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Difference in the Induction of Macrophage Interleukin-1 Production between Viable and Killed Cells of *Listeria monocytogenes*

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T-cell-mediated immunity to *Listeria monocytogenes* in mice, as determined by delayed-type hypersensitivity and acquired resistance, was induced by immunization with viable bacteria but not with killed bacteria, even when killed cells were injected in a high dose or repeatedly. T cells obtained from mice immunized with viable *L. monocytogenes* were readily stimulated with killed-bacterial antigens, resulting in T-cell proliferation in vitro and expression of a delayed footpad reaction in vivo. After immunization with killed-bacterial vaccine, T-cell responsiveness to interleukin 2 (IL-2) never developed but a lower level of responsiveness to IL-1 appeared later than with T cells from mice immunized with viable bacteria. When IL-1 production by macrophages was examined in vitro, viable *L. monocytogenes* stimulated a high level of IL-1 release while killed bacteria did not. Avirulent strains which were ineffective in the induction of T-cell mediated immunity were incapable of inducing IL-1 production as well. The impaired ability of killed bacteria to stimulate IL-1 production was confirmed by the level of IL-1 mRNA expression. These results suggested that the ineffectiveness of killed *L. monocytogenes* vaccine is not due to loss or lack of antigenic epitopes but may be ascribed to insufficient induction of IL-1 production in the initial stage of the immune response in vivo.

Protective immunity to facultative intracellular bacteria is T cell mediated and not dependent on a specific antibody (2, 11, 19, 32). Antigen-specific induction of acquired cellular resistance can be achieved by immunization of animals with a sublethal dose of viable bacteria, including *Mycobacterium tuberculosis* (27, 37, 39), *M. bovis* BCG (3, 40), *Salmonella typhimurium* (4, 24), *Brucella melitensis* (41), *B. abortus* (13), *Legionella pneumophila* (5), etc. In contrast to the effectiveness of immunization with viable bacteria, killed cells of these kinds of bacteria seem to be incapable of inducing acquired cellular resistance.

Listeria monocytogenes is one of the facultative intracellular bacteria with which the mechanisms of T-cell-mediated protective immunity have been most widely studied. It seems to be a generally accepted idea that protective immunity to *L. monocytogenes* cannot be induced by immunization with killed-bacterial vaccine. There were some reports showing induction of acquired resistance after immunization with killed *L. monocytogenes* by addition of some kind of immunosuppressive agent (45) or in a particular strain of mice (46); however, this observation was attributed to possibly contaminating viable bacteria in the vaccine preparation (48). There is no convincing experimental data to show why killed-bacterial vaccine is ineffective in the induction of T-cell-mediated immunity, although a specific antibody response may be readily obtained by vaccination with a variety of nonliving bacterial antigens.

The ineffectiveness of killed-bacterial vaccine may be simply attributable to loss of T-cell-stimulating antigens during preparation of killed cells or to lack of expression of de novo-synthesized highly immunogenic epitopes in vivo. These possibilities appear to be unlikely, since killed bacteria or culture supernatants are usually good antigens for stimulation of T cells from actively immunized mice, resulting in the release of various lymphokines relevant to the expression of cell-mediated immune protection. Another explanation may be an insufficient antigen dose or lack of persistence of the antigen in vivo, because killed bacteria do not multiply in a host and may be rapidly eliminated. However, there has been no report showing that T-cell-mediated immunity was generated by simply increasing the number of killed bacteria or by repeated injections. Induction of a suppressor mechanism is an alternative explanation. Heat-killed cells of BCG or *L. monocytogenes* are reported to induce nonspecific suppressor T cells (16, 49). However, induction of suppressor cells required emulsifying bacteria (water-oil-water form) and was highly dependent on the amount of killed bacteria to be injected. None of the available data seem to give a convincing explanation for the immunological basis underlying the ineffectiveness of killed-bacterial vaccine.

In the present study, we compared the abilities of viable and killed cells of *L. monocytogenes* to induce macrophage interleukin 1 (IL-1) production, which is critical in the initiation of a immune response in vivo.

