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Cytokine Gene Expression in Mice at an Early Stage of Infection with Various Strains of *Listeria* spp. Differing in Virulence

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By using reverse transcription-PCR, cytokine gene expression was examined in mice 24 h after infection with various strains of *Listeria* spp. differing in virulence as determined by in vivo growth and 50% lethal dose values. All the virulent strains of *Listeria monocytogenes* induced the expression of mRNAs specific for interleukin-1α (IL-1α), gamma interferon (IFN-γ), and tumor necrosis factor alpha (TNF-α) in the spleen of mice, while an *L. monocytogenes* strain incapable of producing listeriolysin O and strains of *Listeria innocua* induced the expression of TNF-α mRNA only. The levels of expression of IL-1α and IFN-γ mRNAs were proportional to the levels of listeriolysin O produced by each strain. Those strains which induced the expression of IFN-γ were capable of generating protective immunity in the infected host, suggesting that the virulence-related induction of some cytokine at the initial stage of infection plays a role in the induction of acquired cellular resistance to *L. monocytogenes*.

Listeria monocytogenes is a facultative intracellular bacterium which causes listeriosis in humans and animals. In an animal model of infection, it is known that this microbe multiplies mainly in the liver and spleen, resulting in a fatal infection even in immunocompetent hosts (19, 24). *L. monocytogenes* is able to survive inside macrophages by escaping into the cytoplasm from the phagosome (7, 20, 33, 34). This escape mechanism has recently been ascribed to a 58-kDa listeriolysin O (LLO) (3, 13, 28) and several enzymes encoded by genes which are located adjacent to the *hlyA* gene, coding for LLO (21, 27, 30, 36). All these virulence- or escape-associated genes are known to be under coordinate regulation by the *prfA* gene product (4, 22).

Various cytokines are produced and involved in the innate resistance of the host against infection. Inflammatory cytokines, including tumor necrosis factor (TNF), gamma interferon (IFN-γ), interleukin-1 (IL-1), and IL-6 have been reported to be produced in mice after *L. monocytogenes* infection (12, 17, 29). By using cytokine-specific neutralizing antibodies and recombinant cytokines, it has been shown that some of these endogenous cytokines play an important role in the protection of host in the early stage of infection (6, 8, 9, 18, 25). In most of these studies, all the strains used for infection of mice have been virulent strains of *L. monocytogenes*, and it is not clear whether cytokine involvement is due to the virulence or to in vivo growth of *L. monocytogenes*.

In previous in vitro studies, we have shown that macrophage production of IL-1, one of the inflammatory cytokines, is strongly stimulated by viable cells of *L. monocytogenes* but not by killed bacterial cells or viable bacteria of a nonvirulent strain (23). Furthermore, purified LLO has been shown to be capable of stimulating macrophages to produce IL-1 in vitro (35, 38). It was suggested that virulence factor or virulence factor-dependent growth of bacteria is responsible for IL-1 expression. In this regard, it is of interest to know whether the virulence of *L. monocytogenes* is directly related to the expression of several cytokines in vivo. Using various strains of *L. monocytogenes* differing in virulence and some avirulent strains of *Listeria innocua*, we have examined cytokine gene expression at an early stage of infection.

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MATERIALS AND METHODS

Experimental animals. Male mice of the C3H/He strain (Charles River Japan, Atsugi, Japan), raised and maintained under specific-pathogen-free conditions, were used for experiments at 7 to 10 weeks of age. Outbred mice of the ICR strain were used for the 50% lethal dose (LD_{50}) assay.

Bacterial strains. *L. monocytogenes* EGD, which has been maintained in this laboratory for a long time, was used as the standard strain throughout the study. *L. monocytogenes* ATCC 15313 was obtained from the American Type Culture Collection. *L. monocytogenes* 3388 and *L. innocua* 10, 2740, and 2769 were provided by B. S. Ralovich, Institute of Public Health and Epidemiology, Pecs, Hungary. *L. monocytogenes* JM, F4, and L461 were obtained from T. Nagai, Sapporo Clinical Laboratory Center, Sapporo, Japan.

