

INDUCTION OF A CELLULAR IMMUNE RESPONSE TO A FOREIGN ANTIGEN BY A RECOMBINANT *Listeria monocytogenes* VACCINE¹

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A recombinant strain of *Listeria monocytogenes* that stably and constitutively expresses *Escherichia coli* β -galactosidase was used as a live vaccine vector. BALB/c mice were immunized orally or parenterally with the recombinant *L. monocytogenes*, and their cellular and humoral immune responses to β -galactosidase were measured. Spleen cells taken 1 week after oral inoculation or 5 weeks after oral or parenteral inoculation (with a boost at 4 weeks) showed β -galactosidase-specific CTL responses. The CTL line derived from mice immunized i.p. was also shown to be class I restricted and Thy-1.2⁺, CD8⁺, and TCR $\alpha\beta$ ⁺. All mice immunized with the recombinant *L. monocytogenes* had positive delayed-type hypersensitivity responses to heat-killed *L. monocytogenes*, but only 15% had a positive delayed-type hypersensitivity reaction to β -galactosidase. Individual serum samples from mice immunized i.p. or i.v. were tested for antibody to β -galactosidase. Approximately 11% had low positive titers for β -galactosidase antibodies. These results demonstrate that both oral and parenteral immunization with recombinant *L. monocytogenes* results in a cellular immune response to the foreign protein, which is primarily a specific CD8⁺ CTL response.

In recent years there has been a concerted effort to develop live recombinant vaccine vectors, both bacterial and viral, that could be used to induce immunity to infectious disease agents. It is clearly an essential requirement in the development of such vectors that they be capable of targeting the induction of cellular immune responses in general and the induction of class I-restricted T cells in particular. Indeed, class I-restricted CTL are crucial in eliminating many viral and bacterial infections (1). In addition, strong humoral responses by the host appear to be ineffective in controlling many of the serious diseases for which there are currently no effective vaccines, e.g., malaria and AIDS. Thus, central to the efficacy of a recombinant bacterial or viral vector

vaccine is its ability to target those pathways by which class I-restricted cytotoxic T cells are invoked.

Recent advances in the cell biology of Ag processing and presentation have shown that there are distinct differences in the cellular compartments in which Ag degradation and association with the class I or class II MHC molecules occur (2). These differences have important consequences for cytotoxic vs helper T cell recognition of protein Ag. The generation of the antigenic peptide for class II-restricted T cell recognition of Ag takes place in a membrane-bound endosomal compartment. Thus, the T cell response to priming by soluble protein Ag almost always results in class II-restricted T cell recognition. For class I-restricted recognition, antigenic peptides are derived from intracellular processing of newly synthesized proteins within the cell cytoplasm (3, 4). The sources of these newly synthesized proteins are usually infectious agents that have colonized the cell. It is possible, however, to raise a class I-restricted cytotoxic T cell response to soluble protein Ag by introducing the Ag into the cytosolic compartment of the cell by artificial means, such as osmotic lysis of pinosomes (3, 5). Viral or bacterial vectors that target a foreign protein to the cytosolic compartment should also result in Ag processing and presentation by class I MHC.

Recombinant *Salmonella* and BCG³ vaccines have already been described that elicit humoral (6-13) and cellular (8, 12-15) immune responses to viral (8, 9, 13, 15), bacterial (6, 7, 10-13, 16), and parasitic proteins (14). *Listeria monocytogenes* is an attractive alternative candidate for a recombinant vaccine vector. *L. monocytogenes* has been clearly shown to live in the cytoplasm of the host cell (17) and to elicit strong CD8⁺ T cell responses (18-20). It is a ubiquitous, Gram-positive, facultative intracellular bacteria that has been used for decades to study cell-mediated immune responses (21, 22). Studies on the cell biology of *L. monocytogenes* have shown that it enters the host cell and is taken up in a phagosome. However, unlike many intracellular bacteria such as *Salmonella* (23) and BCG (24, 25), *L. monocytogenes* escapes from the phagosome into the cytoplasm of the cell by disrupting the phagosomal membrane, primarily through the action of hemolysin (17). These studies also demonstrated that, after the initial infection of the host, *L. monocytogenes* grows and spreads from cell to cell primarily in the host cytoplasm.

It has also been well established that live *L. monocytogenes* infection elicits a strong protective cellular immune response, with the induction of both CD4⁺ and

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³ Abbreviations used in this paper: BCG, *Mycobacterium bovis* bacillus Calmette-Guérin; DTH, delayed-type hypersensitivity; HKLM, heat-killed *Listeria monocytogenes*.