MATERIALS AND METHODS

Bacterial strains. *L. monocytogenes* EGD, originally obtained from G. B. Mackaness (Trudeau Institute, Saranac Lake, N.Y.), was maintained in our laboratory and used as the standard strain throughout this study. Other strains used were ATCC 15313 and 2769, which was provided by B. S. Ralovich (Institute of Public Health and Epidemiology, Pécs, Hungary). Strain EGD is a virulent strain with a 50% lethal dose of 2×10^5 CFU, while the others are avirulent strains with 50% lethal doses of over 10^8 CFU. Bacteria were grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.), washed repeatedly with phosphate-buffered saline, and then adjusted to the desired concentration with a colorimeter. The exact bacterial number was determined by

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plating the diluted bacterial suspension on tryptic soy agar plates and counting the colonies after cultivation for 24 h.

Preparation of killed and disrupted bacteria. Killed cells of *L. monocytogenes* were prepared by heating a viable bacterial suspension with a known concentration for 120 min at 74°C (33). Disruption of viable bacteria was performed with a Dynomill. Washed viable cells of strain EGD suspended in phosphate-buffered saline were mixed with 2 volumes of glass beads and treated for 5 min in a Dynomill. After disruption, the material was centrifuged at 4,500 × *g* for 15 min to remove nondisrupted cells, and the supernatant was used as disrupted bacteria.

Mice. Male C3H/He mice raised and maintained in a specific-pathogen-free condition were used at ages of 7 to 11 weeks. C3H/HeJ mice were used as sources of thymocytes for IL-1 bioassays.

Induction of IL-1 production. Peritoneal exudate cells (PEC) were harvested 3 days after intraperitoneal injection of 10% Proteose Peptone (Difco). Cells were washed and suspended in RPMI 1640 medium supplemented with 10% NU-SERUM (Collaborative Research, Inc., Waltham, Mass.), 10 U of penicillin G per ml, and 10 μg of streptomycin per ml. After 90 min of incubation in a 5% CO_2 atmosphere on a six-well plate (Falcon 3046; Becton Dickinson Labware, Oxnard, Calif.), nonadherent cells were washed away by repeated pipetting in warm Hanks balanced salt solution. Over 95% of the adherent cells thus prepared were revealed to be macrophages by ingestion of latex particles (Dow Chemical Co., Midland, Mich.). *L. monocytogenes* cells (10^9) or an equivalent amount of disrupted bacteria were added to 2×10^7 macrophages adhering to each well in RPMI 1640 medium without antibiotics. The plate was incubated for 45 min to allow the macrophages to phagocytose the bacteria, and then penicillin G and streptomycin were added to the wells. Culture supernatants were obtained 48 h after stimulation, dialyzed against phosphate-buffered saline and then RPMI 1640 medium, and stored at −20°C until being bioassayed for IL-1.

Determination of IL-1 activity. IL-1 activity was determined as the ability to enhance the proliferation of C3H/HeJ thymocytes in a suboptimal concentration of phytohemagglutin P. The medium in this assay consisted of RPMI 1640 supplemented with 10% NU-SERUM, penicillin G (100 U/ml), streptomycin (100 μg/ml), and 5×10^{-5} M 2-mercaptoethanol. Thymocytes (5×10^6) were cultured for 3 days in 5 μg of phytohemagglutin per ml of serially diluted supernatant. Activity was expressed as counts per minute of [^{3}H]thymidine incorporated for the final 18 h of culture. Membrane-associated IL-1 activity was measured as described by Kurt-Jones et al. (25). Briefly, macrophages stimulated with *L. monocytogenes* cells were fixed with 1% paraformaldehyde for 15 min at room temperature, washed extensively, incubated for 24 h, and subjected to a thymocyte proliferation assay.