Preparation of bacteria. The bacteria were grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.) at 37°C for 16 h, washed repeatedly, suspended in phosphate-buffered saline, and stored at −70°C. Killed cells of *L. monocytogenes* were prepared by heating a viable bacterial suspension of a known concentration at 74°C for 90 min (23).

Determination of LD_{50}. Groups of ICR mice were intravenously infected with graded doses of each strain, and deaths were recorded every day. After 14 days of observation, the mortality rate was calculated for each group of five to six mice, and the LD_{50} was determined.

Bacterial counts in vivo. To assess the virulence of each strain in terms of growth in infected mice, bacteria were intravenously injected at the dose of 8×10^3 to 10×10^3 CFU. Spleens were homogenized on days 2 and 3 after infection, and viable bacteria were enumerated by the pour plate method after serial dilution. In a challenge experiment, mice were immunized with 4×10^3 to 5×10^3 CFU of bacteria for each strain and then challenged with 10^4 CFU of *L. monocytogenes* EGD 7 days after the immunizing infection. Viable bacteria in the spleen were enumerated 2 days after the challenge, and the

$\log_{10}$ difference in bacterial numbers between each group and nonimmune control mice was taken as the level of protection.

Titration of hemolysin-producing ability. Each strain of bacteria was subcultured overnight in brain heart infusion broth (Eiken Chemical Co. Ltd., Tokyo, Japan), and 100-μl aliquot was inoculated into 20 ml of fresh broth. After 18 h of culture at 37°C with shaking, culture supernatants were obtained by centrifugation at 1,500 × *g* for 30 min. L-Cysteine was added to a final concentration of 10 mM, and serial dilutions were made. In a U-bottomed microtiter plate, diluted samples were mixed with an equal volume of a 2% suspension of sheep erythrocytes and incubated for 45 min at 37°C. The reciprocal of the highest dilution which gave complete hemolysis was taken as the titer, and the result was expressed as the mean of three assays ± standard deviation.

RNA extraction. Mice were intravenously injected with 2×10^3 viable *L. monocytogenes* or *L. innocua* of each strain. One day after the infection, total RNA was extracted from the spleen as described before (5). In brief, mice were bled to death, and spleens were taken and homogenized at room temperature in 5 ml of solution D (4 M guanidinium isothiocyanate, 0.5% *N*-lauroylsarcosine, 25 mM sodium citrate, 100 mM 2-mercaptoethanol) in a glass Teflon homogenizer and transferred to a 12-ml polypropylene tube. Subsequently, 0.4 ml of 2 M sodium acetate (pH 4), 4 ml of water-saturated phenol, and 0.8 ml of chloroform-isoamyl alcohol mixture were added to the homogenate, with thorough mixing by inversion after the addition of each reagent. The final suspension was vortexed vigorously for 20 s and cooled on ice for 15 min. Samples were centrifuged at 10,000 × *g* for 20 min at 4°C. After centrifugation, RNA present in the aqueous phase was transferred to a fresh tube, mixed with 3 ml of isopropanol, and placed at −20°C overnight to precipitate RNA. Sedimentation at 10,000 × *g* for 20 min was performed again, and the resulting RNA pellet was dissolved in 2 ml of solution D, transferred to a new centrifuge tube, and precipitated with 2 ml of isopropanol at −20°C for 2 h. After centrifugation for 15 min at 4°C, the RNA was resuspended in 75% ethanol, precipitated, vacuum dried, and dissolved in 50 μl of distilled water. The RNA yield was determined by the A_{260}, and the purity of the RNA was assessed on the basis of the A_{260}/A_{280} ratio with a nucleic acid spectrophotometer (GeneQuant; Pharmacia).