CD8⁺ T cells (18–20, 26–34). CD4⁺ T cells may act primarily through the secretion of lymphokines (30, 32). CD8⁺ T cells are important in the transfer of resistance (19, 20, 31, 33) and the *in vivo* elimination of bacteria (29, 34). The CD8⁺ T cells may act through both cytolysis of *L. monocytogenes*-infected targets (18, 20) and the secretion of lymphokines (18). A recent study suggests that the CD4⁺ T cells can respond to both live *L. monocytogenes* and HKLM that enters the exogenous pathway of Ag presentation. In contrast, it was shown with non-hemolytic mutants that the generation of CD8⁺ T cells, which are necessary for complete immunity to *L. monocytogenes*, is dependent on the intracellular growth of *L. monocytogenes* in the cytoplasm (35).

The studies presented here were designed to determine whether *L. monocytogenes* is effective as a live recombinant vaccine vector to present a foreign Ag to a host and elicit immune responses to that Ag. The unique ability of *L. monocytogenes* to escape phagosomal restriction and to live in the cytoplasm suggests that this bacteria may be particularly effective as a vector for targeting the class I-restricted pathway of Ag processing for cytotoxic T cell recognition. We have used a recombinant strain of *L. monocytogenes*, which expresses *Escherichia coli* β -galactosidase, and measured humoral and cellular immune responses to β -galactosidase after oral and parenteral administration of the bacteria. We show that immunization by either route results in class I-restricted cytotoxic T cell responses, although humoral responses to the foreign protein are only weakly engendered. Thus, *L. monocytogenes* has potential as a vaccine vector for cell-mediated immune responses.

MATERIALS AND METHODS

Bacteria. *L. monocytogenes* strain 10403S belongs to serotype 1 and has an LD₅₀ of 3×10^4 in BALB/c mice. Strain DP-L967 contains a derivative of transposon Tn917-lac and constitutively expresses *E. coli* β -galactosidase (36). DP-L967 also has an LD₅₀ of 3×10^4 in BALB/c mice. Bacteria were grown on brain heart infusion agar and broth (Difco Laboratories, Inc., Detroit, MI) at 37°C as needed. Heat-killed preparations of 10403S (HKLM-10403S) and DP-L967 (HKLM-DP-L967) were made by incubating live *L. monocytogenes* cultures in an 80°C water bath for 1 h and then washing with PBS.

Mice. Female BALB/c (H-2^d) mice were purchased from Charles River Laboratories (Wilmington, MA) at 6 to 8 weeks of age and received water and food *ad libitum*.

Immunizations. Mice were inoculated either i.p. or i.v. with approximately 0.1 LD₅₀ of viable *L. monocytogenes* 10403S or DP-L967 in sterile saline or orally with 2×10^8 bacteria in sterile water. Some mice were boosted four times with approximately 1 LD₅₀ weekly or given a single boost 4 weeks after the initial injection. For some experiments, mice received either 1×10^8 HKLM-10403S or 1×10^8 HKLM-DP-L967 i.p. and were given a boost of the same dose 4 weeks later.

Additional mice were inoculated i.v. with 2.2×10^7 irradiated syngeneic spleen cells that had been loaded with *E. coli* β -galactosidase (grade VIII; Sigma Chemical Co., St. Louis, MO). Spleen cells were cytoplasmically loaded with 10 mg/ml β -galactosidase in hypertonic medium, by using osmotic lysis of pinosomes, as previously described (3, 37).

Tumor cell lines and transfectants. The tumor cell lines P815 (DBA/2, H-2^d mastocytoma), P13.1 (a P815 transfectant producing β -galactosidase), E22 [an EL4 (C57BL/6, H-2^b thymoma) transfectant producing β -galactosidase], and C3-4 (a transfectant of the BALB/c hybridoma igm 662, which expresses cytoplasmic β -galactosidase) (38) were all generously provided by Drs. Mark Moore (Genentech, San Francisco, CA) and Frank Carbone (Monash Medical School, Prahran, Australia) (3, 39). P13.1, E22, and C3-4 were maintained in selective medium with 400 μ g/ml Geneticin (G-418 sulfate; GIBCO, Grand Island, NY).

SDS-PAGE. Whole-cell lysates from overnight cultures of *L. monocytogenes* 10403S or DP-L967 were prepared by incubating the

pellet in 10 mM Tris, 1 mM EDTA, pH 8.0, with 2 mg/ml lysozyme, for 1 h, and then boiling in SDS sample buffer with 2-ME. The samples and molecular weight markers (Bio-Rad, Melville, NY) were run on 7.5% SDS-PAGE and stained with Coomassie brilliant blue.