Macrophage RNA extraction and detection of IL-1 transcript. Three hours after stimulation of adherent macrophages with viable or killed *L. monocytogenes*, cells were collected and RNA was extracted by the guanidinium thiocyanate and CsCl gradient centrifugation procedure (7). Five micrograms of total RNA was glyoxylated and electrophoresed through 1% agarose in 10 mM phosphate buffer (pH 7.0) and then transferred to a nylon membrane (Hybond-N; Amersham Ltd., Amersham, United Kingdom). The filter was hybridized with a ^{32}P-labeled, nick-translated IL-1 cDNA probe from pIL-1 1301 (29) for 16 h at 65°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0)–5× Denhardt solution (1× Denhardt solution is 0.02% polyvinylpyrrolidone plus 0.02% bovine serum albumin plus 0.02% Ficoll)–10% dextran sulfate–1% sodium dodecyl sulfate–100 μg of herring sperm DNA per ml and washed at 65°C twice in 2× SSC–0.1% sodium dodecyl sulfate and once in 1× SSC–0.1% sodium dodecyl sulfate. Ethidium bromide staining of rRNA served as a molecular size marker. The filter membrane was exposed to Kodak X-OMAT film at −85°C, and the autoradiogram was developed.

Immunization of mice and assessment of DTH and protection. Groups of mice were intravenously immunized with either 2×10^3 viable *L. monocytogenes* EGD cells or 1×10^3 heat-killed bacteria and used for experiments 6 to 7 days later. Delayed-type hypersensitivity (DTH) was assessed as a delayed footpad reaction 24 h after elicitation with 5×10^7 killed bacteria. Footpad swelling was measured by a dial thickness gauge caliper and expressed in 0.1-mm units as previously described (47). Protective immunity was determined by counting the number of bacteria in spleens 2 days after intravenous challenge with 10^4 *L. monocytogenes* bacteria.

Functional maturation of T cells in immune mice. Spleen T cells were obtained by passage of a single-cell suspension twice over a nylon wool column. T cells were examined for responsiveness to recombinant human IL-1 (rIL-1; Genzyme, Boston, Mass.) or rIL-2 (TGP-3), which was provided by the Central Research Division, Takeda Chemical Industries, Ltd., Osaka, Japan. Responsiveness to IL-1 was assessed as the ability to produce IL-2 in culture after stimulation with an appropriate dose of IL-1. IL-2 activity was determined by using an IL-2-dependent cell line, HT-2. T-cell responsiveness to IL-2 was determined by culturing cells for 3 days in 1 U of rIL-2 per ml and counting the [^{3}H]thymidine radioactivity incorporated during the final 18 h.

Proliferation of immune T cells after stimulation with killed-bacterial antigens. Mice were immunized with viable *L. monocytogenes* EGD, and 10% Proteose Peptone was injected intraperitoneally 6 days after immunization. PEC were harvested 3 days later, and peritoneal exudate T-lymphocyte-enriched cells (PETLEs) were obtained by using a nylon wool column. Killed bacteria (10^7) were added to 2×10^5 PETLEs in a 96-well flat-bottom plate, and the proliferative response was determined with or without 5% normal adherent PEC as antigen-presenting cells (APC).

RESULTS

Ineffectiveness of killed-bacterial vaccine. Mice were immunized with viable or killed *L. monocytogenes* and examined for development of DTH and acquired resistance. Seven days after immunization with viable bacteria, mice showed a high level of DTH and enhanced elimination of challenge bacteria from the spleen. In mice immunized with killed bacteria, neither DTH nor acquired resistance could be observed, even after vaccination with a high dose or after repeated immunization, which were used to make the amount of antigen in vivo comparable to that in viable-bacterium-immune mice (Table 1). A cell-mediated immune response could not be detected, even after 14 days, in the killed-bacterium-immune group (data not shown). Killed-bacterial vaccine was thus ineffective in the induction of DTH or acquired resistance.

