Delivery of a Viral Antigen to the Class I Processing and Presentation Pathway by *Listeria monocytogenes*

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Summary

Listeria monocytogenes is a facultative intracellular pathogen that grows in the cytoplasm of infected host cells. We examined the capacity of *L. monocytogenes* to introduce influenza nucleoprotein (NP) into the class I pathway of antigen presentation both in vitro and in vivo. Recombinant *L. monocytogenes* secreting a fusion of listeriolysin O and NP (LLO-NP) targeted infected cells for lysis by NP-specific class I-restricted cytotoxic T cells. Antigen presentation occurred in the context of three different class I haplotypes in vitro. A hemolysin-negative *L. monocytogenes* strain expressing LLO-NP was able to present in a class II-restricted manner. However, it failed to target infected cells for lysis by $CD8^+$ T cells, indicating that hemolysin-dependent bacterial escape from the vacuole is necessary for class I presentation in vitro. Immunization of mice with a recombinant *L. monocytogenes* strain that stably expressed and secreted LLO-NP induced NP-specific $CD8^+$ cytotoxic T lymphocytes. These studies have implications for the use of *L. monocytogenes* to deliver potentially any antigen to the class I pathway in vivo.

Listeria monocytogenes is a gram-positive facultative intracellular bacterium which has been used for decades as a model pathogen for the study of cell-mediated immunity (1; for reviews see references 2–4). The immune response to *L. monocytogenes* is considered a paradigm of T cell–mediated immunity, as both the $CD4^+$ and $CD8^+$ T cell subsets contribute to the resolution of infection, whereas antibody plays no measurable role (2). Adoptive transfer studies have implicated $CD8^+$ cells as the most critical effector T cell subset (5, 6) that secretes cytokines (7, 8) and actively lyses infected cells in vitro (9, 10). The induction of protective immunity to *L. monocytogenes* requires administration of live hemolytic bacteria (11, 12).

During the past several years, a combination of genetic approaches and in vitro analysis of mutants using tissue culture models of infection have led to a detailed description of the cell biology of *L. monocytogenes* infection (13–15). The major findings are that, subsequent to internalization, bacteria are found in vacuoles and shortly thereafter are free in the host cell cytoplasm. Once in the cytoplasm, bacteria grow rapidly and exploit a host system of actin-based motility to move from cell to cell without exposure to the extracellular environment. Its intracytoplasmic life cycle makes *L. monocytogenes* an attractive candidate to introduce foreign antigens directly into the class I pathway of antigen presentation.

Entry of *L. monocytogenes* into the host cytoplasm is facilitated by the secreted, pore-forming hemolysin listeriolysin O (LLO)[1] (14, 16). Like killed *L. monocytogenes*, LLO-negative mutants fail to enter the cytoplasm and induce protective immunity (8, 11). Although both LLO-positive and -negative *L. monocytogenes* target the class II pathway in infected macrophages, only macrophages infected by LLO-positive bacteria are targeted for lysis by $CD8^+$ T cells (8, 9). These results are consistent with a requirement of bacterial entry into the cytoplasm to induce $CD8^+$ T cells. However, LLO itself is a target of the *L. monocytogenes*–specific immune response (17, 18), and recently, both class I- and class II–restricted T cell epitopes of LLO have been identified (18, 19).

In this study, *L. monocytogenes* was engineered to secrete a fusion protein consisting of LLO and a model antigen, influenza nucleoprotein (NP). NP is an extensively studied antigen that plays a central role in the influenza-specific immune response in mice (20). A significant proportion of the influenza-specific CTL response (~50% in BALB/c mice) are directed against NP epitopes (21). These CTLs are protective upon adoptive transfer (20, 22) and are crossreactive among all influenza A strains (21, 23). The class I- and II–restricted epitopes of NP are defined for a variety of MHC haplotypes (24–27). NP-specific CTLs are easily raised in mice by immunization with the virus. In vitro stimulation of immune

[1] *Abbreviations used in this paper:* BMM, bone marrow–derived macrophages; LLO, listeriolysin O; NP, nucleoprotein.

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splenocytes with the relevant class I–restricted NP peptide results in formation of a highly specific effector CTL population (28).

The results of the present study show that *L. monocytogenes* can be engineered to secrete a fusion protein containing viral antigenic determinants that are appropriately processed into peptide epitopes in host cells and presented in vitro in the context of three different MHC class I haplotypes. Influenza NP–specific CTLs were able to recognize in a class I–restricted manner and lyse cells infected with recombinant *L. monocytogenes*. Action of LLO and escape of *L. monocytogenes* from the vacuole were essential for presentation and recognition. NP-specific CTLs were induced in vivo after immunization with bacteria that secreted the fusion protein.

Materials and Methods

Bacterial Strains and Growth Conditions. The *L. monocytogenes* strains used in this study were derived from the wild-type strain 10403S and are described in Table 1. Strain 10403S, a hemolytic strain of *L. monocytogenes*, belongs to serotype 1 and has an LD_{50} of $\sim 3 \times 10^4$ in BALB/c mice (29). Bacteria were grown in brain-heart infusion medium (BHI; Difco Laboratories, Detroit, MI), broth, and agar. Strains containing plasmids were maintained and grown in BHI broth and agar supplemented with chloramphenicol at a concentration of 10 or 25 μg/ml for DP-L2028.

Constructions/Plasmids. Plasmid pAM401 is a shuttle vector able to replicate in both gram-negative and -positive bacteria (30). It contains a gram-positive chloramphenicol resistance gene and gram-negative tetracycline resistance determinant. To construct plasmid pDP1659, the DNA fragment encoding the first 420 amino acids of the hemolysin gene along with the promoter and the upstream regulatory sequences was PCR amplified with *L. monocytogenes* genomic DNA used as a template and ligated into pUC19. PCR primers used were 5′-GGCCCGGGCCCCCTCCTTTGAT-3′ and 5′-GGTCTAGATCATAATTTACTTCATCC-3′ (Operon Technologies Inc., Alameda, CA). The DNA fragment encoding the NP gene was similarly PCR amplified with linearized plasmid pAPR501, a gift of Dr. Peter Palese (Mt. Sinai Medical School, New York) used as a template (31), and subsequently ligated as an in-frame translational fusion into pUC19 downstream of the hemolysin gene fragment. PCR primers used were 5′-GGTCTAGAGAATTCC-AGCAAAAGCAG-3′ and 5′-GGGTCGACAAGGGTATTTTTC-TTTAAT-3′. The whole fusion was then recloned into the EcoRV and SalI sites of pAM401.