RT-PCR. Preparation of cDNA by reverse transcription (RT) was performed in the following way. Total RNA extracted as described above (10 μg in a volume of less than 10 μl) was mixed with 4 μl of RT buffer, 2 μl of 0.1 M dithiothreitol, 0.5 μl of RNasin (Promega, Madison, Wis.), 1 μl of 10 mM deoxynucleoside triphosphates (dNTPs) (Pharmacia Biotechnology AB, Uppsala, Sweden), 2 μl of random primer (Pharmacia), 0.5 μl of reverse transcriptase (Gibco-BRL, Life Technologies, Inc., Gaithersburg, Md.), and distilled water to make the final volume 20 μl. The mixture was incubated at 42°C for 60 min and boiled at 95°C for 3 min. After that, samples were held at −20°C until use for PCR.

PCR amplification was performed by using a TP cycler-100 (Toyobo, Osaka, Japan). The reaction mixture consisted of 5 μl of sample cDNA, 5 μl of PCR amplification buffer, 2 μl of 25 mM $MgCl_2$, 4 μl of 2.5 mM dNTPs, 0.3 μl of *Taq* DNA polymerase (5 U/μl; Promega), 2 μl of 20 mM primer, and 29.7 μl of double-distilled water to make the final volume 50 μl. All the PCR primers were made to our order by Kurabo Biomedicals (Osaka, Japan). The following oligonucleotides were used: for IL-1α, 5′-CTCTAGAGCACCATGCTACAGAC-3′ and 5′-TGGAATCCAGGGGAAACACTG-3′; for IFN-γ, 5′-AGCGGCTGACTGAACTCAGATTGTAG-3′ and 5′-GTCACAGTTTTCAGCTGTATAGGG-3′; and for TNF-α, 5′-GGCAGGTCTACTTTGGAGTCATTGC-3′ and 5′-ACATTCGAGGCTCCAGTGAATTCGG-3′. The predicted sizes of amplified products for IL-1α, IFN-γ, and TNF-α were 288, 213, and 309 bp, respectively. Mineral oil was overlaid on the mixtures to prevent evaporation. One PCR cycle was run under the following conditions: DNA denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and DNA extension at 72°C for 1 min. After 25 cycles of amplification, the reaction was terminated at 72°C for 7 min. The PCR products were stored in the cycler at 4°C until they were collected.

TABLE 1. Characteristics of *Listeria* strains used in this study

Species	Strain	Serotype	LD_{50} ($\log_{10}$/CFU)	Mean LLO titer[a] (HU/ml) ± SD
L. monocytogenes	EGD	1/2a	4.87	75 ± 4
	JM	1b	4.80	100 ± 14
	3388	4b	5.01	71 ± 6
	F4	4b	5.23	18 ± 2
	L461	3b	7.54	23 ± 2
	ATCC 15313	1b	8.00	4 ± 0
L. innocua	10	4ab	8.97	5 ± 3
	2740	6b	>9.00	6 ± 2
	2769	6b	>9.00	7 ± 3

[a] The reciprocal of the highest dilution of culture supernatant showing complete hemolysis of a 2% suspension of sheep erythrocytes.

Agarose gel electrophoresis. The PCR products were analyzed by agarose gel electrophoresis with a horizontal 1.0% agarose gel (low-melting-temperature agarose L; Wako Pure Chemicals, Tokyo, Japan) in 1× TAE buffer supplemented with 0.005% ethidium bromide for DNA staining. Undiluted PCR product (10 μl) plus 2 μl of bromophenol blue was applied to each well. Gels were run in 1× TAE buffer at 50 V for 1.5 h. The PCR products were visualized and photographed on a UV transilluminator.

Northern (RNA) blotting. Ten micrograms of total RNA extracted as described above were glyoxylated, electrophoresed through 1.0% agarose, and transferred to a nylon membrane (Hybond N; Amersham International Plc., Amersham, United Kingdom). The filter was hybridized with ^{32}P-labeled IL-1α probe, which was kindly provided by Y. Yoshikai (Nagoya University, Nagoya, Japan) at 65°C for 16 h in hybridization buffer consisting of one hybridization buffer tablet (Amersham) per 10 ml of distilled water and 100 μg of denatured herring sperm DNA (Sigma) per ml. The filter was washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% sodium dodecyl sulfate at 65°C and exposed to Kodak X-OMAT film at −85°C in the presence of intensifying screens.