Generation of CTL effector populations. Spleen cells (3 to 3.5×10^7) from normal mice or mice that had been injected 1 to 5 weeks earlier with β -galactosidase-loaded spleen cells or *L. monocytogenes* were incubated for 5 days with 1.5 to 3×10^8 irradiated (20,000 rad) C3-4 cells in 25-cm² flasks (Costar, Cambridge, MA), in RPMI 1640 (JRH Biosciences, Lenexa, KS) with 10% FCS (Hyclone Laboratories, Inc., Logan, UT), 100 U/ml penicillin (Sigma), 100 mcg/ml streptomycin (Sigma) and 50 μ M 2-ME (Sigma), at 37°C in 10% CO₂/air. Effector populations were purified over Lympholyte M (Accurate Chemical and Scientific Corp., Westbury, NY), and 5×10^6 cells were restimulated weekly with 2×10^7 irradiated (3000 rad) syngeneic spleen cells and 2×10^6 irradiated C3-4 cells, in 10 ml of RPMI medium with 5% supernatant from Con A-stimulated rat spleen cells and 50 mM methyl- α -D-mannopyranoside (Sigma), in 25-cm² flasks.

Chromium-51 release cytotoxicity assay. Target cells (P815, P13.1, or E22) were labeled for 1.5 h with 200 μ Ci of ⁵¹Cr (as sodium chromate; ICN Radiochemicals, Irvine, CA) and washed, and 1×10^4 cells were added to each well in U-bottomed 96-well microtiter plates (Costar). Serial dilutions of effector cells were added to yield E:T ratios from 100:1 to 0.75:1, in a total volume of 0.2 ml of RPMI-10% FCS medium. After a 4- to 6-h incubation, 100 μ l of supernatant were harvested and specific lysis was determined as % specific lysis = [(experimental cpm – spontaneous cpm)/(total cpm – spontaneous cpm)] $\times$ 100. All determinations were done in triplicate. Total cpm was determined by target cell disruption with 1% Triton X-100, and spontaneous release was <15% in all experiments.

DTH. The DTH response to *Listeria* Ag or β -galactosidase was determined in the footpads of control mice or mice immunized with *L. monocytogenes* 10403S or DP-L967. The right hind footpad was injected with either 5×10^7 HKLM in 25 μ l of saline or 20 μ g of β -galactosidase in 25 μ l of saline. The left hind footpad received 25 μ l of saline. Twenty-four and forty-eight hours later, the thickness of the right and left footpads was measured with dial gauge calipers (Mitutoyo Mfg. Co., Ltd., Japan). The mean of three separate measurements was taken as the thickness for each footpad. Results are expressed as the mean increase in thickness of the Ag (HKLM or β -galactosidase)-injected footpad, compared with the saline-injected footpad.

ELISA. Antibodies to β -galactosidase in serum of mice inoculated with *L. monocytogenes* 10403S or DP-L967 were measured in a standard ELISA. Serum was collected from the tail vein at various times after injection and stored at 4°C until tested. EIA/RIA plates (96 well; GIBCO) were coated overnight at 4°C with 10 μ g/ml Ag. Serial dilutions of serum samples (1:10 to 1:1,000,000) were tested with goat anti-mouse IgG peroxidase conjugate (1:1000; Sigma) and the peroxidase substrate o-phenylenediamine (1 mg/ml; Sigma), with hydrogen peroxide. Plates were read at 450 nm with a V max kinetic microplate reader (Molecular Devices Corporation, Palo Alto, CA).

RESULTS

Expression of *E. coli* β -galactosidase in *L. monocytogenes* strain DP-L967. *L. monocytogenes* strain DP-L967 is a derivative of strain 10403S, which contains a chromosomal copy of the *E. coli* lac-Z gene and was generated by transposon insertion, as recently described (36, 40). Whole-cell lysates of DP-L967 show the expected protein of 115 kDa on Coomassie brilliant blue-stained SDS-PAGE (Fig. 1) and by Western blot analysis (data not shown). The β -galactosidase was clearly a minor component of the total cell protein. However, it was expressed in a constitutive and stable manner under all conditions tested. Bacteria isolated from the liver and spleens of mice 1 week after injection of DP-L967 continued to express β -galactosidase (data not shown). Expression of β -galactosidase did not effect hemolysin activity or growth rate of DP-L967 (40).

Generation of β -galactosidase-specific CTL from mice immunized with *L. monocytogenes* strain DP-L967. BALB/c mice were immunized i.p. with either *L. monocytogenes* strain 10403S or DP-L967 and boosted at 1 month, and their spleens were harvested 1 week later.