To obtain pDP1669, the pUC19 plasmid containing the fusion was treated with PstI and religated. This resulted in excision of the DNA sequence that codes for the COOH-terminal part of NP past amino acid 180. Subsequently, this shorter fusion was recloned into the EcoRV and SphI sites of pAM401.

Plasmids pDP1659 and pDP1669 were introduced into *L. monocytogenes* strain 10403S by electroporation of penicillin-treated bacteria as described (32), and the resulting transformants were designated DP-L1659 and DP-L1669, respectively. Plasmid pDP1659 was similarly introduced in DP-L2161, resulting in strain DP-L2320.

Plasmid pDP2028 was constructed by cloning the *prfA* gene into the SalI site of pDP1659. Transformation of the prfA(−) strain DP-L1075 (33, 34) with pDP2028 resulted in strain DP-L2028, which secreted the fusion protein stably in vitro and in vivo.

Cell Lines. The mouse monocyte–macrophage tumor cell line J774 ($H\text{-}2^d$) and fibroblastoma tumor cell line L929 ($H\text{-}2^k$) were maintained in DME supplemented with glucose (4500 mg/l), fetal bovine serum 7.5%, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Two derivatives of the L929 cell line, one transfected with the K^d molecule and one transfected with the D^b molecule, were provided by Dr. Laurence Eisenlohr (Thomas Jefferson University, Philadelphia, PA) (35) and Dr. James Sheil (West Virginia University, Morgantown, WV), respectively. These lines are referred to as L929-K^d and L929-D^b. L929-D^b was originally isolated by Dr. Stanley G. Nathenson (Albert Einstein College of Medicine, Bronx, NY).

The mouse mastocytoma cell line P815 ($H\text{-}2^d$) was maintained in RPMI 1640 medium supplemented with 7.5% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin.

NP 10-3.1, an NP-specific $CD4^+$ class II ($I\text{-}A^d$)–restricted hy-

Table 1. *Bacterial Strains and Relevant Characteristics*

L. monocytogenes strain	Characteristics/plasmids	Phenotype	References
10403S	Wild-type/none	Wild-type	(6)
DP-L1659	pDP1659, encoding LLO aa 1–420, fused to NP aa 1–498	Secretes a full-length fusion protein (105-kD)	This study
DP-L1669	pDP1669, encoding LLO aa 1–420, fused to NP aa 1–180	Secretes a truncated fusion protein (180 aa of NP).	This study
DP-L2161	Deletion of the chromosomal *hly* gene	Nonhemolytic; fails to escape the vacuole	(55)
DP-L2320	DP-L2161 (pDP1659)	Nonhemolytic; secretes the full-length fusion protein	This study
DP-L1075	Transposon insertion in the *prfA* gene	Low expression of LLO and other virulence-related genes	(33), (34)
DP-L2028	DP-L1075 (pDP1659-*prfA*)	Secretes high amounts of the LLO-NP fusion due to the presence of *prfA*	This study

bridoma, was a gift of Dr. Charles Hackett (Immulogic Co., Palo Alto, CA) (36). It recognizes a peptide spanning amino acids 50–65 of NP and was maintained in DME/high glucose medium as above, supplemented with 5 × 10^{-5} M 2-ME (Sigma Chemical Co., St. Louis, MO).

Generation of CTL Effector Populations. Spleen cells (10^8) from mice immunized intravenously with ~1,000 hemagglutinin units (HAU) of influenza A/PR/8/34 virus at least two wk before killing were purified by passage over Lympholyte M (Cedarlane Laboratories, Ltd., Ontario, Canada) and incubated for 5 d in the presence of ~10^{-6} M of the appropriate peptide corresponding to the K^d, K^k, or D^b epitope, as described in reference 28. The optimal concentration of the peptide for maximal expansion of the T cell population was determined by serial dilution. Splenocytes were incubated in a 25-cm^2 flask containing 20 ml of RPMI 1640 medium (JRH Biosciences, Lenexa, KS) with 10% FCS (Hyclone Laboratories Inc., Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin (both, Sigma Chemical Co.) at 37°C in 10% CO_2/air. The NP peptide 147–158/R156$^-$ corresponding to the K^d-restricted epitope (37) was added to the influenza-immune BALB/c splenocyte cultures; NP peptides 50–63 and 366–374 corresponding to the K^k- and D^b-restricted epitopes (24) were added to splenocytes derived from influenza-immune B10.BR and C57BL/6 mice, respectively.

To induce influenza-specific CTL by in vivo immunization with *L. monocytogenes* DP-L2028, BALB/c mice were immunized intravenously with either wild-type bacteria (10403S) at a dose of 2.4 × 10^4 CFU/mouse (~1 LD_{50}) or with DP-L2028 at three different doses, 3.2 × 10^4, 3.2 × 10^5, or 3.2 × 10^6 CFU/mouse (0.001, 0.01, and 0.1 LD_{50}, respectively). After 8 d, spleens were removed, and splenocytes were purified over Lympholyte-M and cultured in a 96-well plate for 4 d in the presence of 10^{-6} M of NP peptide 147–158/R156$^-$. The number of splenocytes per well is indicated (see Fig. 8) (2.5 × 10^5 to 2 × 10^6, in triplicates). At the end of the incubation period, cells were spun down, resuspended in 100 μl of fresh medium, and labeled target cells were added as described in the ^{51}Cr release assays.

Synthesis of Peptides. Peptides were synthesized as previously described (38, 39) by use of a standard manual solid-phase synthesis procedure on a polystyrene-co-1% divinylbenzene resin and *tert*-butyloxycarbonyl for all N-protection of amino acids. Couplings were carried out using *N-N''*-diisopropylcarbodiimide and were monitored by ninhydrin reaction (40). Simultaneous resin cleavage and side-chain deprotection was achieved by the high–low hydrogen fluoride method (41). The crude products were purified to 98% purity by reverse phase HPLC as previously described (38, 39), and peptide compositions were verified by amino acid analysis.