RESULTS

Virulence of each strain of *L. monocytogenes* and *L. innocua*. The virulence of the bacteria was determined by an LD_{50} assay, bacterial growth in the spleen of mice, and hemolysin production in culture. The profile of each strain with respect to these virulence parameters is shown in Table 1 and Fig. 1. From the LD_{50} value, four strains of *L. monocytogenes* could be regarded as highly virulent strains, while two other strains of *L. monocytogenes* (L461 and ATCC 15313) and all three strains of *L. innocua* seemed to be of low or no virulence. When mice were infected with 8×10^3 to 10×10^3 viable bacteria of each strain, all the *L. monocytogenes* strains except ATCC 15313 exhibited multiplication in the spleen, while *L. monocytogenes* ATCC

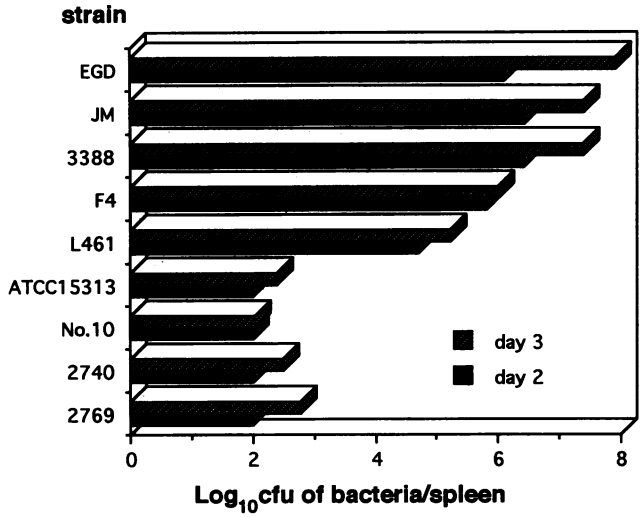

FIG. 1. Bacterial growth in the spleens of mice infected with approximately 10^4 CFU of each strain. Solid and hatched bars indicate counts of viable bacteria recovered on days 2 and 3 of infection, respectively. The standard deviation for each group never exceeded 10% of the mean value.

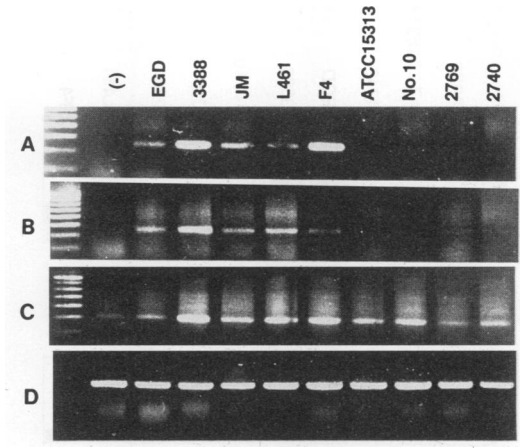

FIG. 2. PCR detection of mRNAs specific for IL-1α (A), IFN-γ (B), TNF-α (C), and β-actin (D). Total RNA extracted from the spleens of mice infected with each strain of bacteria 24 h before was reverse transcribed to cDNA and subjected to PCR amplification with primer pairs specific for each cytokine. PCR products were run on agarose gels after 25 cycles of amplification. Strains are indicated at the top of the figure; lane (—), spleens from normal, noninfected mice. Molecular size standards (100-bp ladder) are shown at the left.