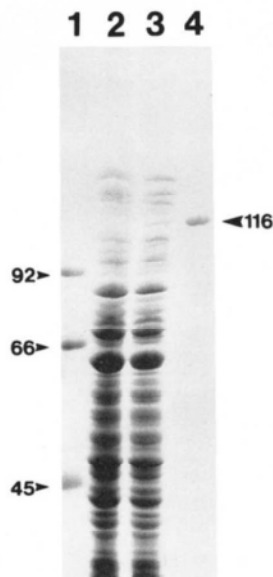


Figure 1. Expression of β -galactosidase in *L. monocytogenes* strain DP-L967. SDS-PAGE analysis of m.w. standards (lane 1), *L. monocytogenes* strain 10403S (lane 2), *L. monocytogenes* strain DP-L967 (lane 3), and purified β -galactosidase (lane 4). Whole-cell lysates were run on 7.5% SDS-PAGE and stained with Coomassie brilliant blue.

Cells were restimulated weekly in vitro with a β -galactosidase-expressing transfectant tumor line (C3-4) and tested for their ability to lyse β -galactosidase targets specifically. As a positive control, cells from mice that had been injected 8 days previously with syngeneic spleen cells cytoplasmically loaded with β -galactosidase were also stimulated in vitro with C3-4 cells. Cells from mice immunized i.p. with *L. monocytogenes* expressing β -galactosidase (DP-L967) showed a β -galactosidase-specific CTL response against P13.1 targets (Fig. 2A). In contrast, cells from mice immunized with wild-type *L. monocytogenes* (10403S) did not generate a specific CTL response but showed some low level background killing against both P815 and P13.1 targets (Fig. 2A). As shown previously (8), cells from mice injected with β -galactosidase-loaded spleen cells show a specific CTL response to the β -galactosidase-expressing target (Fig. 2A).

Mice were also twice inoculated orally with live *L. monocytogenes* strain 10403S or DP-L967, to determine whether CTL could be elicited through the natural route of infection. Spleen cells taken 1 week after the second inoculation were restimulated weekly with C3-4 cells, as described above, and tested for cytolytic activity. It was found that cells from mice given the recombinant *L. monocytogenes* (DP-L967), but not wild-type *L. monocytogenes* (10403S), specifically killed β -galactosidase targets (Fig. 2B). Similar results were seen with spleen cells taken 1 week after a single oral inoculation with DP-L967 and restimulated with C3-4 cells (Fig. 3B). These results demonstrate the efficacy of oral inoculation for the generation of a specific CTL response.

A consistent finding in these studies was that it took approximately 4 to 5 weeks of in vitro restimulation with C3-4 cells to select the β -galactosidase-specific effector population. This was true after both i.p. (Fig. 3A) and oral (Fig. 3B) inoculations. As shown in Figure 3, after 2 to 3 weeks in vitro there was low level nonspecific killing by cells from mice immunized with either the recombinant *L. monocytogenes* (DP-L967) or the wild-type *L.*

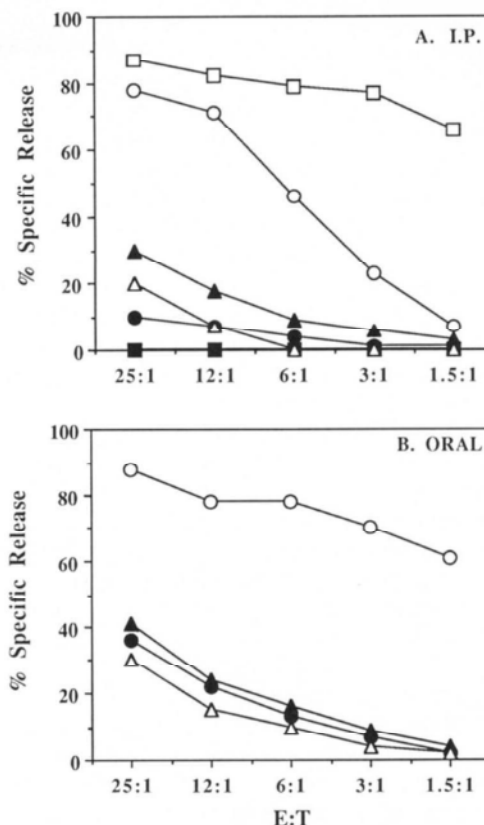


Figure 2. Generation of β -galactosidase-specific CTL. A, cytotoxic activity of spleen cells from mice immunized once i.v. with syngeneic β -galactosidase-loaded spleen cells (squares), twice i.p. with *L. monocytogenes* 10403S (triangles), or twice i.p. with *L. monocytogenes* DP-L967 (circles). Cells were maintained in vitro for 3 to 5 weeks before testing for lysis of ^{51}Cr -labeled P815 (closed symbols) or P13.1 (open symbols). B, cytotoxic activity of spleen cells from mice immunized twice orally with *L. monocytogenes* 10403S (triangles) or with *L. monocytogenes* DP-L967 (circles). Cells were maintained in vitro for 3 weeks with C3-4 stimulator cells before testing for lysis of ^{51}Cr -labeled P815 (closed symbols) or P13.1 (open symbols).