^{51}Cr Release Assays. The ^{51}Cr release assay in J774 cells was adapted from that previously described (18). Target (J774) cells were labeled for 1 h with ~100 μCi radioactive Na_2CrO_4 (ICN Radiochemicals, Irvine, CA), washed, added (1–2 × 10^4/well) to a flat-bottom 96-well plate (Costar Corp., Cambridge, MA) in antibiotic-free medium, and allowed to adhere for 45 min. Overnight cultures (2 ml) of the various *L. monocytogenes* strains were washed in PBS and added to the target cells to achieve an infection of 5–10 bacteria/cell. In some cases, the number of bacteria/cell was varied as discussed in the text. Infection was allowed to proceed for 40 min, then cells were washed in medium containing gentamicin (30 μg/ml) to kill extracellular bacteria. After 1.5–2 h, supernatants were replaced with medium containing the bacteriostatic antibiotic tetracycline (15 μg/ml) to inhibit further intracellular bacterial growth, which resulted in high spontaneous ^{51}Cr release. Serial dilutions of effectors were then added at the indicated E/T ratios. After 3–4 h, 100 μl of supernatant was removed, and specific lysis was calculated as 100× $[(X-S)/(T-S)]$, where X is the experimental counts per minute, S is the spontaneous counts per minute, and T is the total (1% Triton-induced) counts per minute. All determinations were done in quadruplicate. As a positive control, J774 cells were pulsed for 1 h with ~10^{-4} M of the 147–158/R156$^-$ NP peptide, which corresponds to the K^d-restricted epitope before the addition of the effectors. Spontaneous release typically ranged between 18 and 33%. Data shown are representative of several experiments with similar results. For the experiment described in Fig. 8, P815 cells were either pulsed with the NP peptide (~10^{-4} M for 1 h) or left untreated, labeled with ^{51}Cr, and added as targets at a density of 10^4 cells/well. After 4 h incubation, 100 μl of supernatant was removed and the percentage of specific lysis was calculated as described above.

Bacterial Release Assay. The in vitro cytotoxicity assay was adapted from Barry et al. (9). In this study, NP-specific cytotoxic cells (effectors) were generated as described above. Targets were either the J774 or the L929 cell lines grown on round 12-mm-diameter glass coverslips (Propper Manufacturing Co. Inc., Long Island City, NY) at a density of ~1.5 × 10^6 cells/60-mm dish holding 15 coverslips. After overnight incubation at 37°C, at which time each coverslip contained monolayers of ~10^5 cells, the culture supernatant was removed and replaced with 6 ml antibiotic-free medium containing *L. monocytogenes*. 2-ml overnight bacterial cultures were washed and resuspended in PBS, then used to infect the target cells. In the case of the J774 cells, 4 × 10^6 CFU/dish (10403S), 1.5 × 10^7 CFU/dish (DP-L1659, DP-L1669), or 5–10 × 10^8 CFU/dish (DP-L2320) were added and allowed to infect cells for 30 min. In the case of the various L929 cells, bacteria were added at a ratio of 1.2 × 10^8 CFU/dish (10403S) or 1.2 × 10^9 CFU/dish (DP-L1659, DP-L1669) and allowed to infect cells for 60 min. The infection was adjusted so that the final number of CFU/coverslip would be the same for all strains. After the indicated times of infection, target cells were washed three times with PBS and kept in 6 ml of antibiotic-free medium for another 30 min before addition of gentamicin (30 μg/ml) to prevent extracellular growth of bacteria. After dishes were incubated for 2–3 h, coverslips were transferred to 24-well dishes (one coverslip/well) with 1 ml of medium containing gentamicin (30 μg/ml) and tetracycline (15 μg/ml) to inhibit further bacterial growth. Effectors were then added at the indicated E/T ratios. 3–4 h after the addition of the effector cell populations, the numbers of CFU/coverslip were determined by removing the coverslip from the well, hypotonically lysing the host cells in sterile water, and plating 10-fold serial dilutions of the lysate on Luria broth agar plates. Cytotoxic activity of the effectors results in exposure of the bacteria to gentamicin after lysis of the infected target cells and decreased numbers of CFU/coverslip. Cytotoxicity was expressed as the percentage decrease in mean CFU/coverslip in the presence of effector cells relative to mean CFU counts in similarly treated coverslips not exposed to effector cells. All experiments were done on triplicate coverslips and performed several times with similar results to the data presented in the figures. DP-L1659, DP-L1669, DP-L2320, and DP-L2028 were kept under chloramphenicol selection throughout the experiments.

SDS-PAGE/Western Immunoblotting. TCA precipitation of culture supernatant fluid and subsequent SDS-PAGE (7% polyacrylamide) was performed as previously described (29). For Western blotting, proteins were transferred to nitrocellulose (Schleicher & Schuell, Inc., Keene, NH) with an electroblotter (TRANSPHOR; Hoefer Scientific Instruments, San Francisco, CA). All subsequent steps were performed in 50 mM Tris, pH 7.5, 2 mM EDTA, 0.15 M NaCl, 0.5% NP-40, and 1% calf serum. The nitrocellulose filter was first treated for 1 h with 5% skimmed milk, then incubated

for 1 h with either a rabbit polyclonal anti-LLO antiserum (a gift of Dr. Pascale Cossart, Institut Pasteur, Paris, France) or a mouse monoclonal anti-NP antibody (H19-524-4; a gift of Dr. W. Gerhardt, The Wistar Institute, Philadelphia, PA). The nitrocellulose filters were then washed and reacted with ^{125}I-labeled protein A or ^{125}I-labeled sheep anti-mouse Ig (Amersham Corp., Arlington Heights, IL), respectively. After extensive washing, the filter was exposed to x-ray film at $-70°C$ in the presence of two intensifying screens.

Cell Separations. Separation of CD4$^+$ or CD8$^+$ cells was done with the MACS streptavidin-conjugated iron microbeads as described in reference 42. Spleen cells were obtained from BALB/c mice immunized with influenza A/PR/8/34 as described above, purified over Lympholyte M and stimulated in vitro with the 147–158/R156$^-$ NP peptide as described above for 5 d. At that time, 2 × 10^7 live cells were washed and resuspended in 1 ml PBS containing 1% BSA (Sigma Chemical Co.). 60 μl of either biotin-conjugated rat anti-mouse CD8α mAb or biotin-conjugated rat anti-mouse CD4 mAb (GIBCO BRL, Gaithersburg, MD) was added to the cell suspension and incubated on ice for 25 min. Cells were washed, resuspended in 180 μl PBS/BSA 1%, and then 20 μl of streptavidin-conjugated iron microbeads (Miltenyi Biotech Gmbh, Bergisch Gladbach, Germany) were added and incubated on ice for 15 min. At the end of the incubation, 2 μl of streptavidin-FITC conjugate (GIBCO BRL) was also added, and incubation proceeded for another 5 min. Cells were washed, resuspended in 500 μl PBS/1% BSA, and passed through the MACS iron-wool separation column in magnetic field. The column was rinsed with 1.5 ml PBS/1% BSA and the eluates collected. These cells represent populations that were depleted of CD4$^+$ or CD8$^+$ cells. Subsequently, retained cells were recovered by removing the column from the magnetic field and washing it with 1.5 ml PBS/BSA 1%. These cells represent the CD4$^+$- or CD8$^+$-enriched populations. Depletion or enrichment was confirmed by FACS® analysis (Becton Dickinson & Co., Mountain View, CA). Depleted populations were >90% negative for CD4$^+$ or CD8$^+$ cells, respectively. Enriched cell populations were 89% CD4$^+$ or 82% CD8$^+$, respectively. The resulting populations were applied to a bacterial release assay as described above. The results presented are representative of three experiments performed.