T-cell maturation in immune mice. Appearance of T-cell responsiveness to IL-2 is one way to measure an active immune response. When spleen T cells from viable-bacteri-

TABLE 1. DTH and acquired resistance in mice immunized with killed or viable *L. monocytogenes* EGD

Immunization (i.v.) of mice (no. of bacteria)[a]	Mean ± SD DTH U	Mean ± SD $\log_{10}$ CFU/ spleen	$\log_{10}$ acquired resistance[b]
None	2.4 ± 0.8	6.42 ± 0.14	±0
Viable bacteria (2×10^3)	14.6 ± 3.1[c]	2.25 ± 0.32[d]	+4.17
Killed bacteria (1×10^8)	3.4 ± 1.1	6.37 ± 0.10	+0.05
Killed bacteria (1×10^7)[d]	2.8 ± 1.0	6.52 ± 0.18	−0.10

[a] Each group consisted of six mice.
[b] $\log_{10}$ difference from the nonimmune control group.
[c] Significantly different ($P < 0.001$) from the nonimmune control group.
[d] This group of mice was immunized with 10^7 killed bacteria on days 0, 2, and 4.

um-immune mice were stimulated with rIL-2, a high level of responsiveness became detectable 6 days after immunization; in contrast, T-cell responsiveness to IL-2 never developed in mice immunized with killed bacteria (Fig. 1).

As another parameter of T-cell stimulation after immunization, we examined T-cell responsiveness to rIL-1, which may be a result of expression of IL-1 receptors on T cells. IL-2 release from T cells in response to rIL-1 was measured, since there was no method available to directly detect the expression of IL-1 receptor on T cells. T cells from viable-bacterium-immune mice became highly responsive to rIL-1 as early as day 3 after immunization, and responsiveness declined on day 6 (Fig. 2). On day 3, there was no response in T cells from killed-bacterium-immune mice. A marginal but significant level of responsiveness was detected on day 6 in this group. These results suggested that in mice immunized with killed bacteria, the immune response was arrested at the initial stage of its development.

Ability of several *L. monocytogenes* strains to elicit immune T cells. PETLEs were obtained from mice immunized with viable *L. monocytogenes* EGD. They were stimulated with killed-bacterial antigens of several strains in vitro in an appropriate concentration of normal macrophages as APC. All antigens were equally effective in eliciting a proliferative response in T cells from EGD-immune mice (Table 2). ATCC 15313 and strain 2769 were both avirulent and ineffective in the induction of DTH or acquired resistance in mice, even after vaccination with viable bacteria (data not shown). In this regard, these two bacterial strains resembled killed cells of strain EGD. These antigens were also tested for the ability to elicit DTH in mice immunized with viable strain EGD. Very high levels of DTH were observed after 24 h, and there was no significant difference between the

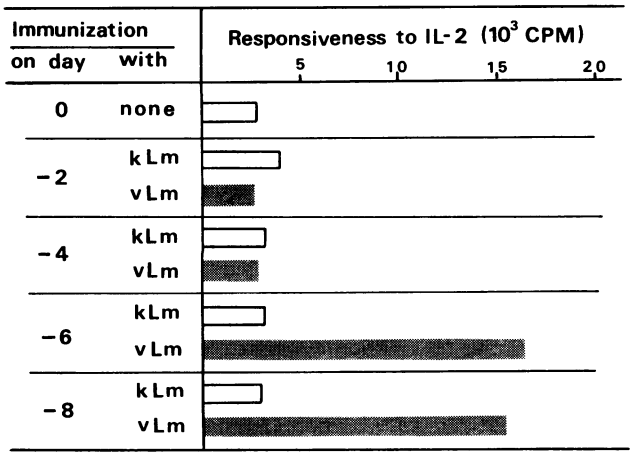

FIG. 1. Kinetic development of T-cell responsiveness to IL-2 in mice after intravenous immunization with 10^8 killed (kLm) or 2×10^3 viable *L. monocytogenes* (vLm) bacteria. Nylon wool-passed spleen T cells from mice immunized at various times before were stimulated with rIL-2, and the proliferative responses were expressed in counts per minute of [^{3}H]thymidine incorporated. Mean counts per minute, of quadruplicate wells are shown; the standard deviation was less than 15% of the mean in each case.

