo during murine listeriosis. Infection with live, virulent bacteria is required for monokine and lymphokine messenger RNA accumulation in the spleen. J. Immunol. **149:**3040–3044.
29. **Romani, L., A. Mencacci, L. Tonnetti, R. Spaccapelo, E. Cenci, S. Wolf, P. Puccetti, and F. Bistoni.** 1994. Interleukin-12 but not interferon-γ production correlates with induction of T helper type-1 phenotype in murine candidiasis. Eur. J. Immunol. **24:**909–915.
30. **Sandström, G.** 1994. The tularemia vaccine: a review. J. Chem. Tech. Biotechnol. **59:**315–320.
31. **Sandström, G., A. Sjöstedt, T. Johansson, K. Kuoppa, and J. C. Williams.** 1992. Immunogenicity and toxicity of lipopolysaccharide from *Francisella tularensis* LVS. FEMS Microbiol. Immunol. **105:**201–210.
32. **Sandström, G., A. Tärnvik, and H. Wolf-Watz.** 1987. Immunospecific T-lymphocyte stimulation by membrane proteins from *Francisella tularensis*. J. Clin. Microbiol. **25:**641–644.
33. **Sandström, G., A. Tärnvik, H. Wolf-Watz, and S. Löfgren.** 1984. Antigen from *Francisella tularensis*: nonidentity between determinants participating in cell-mediated and humoral reactions. Infect. Immun. **45:**101–106.
34. **Scott, P., P. Natovitz, R. L. Coffman, E. Pearce, and A. Sher.** 1988. Immunoregulation of cutaneous leishmaniasis. T cell lines that transfer protective immunity or exacerbation belong to different T helper subsets and respond to distinct parasite antigens. J. Exp. Med. **168:**1675–1684.
35. **Sjöstedt, A., J. W. Conlan, and R. J. North.** 1994. Neutrophils are critical for host defense against primary infection with the facultative intracellular bacterium *Francisella tularensis* in mice and participitate in defense against reinfection. Infect. Immun. **62:**2779–2783.
36. **Sjöstedt, A., M. Eriksson, G. Sandström, and A. Tärnvik.** 1992. Various membrane proteins of *Francisella tularensis* induce interferon-γ production in both $CD4^+$ and $CD8^+$ T cells of primed humans. Immunology **76:**584–592.
37. **Sjöstedt, A., G. Sandström, and A. Tärnvik.** 1992. Humoral and cell-mediated immunity in mice to a 17-kilodalton lipoprotein of *Francisella tularensis* expressed by *Salmonella typhimurium*. Infect. Immun. **60:**2855–2862.
38. **Surcel, H. M.** 1990. Diversity of *Francisella tularensis* antigens recognized by human T lymphocytes. Infect. Immun. **58:**2664–2668.
39. **Swain, S. L., A. D. Weinberg, M. English, and G. Huston.** 1990. IL-4 directs the development of Th2-like helper effectors. J. Immunol. **145:**3796–3806.
40. **Tärnvik, A.** 1989. Nature of protective immunity to *Francisella tularensis*. Rev. Infect. Dis. **11:**440–451.
41. **Tripp, C. S., S. F. Wolf, and E. R. Unanue.** 1993. Interleukin 12 and tumor necrosis factor α are costimulators of interferon γ production by natural killer cells in severe combined immunodeficiency mice with listeriosis, and interleukin 10 is a physiologic antagonist. Proc. Natl. Acad. Sci. USA **90:**3725–3729.
42. **Vieira, L. Q., B. D. Hondowicz, L. C. C. Afonso, M. Wysocka, G. Trinchieri, and P. Scott.** 1994. Infection with *Leishmania major* induces interleukin-12 production in vivo. Immunol. Lett. **40:**157–161.
43. **Wagner, R. D., N. M. Maroushek, J. F. Brown, and C. J. Czuprynski.** 1994. Treatment with anti-interleukin-10 monoclonal antibody enhances early resistance to but impairs complete clearance of *Listeria monocotogenes* infection in mice. Infect. Immun. **62:**2345–2353.
44. **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.