Presentation to the CD4$^+$ T Cell Hybridoma. Bone marrow-derived macrophages (BMM) or J774 macrophage-like cells were resuspended in antibiotic-free DME/high glucose medium, added to 96-well flat bottom plates (Costar Corp.) at a density of ~10^5/well and left to adhere for 1 h. Overnight cultures of bacteria (10403S, DP-L1659, DP-L2161, or DP-L2320) were washed in PBS and added to the macrophages. 2.5 × 10^6, 5 × 10^6, or 2 × 10^7 CFU were added per well and allowed to infect cells for 40 min. Cells were then washed in medium containing gentamicin (30 μg/ml). After 3 h incubation, tetracycline (15 μg/ml) was added to stop further bacterial growth, and the CD4$^+$- class II-restricted NP-specific T cell hybridoma was added (2 × 10^5/well). After 24 h, the supernatants were collected and assayed for IL-2 activity by their ability to maintain the proliferation of CTL/L cells (American Type Culture Collection, Rockville, MD). CTL/L cells (5 × 10^3/well) were cultured in complete RPMI 1640 medium containing 10% FCS in 96-well flat-bottom plates with 25 μl of supernatants in a total volume of 200 μl for 24 h at 37°C and 10% CO_2. The cultures were then pulsed with [^{3}H]thymidine (0.5 μCi/well) for 8–12 h and harvested on glass filter paper with an automatic cell harvester (Inotech Biosystems International, Inc., Lansing, MI). [^{3}H]thymidine incorporation was quantitated with a liquid scintillation counter (model LS3801; Beckman Instruments, Carlsbad, CA). Stimulation of the hybridoma with macrophages without bacteria resulted in 550 cpm (background) for the experiment described (see Fig. 7). Data presented are representative of three experiments performed with similar results.

Results

Construction of L. monocytogenes Strains Secreting NP. LLO is normally expressed and secreted in a host vacuole by *L. monocytogenes* and is required for escape of the bacteria into the cytoplasm (14, 16). Moreover, LLO itself is a target of the class I- and class II-restricted antilisterial response (17, 19), and infected cells present LLO epitopes in the context of both class I and II MHC molecules (18, 19). Therefore, it was reasoned that a fusion protein consisting of LLO and a foreign protein would be expressed during infection of host cells and potentially target the class I pathway of antigen presentation. Accordingly, DNA fragments encoding either the full-length A/PR/8/34 influenza NP or its first 180 amino acids were cloned as in-frame fusions with LLO. A sequence encoding the first 420 amino acids of LLO, its promoter, and the upstream regulatory sequences was PCR amplified from *L. monocytogenes* chromosomal DNA and ligated to PCR-amplified DNA encoding A/PR/8/34 NP, derived from plasmid pAPR501 (31). The construction resulted in an in-frame fusion plus the addition of two amino acids at the site of the fusion junction. The fusion was cloned into the shuttle plasmid pAM401 and introduced into wild-type *L. monocytogenes* (strain 10403S) by electroporation, resulting in strain DP-L1659. An in-frame deletion of the DNA sequence encoding the COOH-terminal part of NP was generated, resulting in a fusion gene encoding the first 420 amino acids of LLO and the first 180 amino acids of NP. This construction was also introduced into wild-type *L. monocytogenes*, resulting in strain DP-L1669. Lastly, we cloned the *prfA* gene of *L. monocytogenes* into the shuttle vector along with the full-length fusion protein gene and used this plasmid to complement a *prfA(−)* strain, resulting in strain DP-L2028.

The recombinant strains were clearly able to express and secrete the fusion proteins as determined by Western blot analysis of secreted proteins (Fig. 1). The LLO-NP fusion proteins were secreted and migrated on SDS-PAGE as predicted at 105 or 68 kD. Both reacted with the anti-LLO polyclonal antiserum, but only the fusion containing the full-length NP reacted with the anti-NP mAb. It should be noted that the majority of the fusion protein was degraded, as multiple lower molecular mass polypeptides can be seen reacting with the anti-LLO antiserum. The presence of a multicopy plasmid with the fusion gene under the control of the LLO promoter had only a slight effect on bacterial growth rate in vitro. However, it did result in reduced secretion of the chromosomally encoded LLO, but not to the extent that it prevented escape of the bacteria from the vacuole or subsequent intracytoplasmic growth (data not shown).

Specific Lysis of J774 Cells Infected with L. monocytogenes Expressing NP by Influenza-immune Splenocytes. The ability of

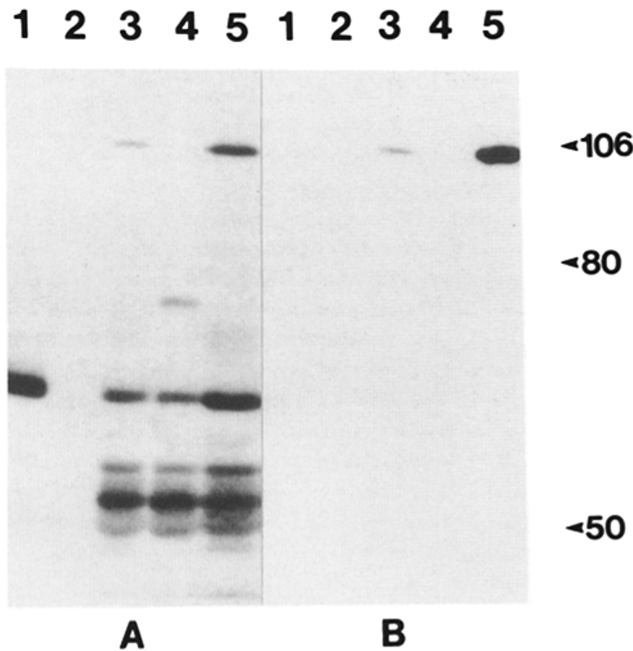

Figure 1. Western blot of *L. monocytogenes*–secreted proteins reacted with antilisteriolysin O antiserum (*A*) or an anti-NP mAb (*B*). Supernatants were derived from cultures of the following *L. monocytogenes* strains: lane *1*, strain 10403S; lane *2*, strain DP-L1075, a mutant strain that expresses minimal levels of listeriolysin O and other virulence-related genes; lane *3*, DP-L1659; lane *4*, DP-L1669; and lane *5*, DP-L2028.

the recombinant strains to deliver NP into the class I pathway of antigen presentation was evaluated by use of the J774 cell line as host cells. This H-2^d macrophage-like cell line was chosen as the primary target cell because it is readily infected by *L. monocytogenes*, but is not listericidal, unlike primary cultures of murine peritoneal macrophages (43). Effector cells were bulk splenocyte cultures of influenza A/PR/8/34-immunized BALB/c mice. Splenocytes were stimulated in vitro for 4–5 d with a synthetic NP peptide corresponding to the K^d-restricted epitope (147–158/R156$^-$) (37).