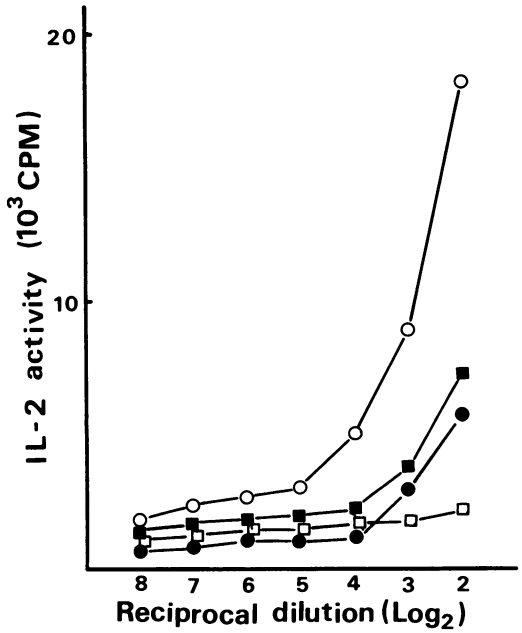

FIG. 2. Development of T-cell responsiveness to rIL-1 in mice after intravenous immunization with 10^8 killed or 2×10^3 viable *L. monocytogenes* bacteria. Response to rIL-1 was determined as the ability to release IL-2 in culture. Nylon wool-passed spleen T cells were obtained 3 (□) or 6 (■) days after immunization with killed bacteria and 3 (○) or 6 (●) days after immunization with viable bacteria. Culture supernatants of T cells stimulated with IL-1 were diluted twofold; and IL-2 activity in each dilution was measured by the proliferation of HT-2 cells. Mean counts per minute of quadruplicate wells are shown; the standard deviation was less than 13% of the mean in each case.

TABLE 2. Ability of killed cells of several *L. monocytogenes* strains to stimulate T-cell proliferation in vitro and to elicit DTH in vivo

Strain used for killed-bacterial antigen	Mean ± SD proliferation of PETLEs (cpm) with[a]:		Mean ± SD DTH U in mice[b]
	No APC	5% APC	
EGD	1,020 ± 260	21,770 ± 2,292	12.4 ± 1.0
2769	1,307 ± 371	21,292 ± 2,338	10.4 ± 1.9
ATCC 15313	1,228 ± 148	17,186 ± 2,602	11.9 ± 2.4

[a] PETLEs were prepared from PEC 3 days after intraperitoneal injection of 10% Proteose-Peptone into mice immunized previously with viable *L. monocytogenes*. Cells were stimulated with killed bacteria for 3 days with or without APC.
[b] DTH was elicited with 5×10^7 killed bacteria in mice immunized with viable *L. monocytogenes* 6 days before, and footpad reactions were measured 24 h later.

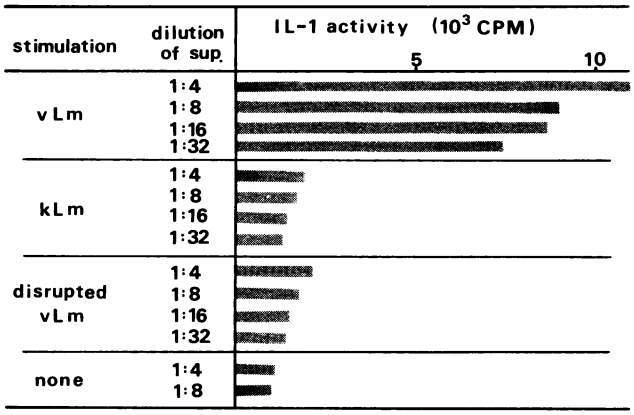

FIG. 3. IL-1 activity in macrophage culture supernatant (sup.) after stimulation with viable (vLm) or killed (kLm) *L. monocytogenes* EGD. C3H/HeJ thymocytes were cultured in a suboptimal concentration of phytohemagglutinin, and final culture supernatant dilutions are indicated. Mean counts per minute of quadruplicate wells are shown; the standard deviation was less than 16% of the mean in each case.

degrees of footpad swelling elicited with the antigens (Table 2). This showed that killed bacteria incapable of inducing active cell-mediated immunity in vivo still possessed antigenicity capable of stimulating immune T cells both in vitro and in vivo.