monocytogenes (10403S). After 4 weeks, cells from mice given the wild-type *L. monocytogenes* continued to show this nonspecific lysis, but these cells decreased in number and died. Cells from mice given the recombinant *L. monocytogenes*, however, continued to grow and by 4 to 6 weeks in vitro had strong β -galactosidase-specific cytolytic activity. Thus, cells from mice injected with the wild-type *L. monocytogenes*, or cells from naive mice (data not shown), do not develop β -galactosidase-specific effectors and do not survive in vitro. These findings were very reproducible, in that, in seven different experiments in which mice were immunized parenterally (five times i.p. or twice i.v.) with either wild-type (10403S) or β -galactosidase-expressing *L. monocytogenes* (DP-L967), spleen cells from the mice immunized with 10403S failed to survive in culture on stimulation with C3-4 cells, whereas spleen cells from mice immunized with DP-L967 consistently survived in culture to produce β -galactosidase-specific CTL lines that displayed the specificity and cytotoxicity shown in Figure 2. Similar results were obtained in three separate experiments in which mice were immunized orally with either 10403S or DP-L967. In addition, we consistently failed to prime normal spleen cells from naive mice with C3-4 cells in vitro. These results suggest that the generation of β -galactosidase-specific CTL is not due to in vitro priming with C3-4 cells

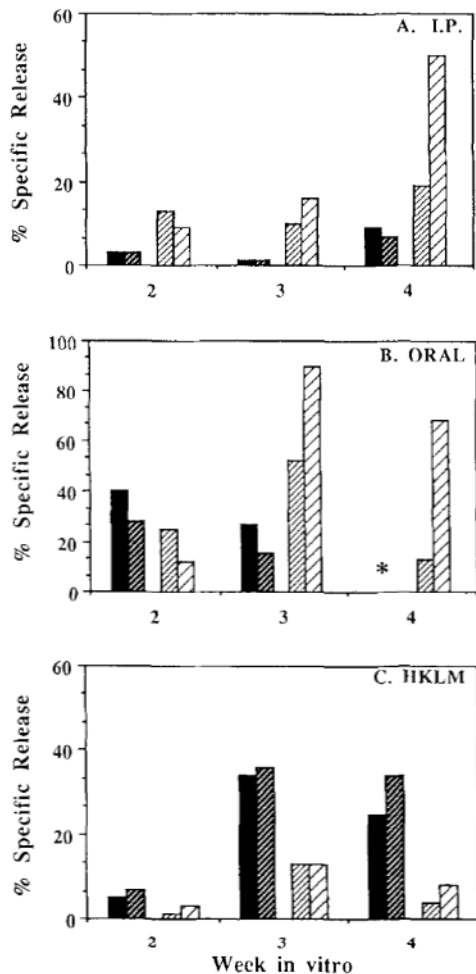


Figure 3. Selection of β -galactosidase-specific CTL in vitro. Cytotoxic activity of spleen cells from mice immunized i.p. (A) or orally (B) with live *L. monocytogenes* 10403S or with *L. monocytogenes* DP-L967, or i.p. with HKLM 10403S or HKLM DP-L967 (C), and restimulated in vitro from 2 to 4 weeks. Oral immunizations were with one dose of *L. monocytogenes*, and mice immunized i.p. received two doses. Specific lysis of cells from 10403S-immunized mice against P815 (■) or P13.1 (▣) and of cells from DP-L967-immunized mice against P815 (▨) or P13.1 (◐) was measured in a 4-h ^{51}Cr release assay. Data shown are for an E:T ratio of 12:1. *, cells had died by week 4.

but depends on in vivo immunization with the recombinant *L. monocytogenes* expressing β -galactosidase. This is consistent with the findings of Carbone and Bevan (5) that a secondary in vitro CTL response to a tumor line transfected with chicken OVA required prior in vivo priming with splenocytes loaded with OVA.