Two different assays were used to evaluate T cell–mediated cytotoxicity. The first assay was a standard ^{51}Cr release assay, comparing specific lysis of target cells infected with wild-type *L. monocytogenes* or strains expressing the fusion proteins (Fig. 2 *A*). It is clear that both recombinant strains targeted the J774 cells for recognition by influenza-specific splenocytes, while wild-type *L. monocytogenes* did not. As a positive control, targets pulsed with the NP peptide corresponding to the H-2^d–restricted NP epitope were also lysed. DP-L1669, which expresses the first 180 amino acids of NP, presented as well as DP-L1659, which expresses the full-length NP. This was expected, since both express the K^d-restricted NP epitope, which spans amino acids 147–155. Targets infected with DP-L2028 were lysed by influenza-immune splenocytes as effectively as those infected with DP-L1659 (data not shown).

The ^{51}Cr release assay has the disadvantage that it requires

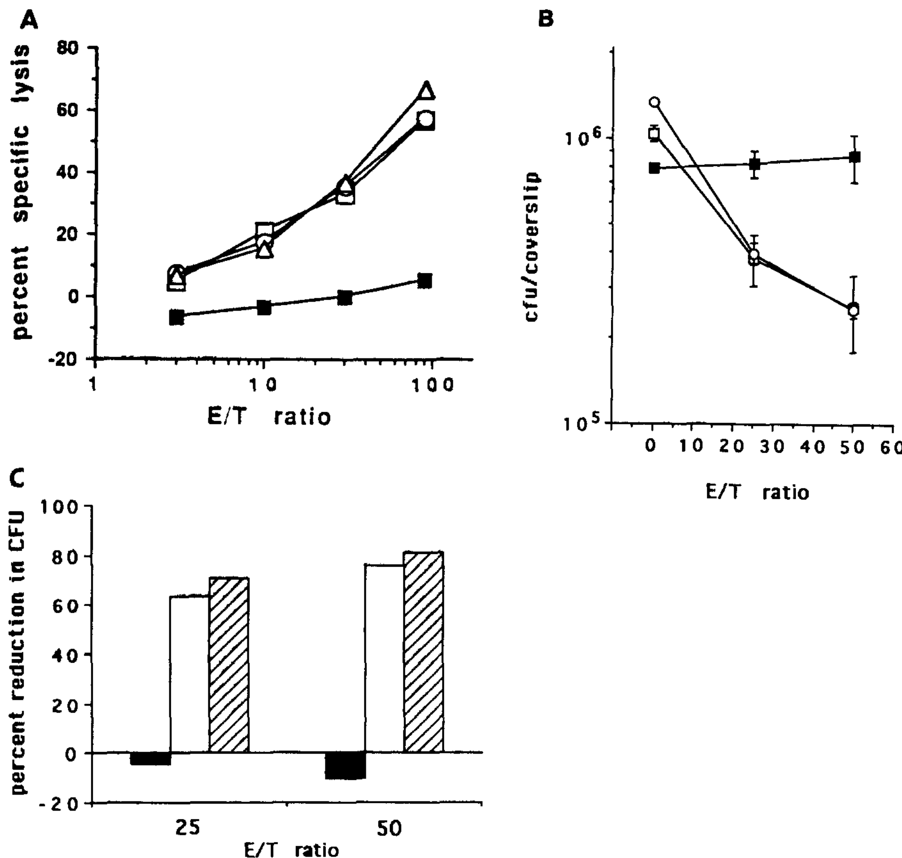

Figure 2. Specific lysis of *L. monocytogenes*–infected J774 cells by NP-specific T cells. (*A*) Lysis was measured by a standard ^{51}Cr release assay. Targets were infected with wild-type *L. monocytogenes* (*solid squares*), DP-L1659 (*open squares*), DP-L1669 (*open circles*), or pulsed with the K^d-restricted NP peptide (*open triangles*). (*B*) Lysis was measured in a bacterial release assay. The mean bacterial CFU/coverslip ± SE is shown. Targets were infected with wild-type *L. monocytogenes* (*solid squares*), DP-L1659 (*open squares*), or DP-L1669 (*open circles*). Reductions in CFU of DP-L1659 and DP-L1669 are significant (p <0.004 by Student's t test), whereas wild-type CFU do not change significantly (p >0.62). (*C*) Results of *B* were expressed as the percentage of reduction in bacterial CFU. Targets infected with wild-type *L. monocytogenes* (*solid bars*), DP-L1659 (*open bars*), or DP-L1669 (*hatched bars*).

the majority of cells to be infected and therefore is not ideal for use with many adherent cell lines that are not readily infected (see below). Another disadvantage is that infection with *L. monocytogenes*, especially at high levels, causes an increase of spontaneous release of ^{51}Cr by cells. Therefore, we adapted a previously described assay (9), which can accurately measure specific lysis even when a small fraction of the cells are infected. This assay, called the bacterial release assay, measures the reduction in bacterial CFU that results after lysis of the target cells by an effector population. The decrease in CFU is caused by gentamicin, which has been added to the tissue culture medium. Gentamicin has no effect on the growth or viability of intracellular bacteria in intact cells (29), but is rapidly bactericidal in lysed cells. Specific lysis results in reduction of bacterial CFU. The results from the bacterial release assay (Fig. 2, *B* and *C*) are comparable to the ^{51}Cr release results, showing that cells infected with the strains expressing NP are targeted for lysis by influenza-specific splenocytes. In Fig. 2 *B*, the mean bacterial numbers per coverslip are presented in the presence or absence of splenocytes. The data are also presented as the percentage of reduction in bacterial CFU (Fig. 2 *C*).