15313 and three strains of *L. innocua* did not (Fig. 1). These four nonvirulent strains showed a very poor ability to produce hemolysin (LLO) in broth culture, less than 10 hemolysis units (HU)/ml in our assay (Table 1). When the culture supernatants were subjected to Western blotting (immunoblotting) with monoclonal antibody to LLO (clone 7D10/E12; unpublished), those obtained from the five strains which grew well in the spleen showed a 58-kDa band, indicating that the hemolysin produced in the culture supernatant was LLO (data not shown). From the results shown in Table 1 and Fig. 1, it appeared that the strain capable of producing more than approximately 20 HU/ml of LLO could multiply in the infected host and exhibit virulence for mice.

Cytokine gene expression in infected spleens. One day after infection of mice with the same number of viable bacteria of each strain, spleens were removed and total RNA was extracted. Extracted total RNA was reverse transcribed, and the levels of IL-1α, IFN-γ, and TNF-α expression were assessed by PCR with primer pairs specific for each cytokine. The predicted sizes of the PCR products for IL-1α, IFN-γ, and TNF-α were 288, 213, and 309 bp, respectively. PCR products for β-actin (348 bp) revealed that almost equal amounts of RNA were subjected to RT-PCR (Fig. 2). Five strains which were highly virulent for mice induced the expression of substantial levels of IL-1α and IFN-γ. In the spleens of mice infected 24 h before with avirulent strains, including *L. monocytogenes* ATCC 15313 and *L. innocua* 10, 2769, and 2740, there was no expression of these two cytokines. In contrast, expression of the TNF-α gene could be observed in the spleens of all mice infected with the various strains irrespective of their virulence for mice (Fig. 2).

We monitored the expression of the cytokine genes in mice for 5 consecutive days after infection with *L. monocytogenes* EGD and ATCC 15313 and *L. innocua* 2740. It was found that the three cytokine genes were expressed for 4 days in mice infected with *L. monocytogenes* EGD, while mRNAs specific for IL-1α and IFN-γ were never expressed during the observation period in mice infected with ATCC 15313 or 2740 (data not shown). These results suggested that the expression of IL-1α and IFN-γ is related to the LLO-producing ability of bacteria but TNF-α expression is not. When mice were injected with the same number (2×10^3) of killed cells of *L. monocytogenes* EGD, the level of cytokine gene expression was the same as in the noninfected controls (data not shown).

Stimulation of macrophage IL-1 production by each strain in vitro and in vivo. To confirm that the ability of *L. monocytogenes* strains to induce IL-1 production is related to LLO-producing ability, we determined IL-1 production by macrophages in vitro, and IL-1α expression in the spleen was examined again by Northern blot analysis. Peritoneal exudate macrophages were stimulated with each strain of bacteria at a multiplicity of infection (MOI) of 50 in the absence of antibiotics for 1 h and then cultured for 3 days in the presence of antibiotics. Supernatants were saved, and IL-1 activity was assayed by the standard thymocyte costimulator assay (23). The IL-1 activity detected in the culture supernatants was almost proportional to the LLO-producing ability of each strain. The Northern blot analysis of IL-1α mRNA expression in the spleens of mice showed a result consistent with the RT-PCR results; IL-1 gene expression was not observed in the spleen of mice infected with *L. monocytogenes* ATCC 15313 or *L. innocua* 10, 2769, and 2740 (Fig. 3).

Generation of protective immunity in mice after infection with each strain. It is known that virulent strains of *L. monocytogenes* can generate T-cell-mediated protective immunity in mice, while avirulent or killed bacteria cannot (1, 11). In order to compare the ability of each strain to generate protective immunity, mice were immunized by intravenous infection with a nonlethal dose. Seven days after the immunization, mice were challenged with the standard strain, *L. monocytogenes* EGD, and the level of protection was assessed by the decrease in the number of viable bacteria in the spleen (Fig. 4). Five strains of *L. monocytogenes* which grew well in vivo (Fig. 1) and induced IL-1 and IFN-γ expression (Fig. 2) were able to generate protective immunity; however, *L. monocytogenes* ATCC 15313 and the three strains of *L. innocua* never induced immunity to eliminate challenge bacteria (Fig. 4).