Immunization with live *L. monocytogenes* is a requirement for generation of β -galactosidase-specific CTL. In separate experiments, mice were inoculated i.p. with HKLM strain 10403S or DP-L967. Variable results were obtained from four experiments with regard to the degree of killing by cells derived from mice inoculated with the two heat-killed strains. Nonspecific killing of both P815 and P13.1 target cell lines was observed after the third week of in vitro restimulation with C3-4 cells (Fig. 3C). The nonspecific killers were derived from mice given either the recombinant *L. monocytogenes* (DP-L967) or the wild-type (10403S). However, these cells did not survive long term in vitro culture with C3-4, regardless of the strain of *L. monocytogenes* used to immunize the mice. This was in contrast to the β -galactosidase-specific CTL lines derived from mice immunized with the live

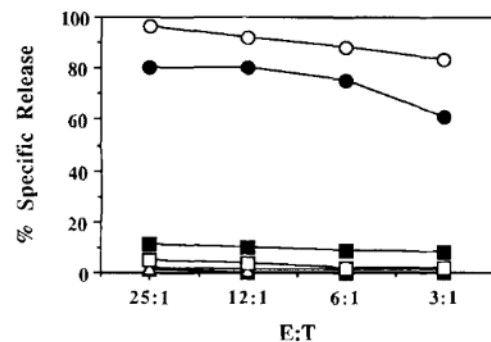


Figure 4. MHC restriction of β -galactosidase-specific CTL. CTL lines from mice immunized i.v. with syngeneic β -galactosidase-loaded spleen cells, selected in vitro for several months (closed symbols), or from mice immunized i.p. with *L. monocytogenes* DP-L967, selected in vitro for 8 weeks (open symbols), were tested for specific lysis against P815 (H-2^d) (triangles), P13.1 (H-2^d, β -galactosidase expressing) (circles), or E22 (H-2^b, β -galactosidase expressing) (squares).

strain DP-L967, which in 10 different experiments remained viable in vitro for months.

Class I restriction of CTL from *L. monocytogenes* DP-L967-primed mice. To further characterize the CTL population from mice primed with *L. monocytogenes* DP-L967, cells were tested for cytolytic activity against an EL-4 transfectant, E22, which expresses β -galactosidase. The CTL lines from mice immunized i.p. with DP-L967, as well as the CTL line from mice primed with β -galactosidase-loaded spleen cells, had significant activity against the P13.1 (H-2^d) targets but were unable to recognize the E22 (H-2^b) target (Fig. 4). Because P13.1 and E22 do not express class II MHC Ag, these results show that the recognition of β -galactosidase is class I MHC H-2^d restricted. The CTL line was primarily Thy-1.2⁺ (99%), CD8⁺ (77%), and TCR $\alpha\beta$ ⁺ (95%), as determined by FACS analysis (data not shown).

DTH responses to β -galactosidase and *L. monocytogenes*. Mice that had been immunized i.p. or orally with wild-type *L. monocytogenes*, or the recombinant, were tested for DTH responses to HKLM or β -galactosidase. All mice given either the wild-type *L. monocytogenes* (10403S) or the recombinant (DP-L967) i.p. had a positive reaction to HKLM from 1 to 4 weeks after immunization (data not shown) and 1 week after a boost at 1 month (Table I). Mice given the recombinant *L. monocytogenes* showed no response to β -galactosidase from 1 to 4 weeks after the initial injection (data not shown). A total of 21 mice that received a boost of the recombinant *L. monocytogenes* at 1 month were tested for footpad reactivity to β -galactosidase 1 week later. Of the 21 mice, four had a significant DTH response to β -galactosidase (Table I). Of mice given the *L. monocytogenes* recombinant orally and tested 1 week later, all had a positive DTH response to HKLM but not to β -galactosidase (Table I). Mice that had received the wild-type *L. monocytogenes* showed no response to β -galactosidase at any time tested.

Poor serum antibody responses to β -galactosidase as a result of immunization with *L. monocytogenes* DP-L967. Individual serum samples were taken from mice at various times after parenteral immunization with the recombinant *L. monocytogenes* (DP-L967) and were tested for antibody to β -galactosidase. Approximately 11% of the samples tested were positive for β -galactosidase antibody at low titers (1:10 to 1:100), but most of the samples were negative (Table II). This was true of samples