The Effector Population Consists of Class I–restricted CD8$^+$ T Cells. To examine the MHC restriction of the effector population, we used infected L929 cells as targets in a bacterial release assay. L929 cells are of the H-2^k haplotype and are class II negative. These cells were not capable of presenting antigen to NP-specific effector cells derived from BALB/c mice (H-2^d). In contrast, a L929 clone transfected with the appropriate class I molecule (K^d) (35) was able to present the K^d epitope consistent with the MHC class I restriction of the effector population (Fig. 3). Furthermore, T cell depletion studies revealed the CD8$^+$CD4$^-$ nature of the effectors (Fig. 4). The bulk primary splenocyte cultures were depleted of or enriched with CD8$^+$ cells or depleted of CD4$^+$ cells. The extent of depletion or enrichment was verified by FACS® analysis. The resulting populations were used in the bacterial release assay described above, and the percentage of reduction in CFU was calculated (Fig. 4). Even though equal numbers of cells were used in each case, Fig. 4 clearly shows that the absence of CD8$^+$ cells resulted in a population of cells incompetent to lyse bacterially infected targets. This was in contrast to the cell population depleted of CD4$^+$ cells, which displayed greater cytotoxicity than whole splenocytes. It is clear that the cytotoxic population is exclusively or predominantly of the CD8$^+$CD4$^-$ phenotype.

H-2^k– and H-2^b–restricted Class I NP Epitopes Are Also Generated by Recombinant L. monocytogenes. The previously described experiments were repeated in the context of H-2^k and H-2^b mouse MHC haplotypes. In the case of the H-2^k haplotype, the K^k class I molecule presents an NP epitope that is found in the region spanning amino acids 50–63 of NP. In the case of the H-2^b haplotype, the D^b molecule presents an NP epitope spanning amino acids 366–374 (24).

The H-2^k–restricted effectors were splenocytes derived from B10.BR mice immunized with influenza A/PR/8/34 and restimulated in vitro with an NP peptide corresponding to the K^k epitope. Targets were L929 cells infected with either wild-type or recombinant *L. monocytogenes* in a bacterial release assay (Fig. 5 *A*). It should be noted that DP-L1659 and DP-L1669 presented equally well, since both secreted fusion proteins contain the K^k-restricted epitope.

The H-2^b–restricted effectors were splenocytes from C57BL/6 mice immunized with A/PR/8/34 and restimulated in vitro with a peptide corresponding to the D^b-restricted epitope. Targets were L929 cells transfected with the appropriate H-2^b molecule (D^b). The results (Fig. 5 *B*) show that DP-L1659 expressing the full-length NP targeted

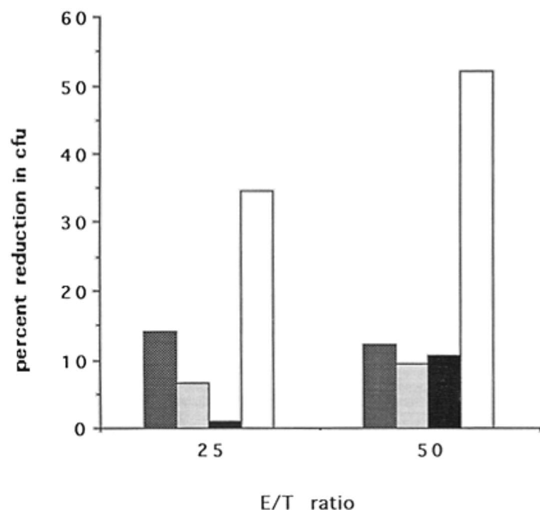

Figure 3. Class I restriction of the effectors. Lysis of infected L929 (H-2^k) cells by BALB/c (H-2^d)-derived CTL occurred only when they were transfected with the appropriate class I molecule (K^d). L929 cells infected with 10403S (*dark stippled bars*); L929 cells infected with DP-L1659 (*light stippled bars*); L929-K^d cells infected with 10403S (*solid bars*); L929-K^d cells infected with DP-L1659 (*open bars*).

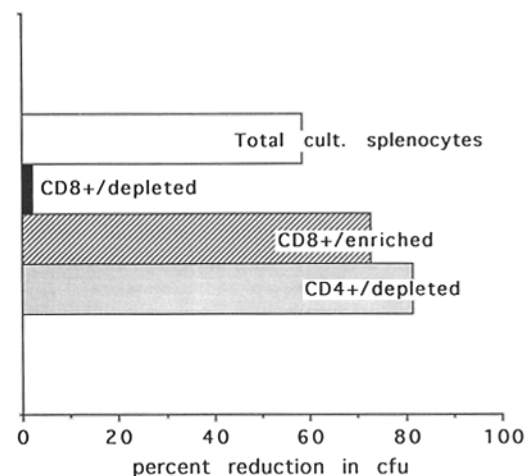

Figure 4. Characterization of the effector population. BALB/c-derived influenza-immune splenocytes were restimulated in vitro with the K^d-restricted NP peptide and used in a bacterial release assay on J774 cells infected with DP-L1659 at an E/T ratio of 15:1. Whole splenocyte culture (*open bar*); CD8$^+$-depleted splenocytes (*solid bar*); CD8$^+$-enriched splenocytes (*hatched bar*); CD4$^+$-depleted splenocytes (*stippled bar*).

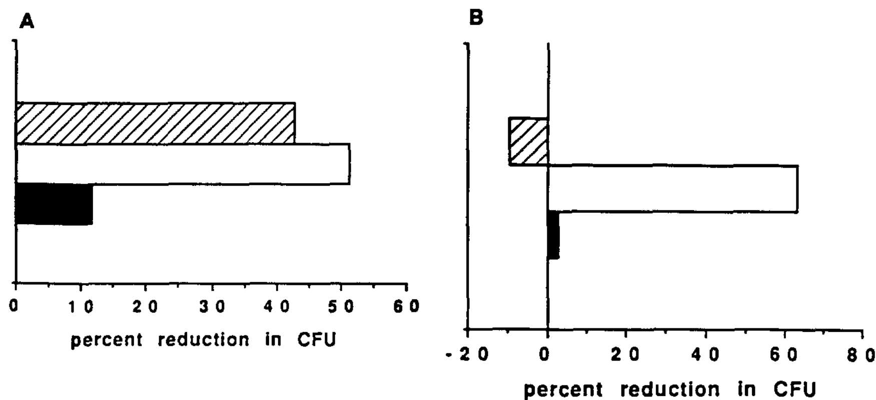

Figure 5. Presentation of NP epitopes in the context of different class I haplotypes. (*A*) L929 cells (H-2^k) were infected and used as targets in a bacterial release assay. Effectors were cultures of splenocytes from influenza-immune B10.BR (H-2^k) mice added at an E/T ratio of 25:1. Targets infected with 10403S (*solid bar*), DP-L1659 (*open bar*), or DP-L1669 (*hatched bar*). (*B*) L929 cells transfected with the D^b molecule were infected and used as targets in a bacterial release assay. Effectors were cultures of splenocytes from influenza-immune C57BL/6 (H-2^b) mice added at an E/T ratio of 40:1. Infected with 10403S (*solid bar*), DP-L1659 (*open bar*), or DP-L1669 (*hatched bar*).

normally, but DP-L1669 failed to target infected cells, since its fusion protein does not contain the D^b-restricted epitope.