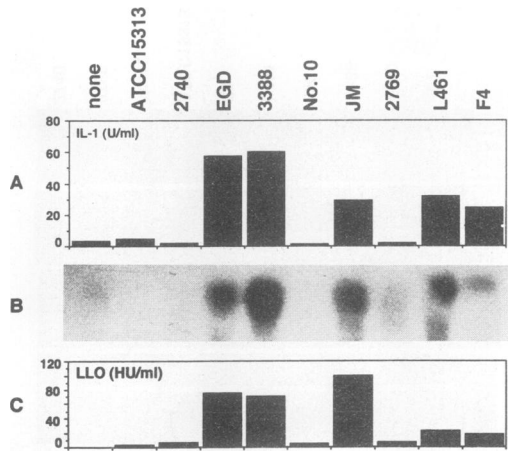

FIG. 3. IL-1-inducing ability of each *Listeria* strain in vitro and in vivo. (A) IL-1 activity, as determined by enzyme immunoassay, in the culture supernatants of peritoneal exudate macrophages after stimulation for 3 days. (B) Northern blot detection of IL-1α mRNA in the spleens of mice 24 h after injection with each strain. (C) The LLO-producing ability in broth culture of each strain shown in Table 1 is shown here for comparison.

DISCUSSION

In the present study, we have examined the expression of IL-1α, TNF-α, and IFN-γ in the spleens of mice infected with various *Listeria* strains. All three cytokines have been shown to be produced at an early stage of infection (10, 25) and involved in the nonspecific resistance of mice to virulent *L. monocytogenes* (16, 31). Among the inflammatory cytokines, IL-6 was not examined because IL-6 appears to contribute very little to resistance (6).

Among the six *L. monocytogenes* strains used in this study, all except ATCC 15313 produced substantial amounts of LLO in broth culture (Table 1). Strain ATCC 15313 had been the type strain of *L. monocytogenes* in *Bergey's Manual of Systematic Bacteriology*, although this strain is not beta-hemolytic (32). When we examined the chromosomal DNA of all the strains by standard PCR, we found that only in this strain of *L. monocytogenes* was there no amplification of the *hlyA* gene, encoding LLO, and the *plcA* gene, encoding phosphatidylinositol-specific phospholipase C, whereas other virulence-associated genes, including *prfA*, *mpl*, and *plcB*, appeared to be intact (26). Biochemical identification in our laboratory confirmed the identification of ATCC 15313 as *L. monocytogenes*, but this strain was avirulent, as judged from both the LD_{50} and growth in the spleen of mice (Table 1, Fig. 1), probably because of the lack of an intact *hlyA* gene.

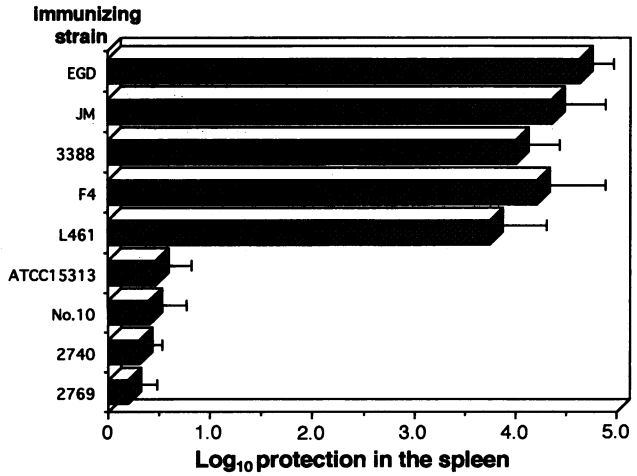

FIG. 4. Ability of *Listeria* strains to generate protective immunity in mice. Mice were immunized with the same number of viable bacteria of each strain and challenged 7 days later with 10^4 viable cells of *L. monocytogenes* EGD. The number of bacteria in the spleen was counted 2 days after the challenge. The mean $\log_{10}$ difference in the number of bacteria between each group and the nonimmunized control is shown. Each group consisted of four or five mice.