TABLE I
DTH to β -galactosidase and *Listeria* Ag

Challenge Ag (dose)	DTH Reactivity (0.1-mm) ^a			Route of Immunization ^b
	Saline ^c	10403S ^c	DP-L967 ^c	
HKLM (5×10^7) ^d	0.216 $\pm$ 0.070 (5)	0.445 $\pm$ 0.09 (7)	0.424 $\pm$ 0.119 (11)	i.p.
β -Galactosidase (20 μ g)	0.000 $\pm$ 0.080 (5)	-0.014 $\pm$ 0.07 (7)	0.237 $\pm$ 0.019 (4)	i.p.
β -Galactosidase (20 μ g)			0.042 $\pm$ 0.052 (17)	i.p.
HKLM (5×10^7)	ND	ND	0.790 $\pm$ 0.142 (3)	Oral
β -Galactosidase (20 μ g)	ND	ND	0.026 $\pm$ 0.087 (5)	Oral

^a Data are the difference (mean $\pm$ SD) in footpad thickness (in 0.1-mm units) between saline-injected footpads and Ag-injected footpads 24 h after injection. Numbers in parentheses are the number of mice per group.

^b Mice were either injected i.p. with 0.1 LD₅₀ of *L. monocytogenes*, boosted with 1 LD₅₀ at 1 month, and tested for DTH reactivity 1 week later or inoculated orally with 2×10^8 bacteria and tested 1 week later.

^c Immunization of mice.

^d Saline-injected mice received HKLM cells of strain 10403S, 10403S-injected mice received HKLM of strain 10403S, and DP-L967-injected mice received HKLM of strain DP-L967.

TABLE II
Antibody response in mice immunized with *L. monocytogenes* strain DP-L967

No. of Days after Immunization ^a	No. of Days after Booster ^b	Total No. of Mice Immunized	No. of Mice with an Antibody Response ^c	
			+	-
6-28		12	2	10
35	7	8	1	7
35	7 ^d	6	0	6
Total		26	3	23

^a Initial immunization was 0.1 LD₅₀.

^b Booster immunization was 1 LD₅₀.

^c Positive antibody response at 1/10 to 1/100 titer gave an OD₄₅₀ of greater than 2 times the preimmune serum OD₄₅₀ value.

^d These serum samples were from mice that received two to four boosts of DP-L967.

taken from mice injected i.p. or i.v. under a number of different immunization protocols. Serum samples from mice that received wild-type *L. monocytogenes* (10403S) were all negative for antibody to β -galactosidase (data not shown).

DISCUSSION

The results of this study show that oral or parenteral immunization of BALB/c mice with *L. monocytogenes* expressing *E. coli* β -galactosidase induced a population of CTL specific for β -galactosidase. The CTL line derived from mice immunized i.p. with the recombinant *L. monocytogenes* was further shown to be Thy-1.2⁺, CD8⁺, TCR $\alpha\beta$ ⁺, and MHC class I H-2^d restricted. These results warrant additional studies to evaluate further the potential of *L. monocytogenes* to act as a live recombinant vaccine vector to induce cellular and humoral immune responses to foreign Ag.

In vitro selection of a population of β -galactosidase-specific effector cells, which demonstrated high levels of specific lysis at low E:T ratios, took approximately 4 to 5 weeks. The β -galactosidase-specific CTL induced by in vivo priming with live recombinant *L. monocytogenes* had significant specific cytotoxicity (76% lysis at an E:T ratio of 12:1) and have been maintained stably in culture for several months. In contrast, control cultures with spleen cells from (a) naive mice, (b) mice immunized with the parental *L. monocytogenes*, or (c) mice immunized with HKLM strains DP-L967 or 10403S did not develop CTL specific for β -galactosidase after in vitro restimulation and died after 4 to 6 weeks. These results demonstrate that in vivo priming with the recombinant live *L.*

monocytogenes was necessary for the generation of the β -galactosidase-specific CTL and CTL generation was not a result of in vitro priming with the C3-4 line. It was not surprising that it took several cycles of in vitro restimulation with C3-4 cells to expand the CTL specific for β -galactosidase, which is only a minor nonsecreted protein expressed by the recombinant *L. monocytogenes* (Fig. 1). We are currently developing expression systems that should increase the amount of protein made and allow it to be secreted. This may enhance the immune response generated to the foreign protein.

To evaluate further the cell-mediated responses, the DTH response to HKLM and β -galactosidase was measured in mice immunized i.p. or orally with the recombinant *L. monocytogenes*. As expected, all *L. monocytogenes*-immune mice had a positive reaction to HKLM. However, only four of 26 mice tested had a significant DTH response to β -galactosidase. Because *L. monocytogenes* is known to elicit strong DTH responses mediated by CD4⁺ T cells (29, 33), it is surprising that only 15% of the mice tested positive for a DTH response to β -galactosidase. Given that CD4⁺ T cells are required to generate CD8⁺ T cells, the demonstration of β -galactosidase-specific CTL in the absence of a demonstrable specific CD4⁺ T cell DTH response to β -galactosidase suggests that the variety of Ag derived from *L. monocytogenes* itself may induce CD4⁺ T cells that provide help in inducing the CD8⁺ T cell response to β -galactosidase.