Presentation Requires Functional LLO. LLO-negative mutants of *L. monocytogenes* fail to escape from the vacuole in J774 cells and are unable to target infected cells for lysis by *L. monocytogenes*-specific $CD8^+$ T cells (8, 9). A LLO-negative mutant of *L. monocytogenes* was transformed with plasmid pDP-L1659, which encodes the LLO-NP fusion protein, resulting in strain DP-L2320, which secreted the fusion protein at similar levels to DP-L1659 (data not shown). It should be noted that the part of the LLO sequence (420 amino acids) that is present in the fusion protein does not confer hemolytic activity. Thus, DP-L2320 is still a LLO-negative mutant that secretes the NP-containing fusion protein but is unable to escape from the vacuole or grow intracellularly. This strain failed to target J774 cells for lysis by influenza-immune splenocytes in either a ^{51}Cr release assay (Fig. 6 *A*) or a bacterial release assay (Fig. 6 *B*), even when the target cells were infected with high levels of bacteria.

One possible interpretation of these results is that the LLO-negative mutant did not present the antigen because the nongrowing bacteria confined in the vacuole failed to express the fusion protein. Accordingly, we asked whether the fusion protein would be presented in a class II–dependent fashion. BMM infected with DP-L2320 or DP-L1659 were equally able to present in a class II–restricted manner to NP 10-3.1 (36), a $CD4^+$ NP-specific T cell hybridoma (Fig. 7). Presentation was reduced when the infection and subsequent incubation was done in the presence of tetracycline, which inhibits bacterial protein synthesis (data not shown). The data suggest that the fusion protein was in fact being made inside the vacuole by DP-L2320. J774 cells infected with DP-L2320 were also able to present NP in a class II–restricted manner (data not shown). These results are consistent with the notion that listerial entry into the cytoplasm is required for delivery of antigen to the class I pathway of antigen presentation.

Generation of Influenza-specific CTL by In Vivo Immunization with DP-L2028. DP-L1659 and DP-L1669 proved to be unstable in vivo. In fact, almost all colonies recovered from organs 48 h after immunization with these recombinants had lost the plasmid. To solve this problem, we complemented a *prfA(−) L. monocytogenes* mutant with a plasmid containing *prfA* and the LLO-NP fusion (pDP2028), thus selecting for the retention of the plasmid in vivo. This strain (DP-L2028) was able to induce potent NP-specific CTL activity, which was easily detectable in secondary splenocyte cultures (Fig. 8). Splenocytes from DP-L2028–immune mice were able to lyse NP peptide–pulsed targets, in sharp contrast to those isolated from 10403S-immune mice. P815 cells that were not peptide pulsed were not lysed by either splenocyte population (data not shown). The NP-specific CTL activity was evident

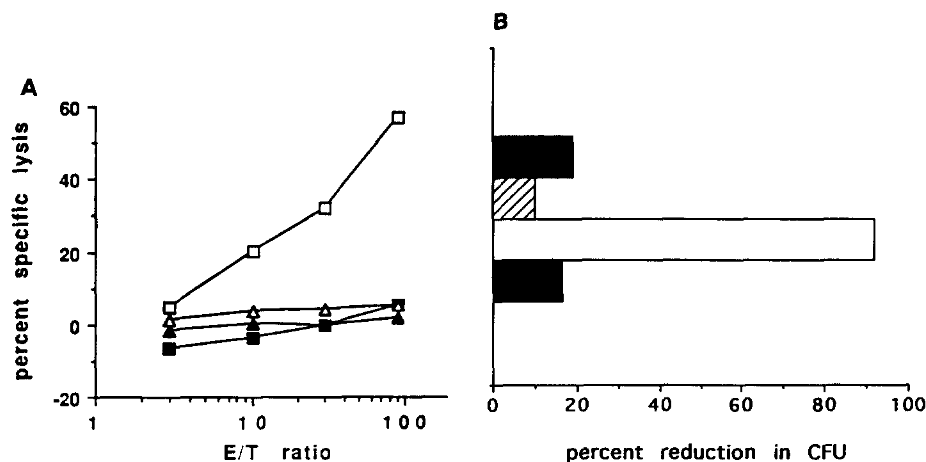

Figure 6. Antigen presentation by nonhemolytic *L. monocytogenes* expressing NP. J774 cells were used as targets in a standard ^{51}Cr release assay (*A*) or a bacterial release assay (*B*) with influenza-immune splenocytes from BALB/c mice used as effectors. (*A*) Targets were J774 cells infected with 10403S (*solid squares*), DP-L1659 (*open squares*), DP-L2320 (*solid triangles*), or DP-L2320 at eight times the usual infection (*open triangles*). (*B*) Targets were J774 cells infected with 10403S (*solid bar*), DP-L1659 (*open bar*), DP-L2320 (*hatched bar*), or DP-L2320 at a high infection rate of ∼30 bacteria/cell (*heavily hatched bar*). The E/T ratio was 50:1.

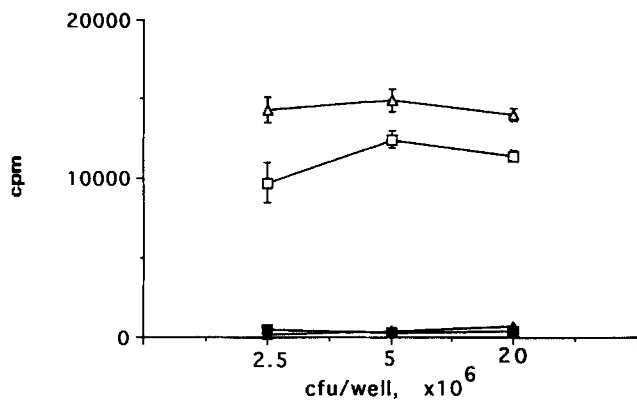

Figure 7. Class II–restricted presentation by LLO-positive and -negative bacteria expressing NP. BMM were infected at three different infection ratios with *L. monocytogenes* strains and used as described in Materials and Methods to stimulate NP 10-3.1, a class II–restricted $CD4^+$ NP-specific T cell hybridoma. Supernatants were used in a CTLL proliferation assay. cpm values ± SD are shown. BMM cells infected with 10403S (*solid squares*), DP-L2161 (*solid triangles*), DP-L1659 (*open squares*), or DP-L2320 (*open triangles*).

even after relatively low immunization doses ($0.001LD_{50}$); it should be noted that DP-L2028 has an LD_{50} of $\sim 3 \times 10^7$ CFU/mouse, compared with 3×10^4 for 10403S.