Macrophage IL-1 production after stimulation with viable or killed cells of *L. monocytogenes*. Macrophage IL-1 production is an indispensable event for generation of cell-mediated immunity, in addition to appropriate handling and presentation of an antigen(s) to reactive T-cell clones. We compared the amounts of IL-1-like activity released from macrophages after stimulation with viable or killed *L. monocytogenes*. When macrophages were stimulated in vitro with viable bacteria, a very high level of IL-1-like activity was detected in culture, as determined by a conventional costimulator assay for thymocyte proliferation (Fig. 3). The culture supernatant rich in the activity did not contain IL-2, as determined by proliferation of HT-2 cells (data not shown). The activity found even in diluted culture supernatant was further confirmed to be IL-1 by its ability to induce IL-2 release from the IL-1-dependent EL-4 subline or by restricted elution of activity on Sephacryl S-200 gel filtration to fractions with molecular weights ranging from 15,000 to 20,000 (data not shown). Compared with IL-1 activity after stimulation with viable bacteria, IL-1 activity in the culture stimulated with the same number of killed cells was significantly lower. To know whether this impaired ability of killed bacteria to induce macrophage IL-1 production was simply caused by heating during the preparation of killed cells, viable bacteria were mechanically disrupted with a Dynomill. In this preparation, it was not possible to cause any loss or modification of antigens which may take place in killing of bacteria by heat or Formalin treatment. When macrophages were stimulated with disrupted cells in a dose equivalent to that of the whole cells used as described above, the amount of IL-1 was very low and almost identical to that found after stimulation with whole cells of killed bacteria (Fig. 3).

Induction of macrophage IL-1 production by viable cells of avirulent strains of *L. monocytogenes*. To determine whether viability of bacteria is essential for macrophage production of a large amount of IL-1, two avirulent strains of bacteria were used for stimulation. Viable cells of *L. monocytogenes* EGD were effective in the induction of macrophage IL-1 production, but killed cells were not. It was interesting that the levels of IL-1 produced after stimulation with viable cells of ATCC 15313 or strain 2769 were far lower than that caused by stimulation with viable cells of strain EGD (Table 3).

TABLE 3. IL-1 activity released in macrophage cultures after stimulation with various strains of killed or viable *L. monocytogenes*

Strain used for stimulation	Mean ± SD IL-1 activity (cpm)[a]
None	1,442 ± 206
Viable EGD	13,580 ± 1,380[b]
Killed EGD	2,288 ± 464[c]
Viable 2769	3,349 ± 268[b]
Viable ATCC 15313	2,980 ± 183[b]

[a] IL-1 activity was determined by thymocyte costimulator assay at a 1/4 dilution of culture supernatant of macrophages stimulated with each type of *L. monocytogenes* cells.
[b] Significantly different ($P < 0.001$) from the control.
[c] Significantly different ($P < 0.05$) from the control.

Membrane-associated IL-1 activity. Membrane-associated IL-1 is believed to be important in the initiation of an immune response (14). The membrane-associated form of IL-1 was determined by using the paraformaldehyde fixation method. Peritoneal exudate macrophages were stimulated in vitro in the manner used to assay soluble IL-1 and fixed 24 h later. Activity measured by thymocyte proliferation was very low, but there was a significant difference between stimulation with viable bacteria and stimulation with killed bacteria (Table 4). Alternatively, viable or killed cells of *L. monocytogenes* EGD were injected into the peritoneal cavities of mice which had received 10% Proteose Peptone 3 days before. PEC were harvested 15 min after in vivo stimulation with bacterial cells, cultured in vitro to obtain adherent cells, and then fixed. A significant level of membrane-associated IL-1 was detected only in macrophages stimulated with viable bacteria (Table 4).