Another explanation for these results is that the antigenic peptide generated by the recombinant *L. monocytogenes* is different from the epitope processed from the whole purified protein used to elicit the DTH response and, thus, is not recognized. *L. monocytogenes*-derived Ag, including β -galactosidase, are produced in the host cytoplasm and may be processed mainly via the endogenous route of processing. Therefore, the antigenic peptides derived by processing of β -galactosidase synthesized by *L. monocytogenes* may differ from the peptides produced by direct endosomal processing of purified β -galactosidase from *E. coli*.

We also determined the humoral response to β -galactosidase in the serum of mice immunized with the recombinant *L. monocytogenes*. Of several samples tested, approximately 11% of the samples had a positive antibody response, although these were of low titer (<1:100). This result is not entirely unexpected, because it has been well established that *L. monocytogenes* does not elicit good humoral responses (41). It is interesting that other investigators, by using *Salmonella* (14) and BCG

(15) as Ag-delivery systems, have also shown relatively poor antibody responses to the cloned Ag. In contrast, other recombinant *Salmonella* strains, including one that expresses intracellular β -galactosidase, have been found to elicit strong antibody responses (6–8, 10–12, 16). One study with *E. coli* expressing viral epitopes in the periplasm showed that presentation at the cell surface was not necessary for the induction of an antibody response but antibody production was dependent on the route of administration (9). They also found that, when the protein was released in a soluble form, much higher levels of antibody were produced under all conditions. We are currently trying to determine whether secretion of the protein by *L. monocytogenes* leads to a stronger antibody response. It will be important for the development of recombinant vaccine systems to determine the optimal conditions to elicit antibody responses. It may be that additional forms of the Ag that are effective in inducing humoral responses, such as conventional nominal Ag, will be necessary adjuncts to vaccine vectors that target cellular responses, for those diseases where strong humoral immunity is also required.

Recently, studies on the cell biology of *L. monocytogenes* infections have shown that, after invasion of a host cell, *L. monocytogenes* escapes from the phagosome into the cytoplasm of the cell, where it replicates (17). It then migrates to the periphery of the cell and forms a pseudopod-like structure, which is internalized by a neighboring cell. The bacteria then break down the surrounding membranes and are again free in the cytoplasm to repeat the cycle of replication and cell to cell spread. This work demonstrates that, after the initial infection, *L. monocytogenes* is found primarily in the cytoplasm of the cell. In recent years, it has been shown that class I-restricted T cell responses are generally directed to endogenously synthesized Ag (2) or Ag that have been artificially introduced into the cytoplasm of the cell (3). Taken together, these studies suggest that the recombinant *L. monocytogenes* elicits class I-restricted CTL responses to the cloned Ag by accessing the class I pathway of processing and presentation through the cytoplasm. A recent study has shown that nonhemolytic mutants of *L. monocytogenes* that cannot escape into the cytoplasm do not elicit class I-restricted T cell responses (35). Conversely, the cytoplasmic listerial Ag may be sequestered from the humoral arm of the immune system, resulting in the poor induction of an antibody response. *L. monocytogenes* mutants that are arrested at different stages of the life cycle will be helpful in elucidating the mechanisms involved in the endogenous pathway of presentation.

Our results demonstrate that recombinant *L. monocytogenes* was able to present a foreign Ag for the induction of an immune response and has potential as a live vaccine vector. *L. monocytogenes* is attractive as a vaccine candidate for several reasons; (a) it naturally infects through the oral route and we have shown that it elicits cell-mediated responses after oral administration, (b) it can be used as a live vaccine, which, unlike killed preparations or protein and adjuvant, results in long lasting cellular immunity, (c) it is a Gram-positive bacteria and so does not have the problems associated with toxicity from endotoxin, which Gram-negative vectors such as *Salmonella* present, and (d) it remains viable on refrigeration and freezing and can be stored indefinitely. An important consideration is the overall safety of using a live bacteria as a vaccine. *L. monocytogenes* is susceptible to antibiotics and, thus, infections, which rarely occur in healthy individuals, can be controlled. However, *L. monocytogenes* can sometimes cause serious or fatal infections. We have developed mutant strains of *L. monocytogenes* that are avirulent but still protective that would be safer as vaccines (36, 42). These mutant strains will be tested as potential vaccine candidates.

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