It was interesting that the ability of the *Listeria* strains to induce in vivo expression of IL-1α and IFN-γ was closely related to their LLO-producing ability (Fig. 2 and 3). The virulence of the bacterial strains as judged from the LD_{50} and in vivo growth was not always proportional to the LLO titer produced in broth culture; however, only the strains capable of producing LLO were able to induce a substantial level of mRNA expression in the infected spleens. This may suggest that LLO-producing ability is a prerequisite for the induction of some cytokines in the host after infection. The present findings of IL-1 expression in vivo is consistent with the results of our previous in vitro study, which showed that avirulent strains or killed cells of virulent strains did not stimulate the production of massive amounts of IL-1 from peritoneal macrophages (23). This fact was reconfirmed in the present study with nine *Listeria* strains (Fig. 3). LLO-producing ability also seemed to be critically required for IFN-γ expression, since no significant IFN-γ mRNA expression was observed in the spleens of mice after infection with non-LLO-producing *L. monocytogenes* ATCC 15313 and three strains of *L. innocua*. As we have recently reported, purified LLO itself was responsible for IL-1 production from macrophages (35, 38). Whether purified LLO induces the expression of IFN-γ in spleen cells should be examined.

In contrast to IL-1 and IFN-γ expression, TNF-α was expressed in virtually all the infected spleens irrespective of the virulence or LLO-producing ability of the infecting strain. TNF is produced upon stimulation with various agents; therefore, TNF mRNA expression may be the result of stimulation by the bacterial cell wall or other components. Administration of 2×10^3 cells of killed bacteria (strain EGD) never resulted in the expression of TNF to the levels observed in the spleens of mice given the same number of viable ATCC 15313 or *L. innocua*. Even after injection of an avirulent strain, small numbers of viable bacteria were recovered from the spleen; therefore, avirulent strains are not exactly the same as killed cells in this respect.

It is known that IFN-γ upregulates the synthesis of TNF-α in vitro (2, 14). However, the fact that TNF expression was observed even in mice injected with avirulent strains lacking IFN-γ-inducing ability suggested that IFN-γ alone is not responsible for the expression of TNF in vivo. Recently, Kuhn and Goebel reported the different ability of various *Listeria* strains to induce cytokines in the P388D1 macrophage cell line (15). Our result is consistent with their result in that most of the inflammatory cytokines were induced only by LLO-producing strains. They also found that non-LLO-producing mutants of *L. monocytogenes* did not induce TNF-α expression in P388D1 cells, which is different from the result obtained in our in vivo experiments. In the in vitro experiment with established cells of the macrophage lineage, there is no possible contribution from other types of cells, including T cells, B cells, natural killer cells, and neutrophils, which have been reported to be

capable of producing TNF-α (37), whereas all these cells are present in the in vivo condition. The discrepancy in TNF induction might be explained by such a difference.

The strains which lack LLO-producing ability were not capable of inducing protective immunity after infection (Fig. 4), while strong protection against rechallenge was generated in mice infected with strains which induced IL-1 and IFN-γ. Although the precise mechanism(s) for the inability of avirulent strains or killed bacteria to induce protective immunity is not clear, it may be argued that cytokines expressed at the early stage of infection play a critical role in the induction of protective T cells. In our previous study, it was postulated that IL-1 was the most important factor in the generation of protective immunity to *L. monocytogenes*; however, administration of recombinant IL-1 failed to induce protective immunity in mice immunized with killed bacteria, although recombinant IL-1 promoted the antigen-specific differentiation of T cells (11). The fact that protective immunity was generated only in mice expressing IFN-γ suggests that the early expression and production of IFN-γ or the simultaneous expression of IL-1 and IFN-γ is a distinct factor required for the generation of protective T cells.

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