Expression of IL-1 mRNA in macrophages. To further confirm the difference in IL-1-inducing ability between killed and viable bacteria, expression of the IL-1 message was examined. PEC were obtained from over 100 mice, and adherent cells were stimulated with viable or killed *L. monocytogenes* or left without stimulation. RNAs were extracted from each group of adherent macrophages, and a constant amount was run on agarose gel and transferred to a nylon membrane. Northern (RNA) hybridization was per-

TABLE 4. Membrane-associated IL-1 activity in macrophages stimulated with killed or viable *L. monocytogenes* EGD

Stimulation	Mean ± SD IL-1 activity (cpm)	
	In vitro stimulation[a]	In vivo stimulation[b]
None	688 ± 116	704 ± 162
Killed bacteria	966 ± 201	1,006 ± 249
Viable bacteria	2,268 ± 482[c]	3,493 ± 571[d]

[a] Bacterial cells were intraperitoneally injected into mice which had been given 10% Proteose Peptone 3 days before, and then PEC were harvested, washed, and fixed with paraformaldehyde for membrane-associated IL-1 assay.
[b] PEC were harvested from mice given Proteose Peptone 3 days before. Bacterial cells were added to adherent PEC in vitro, washed, and fixed with paraformaldehyde.
[c] Significantly different ($P < 0.01$) from the control.
[d] Significantly different ($P < 0.005$) from the control.

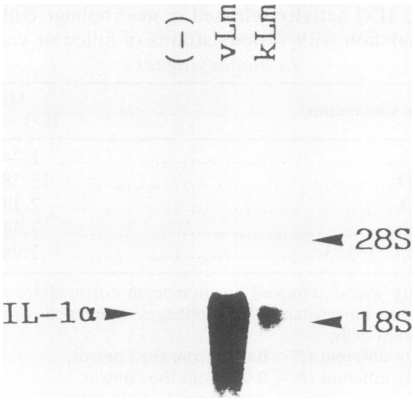

FIG. 4. Northern blot analysis of IL-1-specific transcript in macrophages stimulated in vitro with killed (kLm) or viable (vLm) bacteria or not stimulated (−). Ribosomal 18S and 28S RNAs are marked.

formed with a cDNA probe specific for IL-1. A strongly positive signal for the IL-1-specific transcript was visualized on the autoradiogram only in the lane containing a sample from viable-bacterium-stimulated macrophages (Fig. 4).

DISCUSSION

Since Mackaness established an experimental model over 2 decades ago (31), *L. monocytogenes* has been extensively used to study T cells involved in protective immunity to facultative intracellular bacteria. By means of T cells obtained from mice immunized with viable *L. monocytogenes*, the role of class II antigen in macrophage–T-cell interactions was clearly elucidated (9, 44). Immune T cells or cloned T cells prepared from lymphocytes of infected animals have been shown to be capable of transferring DTH (20, 33), granuloma formation (36), and specific protection (17, 23, 47) and also to be capable of producing gamma interferon (21), migration inhibition factor (43), macrophage chemotactic factor (12), or IL-2 (22). However, there has been no report of the generation of such *Listeria*-specific T cells by immunization with killed bacteria.

In the present study, the ineffectiveness of killed-bacterial vaccine could not be overcome by increased antigen doses or repeated injections. Immunization with killed bacteria never generated DTH, acquired resistance, or T-cell responsiveness to rIL-2, although killed bacteria were an effective antigen for secondary stimulation for T-cell proliferation in vitro and for elicitation of DTH in vivo. A similar result was obtained by using two different avirulent strains of *L. monocytogenes* incapable of inducing a primary cell-mediated immune response. It was suggested that lack or loss of antigenic epitopes did not account for the ineffective induction of an immune response in mice.