Discussion

The results of this study show that *L. monocytogenes* can be engineered to secrete influenza NP, a viral antigen. Infection of cells with hemolytic *L. monocytogenes* secreting NP resulted in efficient in vitro presentation of viral epitopes to $CD8^+$ class I–restricted T cells specific for the viral pathogen. Presentation required the action of listeriolysin and escape from the vacuole, as it did not occur in a LLO(−) mutant. In contrast, expression of the same fusion protein in a LLO(−) background resulted in presentation to class II–restricted NP-specific $CD4^+$ cells. Immunization of mice with *L. monocytogenes* expressing NP resulted in induction of a potent CTL response specific for the viral protein.

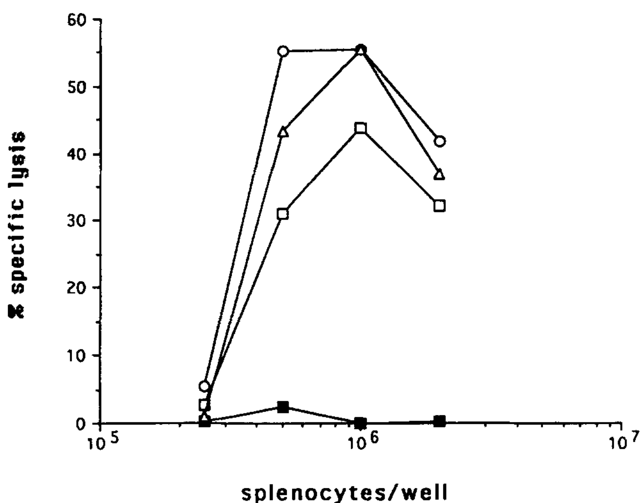

Figure 8. Generation of NP-specific CTL after immunization with *L. monocytogenes* stably expressing LLO-NP. Splenocytes from *L. monocytogenes*–immunized mice were cultured in vitro at the indicated cell densities/well with the K^d-restricted NP peptide and used as effectors against P815 cells pulsed with the peptide in a ^{51}Cr release assay. Splenocytes from mice immunized with 2.4×10^4 CFU 10403S (*solid squares*), 3.2×10^4 CFU DP-L2028 (*open squares*), 3.2×10^5 CFU DP-L2028 (*open circles*), or 3.2×10^6 CFU DP-L2028 (*open triangles*). P815 cells not pulsed with the NP peptide displayed negligible amounts of lysis in all cases (not shown).

The antigen used in this study consisted of an in-frame fusion between LLO and NP, which was readily secreted into the cytoplasm. The data are consistent with its secretion within a vacuole and directly into the host cytoplasm. Presentation of NP epitopes occurred in the context of three different class I haplotypes, which has implications for the ability of this vector to present antigens in outbred populations. It has not been formally proven that bacterial protein secretion was essential for delivery to the class I pathway, but it is very likely that secretion is necessary as intracytoplasmic bacteria are viable and remain intact, so that internal bacterial antigens would have no access to the host cytoplasm. Consistent with this notion, all three *L. monocytogenes* antigens known to be recognized by immune $CD8^+$ T cells are secreted antigens (17, 18, 44). Also, in a previous study (45), we showed that *L. monocytogenes* expressing nonsecreted β-galactosidase was capable of inducing a β-galactosidase– specific $CD8^+$ response in vivo; however, these bacteria were unable to target infected J774 cells for class I–restricted lysis (Schafer, R., D. A. Portnoy, and Y. Paterson, unpublished results).

It is generally accepted that there are two distinct pathways of antigen presentation: the exogenous pathway and the endogenous pathway (46). Our results with $CD8^+$ T cells are consistent with presentation via a cytoplasmic route subsequent to bacterial secretion of de novo synthesized antigen. However, there are a few examples in which antigen can enter macrophages through the exogenous route and still present antigen to $CD8^+$ T cells. Indeed, pathogens, such as *Salmonella*, that reside in vacuoles are able to induce $CD8^+$ T cells in vivo (45), although the same strain of *Salmonella* is not recognized by $CD8^+$ T cells while intracellular during in vitro assays (47–49). One possible explanation for the presentation of exogenous antigen to the class I pathway was recently described in which primary macrophages "regurgitated" processed antigen, which was then presented by neighboring cells (50). In another report, antigen linked to beads was efficiently introduced into the class I pathway upon phagocytosis by macrophages (51). Whereas these latter examples are provocative, it is clear that secretion by *L. monocytogenes* is a highly effective system for the direct delivery of potentially any foreign antigen into the cytoplasm of both phagocytic and nonphagocytic cells. Indeed, nonhemolytic *L. monocytogenes* confined to a vacuole failed to reach the class I pathway and target infected cells for lysis. It is also well established that LLO(−) mutants of *L. monocytogenes* that fail to escape the vacuole also fail to evoke protective immunity against *L. monocytogenes* challenge (11).

During the past few years, a number of different bacterial

species have been proposed and developed as live vaccine vectors, most notably *Salmonella* and Bacillus Calmette-Guérin. Both systems have shown considerable promise in the induction of CD8$^+$ T cells in vivo (47, 52). *L. monocytogenes* is particularly attractive as a vaccine vector for the induction of cell-mediated immunity. It grows in the host cell cytoplasm and delivers antigens directly into the class I pathway of antigen presentation (8). *L. monocytogenes* is a natural inducer of IL-12, which is thought to bias the immune response towards cell-mediated immunity (53, 54). It is also amenable to genetic manipulation, which may facilitate the design of rationally attenuated mutants (13) and the optimal expression of foreign antigens. For example, by use of a *L. monocytogenes* mutant lacking LLO, antigens were delivered exclusively into the class II pathway of antigen presentation. Thus, at least in vitro, foreign antigens can be directed into the class II pathway or to both class I and class II pathways simply by use of the suitable *L. monocytogenes* mutant.

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