Our present result was in part consistent with that reported by Kaufmann (18) with respect to the presence of antigenic epitopes recognizable by protective T cells obtained from mice immunized with *L. monocytogenes* EGD on bacterial cells incapable of inducing a strong immune response. In that report, the difficulty of inducing a strong T-cell response by several strains was attributed to the lack of persistence of viable bacteria in immunized mice. If that is true, the T-cell-mediated immunity may be generated, at least to some extent, by repeated administration of killed-bacterial vaccine; however, it was not possible in the present study. In mice immunized with a high dose of killed-bacterial vaccine, a low level of T-cell responsiveness to IL-1 was observed later. This finding raised the possibility that antigenic epitopes exist on killed bacterial cells but development of a complete immune response was arrested at the initial stage. IL-1 was one of the possible candidates which operate in the initial stage of the immune response. A significantly high level of IL-1 production was induced when macrophages were stimulated in vitro with viable *L. monocytogenes*. The ability to stimulate IL-1 production was markedly impaired after killing of bacteria by heat or mechanical disruption. Even in viable form, avirulent strains were incapable of inducing high levels of IL-1. This difference was further confirmed at the level of mRNA prepared from stimulated macrophages.

IL-1 is known to be produced by and act on various types of cells (38), but its role in T-cell activation after production by macrophages is of major importance (35). IL-1 upregulates the expression of IL-1 receptor on T cells (42), induces IL-2 receptor expression (30), and stimulates IL-2 synthesis by T cells (34), resulting in T-cell proliferation. Ineffective induction of a T-cell response after immunization with killed bacteria or an avirulent strain of viable bacteria (18) seems to be accounted for by insufficient IL-1 production in vivo.

Our results showing ineffective IL-1 induction by killed *L. monocytogenes* contradict the reported results of induction of membrane IL-1 by heat-killed listeriae (25, 26). The activity of membrane-associated IL-1 on killed listeria-pulsed macrophages shown in those reports was significantly higher than that of unpulsed controls; however, the actual count of thymocyte proliferation was at most, twofold higher (25). Besides, a helper T-cell clone, D10.G4, used to assay cells for IL-1 activity in many experiments may be more sensitive to a small amount of IL-1. Our results do not deny the production of a minute amount of IL-1 after macrophage stimulation with killed bacteria. Therefore, we should stress that the implication of the present study is not that killed *L. monocytogenes* cells completely lack IL-1-inducing activity but that the large amount of IL-1 produced only by viable virulent bacteria may contribute to induction of T-cell-mediated immunity in vivo.

It is not clear what is responsible for the stimulation of high-level IL-1 production. Macrophage production of IL-1 in both soluble and membrane-associated forms is triggered by various agents, including bacterial lipopolysaccharide, silica particles, zymosan, and phorbol myristate acetate (8, 28), but it is unlikely that any of these is involved in the stimulation of IL-1 production and induction of T-cell-mediated immunity by *L. monocytogenes*. *L. monocytogenes* hemolysin (listeriolysin) has attracted considerable attention as a major virulence factor (6, 10, 15). Berche et al. showed that only viable cells of a hemolytic strain of *L. monocytogenes* induced T-cell-mediated immunity as determined by DTH or protection against challenge infection. They suggested that besides the major importance of adequate antigen processing, powerful mechanisms that amplify T-cell expansion in vivo may exist (1). The two avirulent strains used in the present study did not produce hemolysin (data not shown), while strain EGD was hemolytic. In our preliminary study, purified hemolysin prepared from the culture supernatant of strain EGD did induce macrophage IL-1 production in vitro. Killed cells of strain EGD are apparently incapable of producing hemolysin. It is plausible that macrophage IL-1 production is stimulated mainly by hemolysin, which is produced only by viable bacteria of virulent strains.

The present study raised the possibility that promotion of IL-1 production in vivo or administration of IL-1 in vivo, along with immunization with killed bacterial vaccine, may induce active T-cell-mediated immunity. A study along this line is under way.

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