INFECTION AND IMMUNITY, May 1996, p. 1870–1872 Vol. 64, No. 5
0019-9567/96/$04.00+0


# Peptide Epitopes from Noncytosolic *Listeria monocytogenes* Can Be Presented by Major Histocompatibility Complex Class I Molecules

HEATHER L. ZWICKEY AND TERRY A. POTTER*

*Division of Basic Immunology, Department of Medicine, National Jewish Center for Immunology and Respiratory Medicine, and Department of Immunology and Cancer Center, University of Colorado Health Sciences Center, Denver, Colorado*

Received 28 November 1995/Returned for modification 8 January 1996/Accepted 20 February 1996

***Listeria monocytogenes* is an intracellular pathogen which escapes the phagosome and resides in the cytosol of the host cell. Using *Listeria innocua* and a mutant strain of *L. monocytogenes* (listeriolysin O negative), which do not enter the cytosol of the host cell, we demonstrate class I presentation of an epitope of p60, a protein secreted by *L. monocytogenes*, to a class I-restricted CD8$^+$ cytotoxic T lymphocyte clone.**

Upon phagocytosis by macrophages, several species of bacteria remain within the phagosome whereas other bacterial species escape into the cytosol. In the case of wild-type *Listeria monocytogenes*, escape into the cytosol is achieved by the secretion of a lysin, listeriolysin O (LLO). In contrast, *Listeria innocua* and various mutant strains of *L. monocytogenes* do not synthesize functional LLO and do not escape the phagosome (2, 13). By comparing the abilities of wild-type and mutant (LLO$^-$) *L. monocytogenes* to sensitize phagocytic antigen-presenting cells (APCs) for lysis by cytotoxic T lymphocytes (CTL), one study concluded that presentation of *L. monocytogenes*-derived peptides by major histocompatibility complex (MHC) class I molecules occurs only if the *L. monocytogenes* bacteria escape into the cytosol (2). A conflicting report (20) observed class I-restricted presentation to an *L. monocytogenes*-specific CTL line raised against heat-killed *L. monocytogenes*. Using a T-cell clone specific for an epitope derived from the *L. monocytogenes* protein p60 (11), we demonstrated that presentation of the p60 epitope by class I molecules occurs regardless of whether bacteria reside in the cytosol (LLO$^+$) or the phagosome (LLO$^-$). We suggest that this secreted protein is able to enter the class I pathway from either location and that therefore class I presentation can be influenced not only by the intracellular location of the pathogen but also by the properties of the antigenic protein produced by the pathogen.

**Bacterial strains.** *L. monocytogenes* 163/85 (LLO$^+$) and 162/85 (LLO$^-$) (5) were grown in tryptic peptone broth (TPB). *L. monocytogenes* 162/85 has a transposon inserted in the LLO gene which causes a truncation in the protein, rendering the LLO secreted from this bacterium nonfunctional. *L. monocytogenes* 162/85 was grown in TPB supplemented with 1 μg of tetracycline per ml in order to maintain selection for the transposon. The inability of the strain to produce functional hemolytic LLO was confirmed by plating on sheep blood agar plates, its inability to escape into the cytosol was confirmed by the failure to polymerize actin upon phagocytosis into APCs (data not shown). Freshly thawed stocks of both *L. monocytogenes* strains were added to 4.5 ml of TPB (a mixture of TPB and tetracycline was used for 162/85) and cultures were grown for 3 h at 37°C.

*L. innocua* SLCC 3379 and SLCC 3423 were obtained from the American Type Culture Collection and were grown in TPB as described above.

**APCs.** The cell lines P815 (mastocytoma) and J774A.1 (macrophage-like) were obtained from the American Type Culture Collection (ATCC CRL 6448 and TIB 67, respectively) and were maintained in antibiotic-free modified Eagle's medium (MEM) supplemented with 7.5% fetal calf serum. Bone marrow macrophages were isolated from the femurs of BALB/c mice and were cultured in media supplemented with granulocyte-macrophage colony-stimulating factor (10% supernatant from CHO cells transfected with the murine granulocyte-macrophage colony-stimulating factor gene) for 7 to 10 days before use.

**Infection of APCs.** APCs ($10^6$) were harvested and resuspended in 10 ml of antibiotic-free MEM. A total of $10^9$ live *L. monocytogenes* bacteria (1 ml of a 3-h log-phase culture) were washed three times in phosphate-buffered saline (PBS) and resuspended in PBS. Ten microliters of the washed bacteria ($10^7$ bacteria, i.e., a multiplicity of infection of 10 bacteria per APC) were added to the APCs. After 30 min (for the J774 cell line and the bone marrow macrophages) or 3 h (for the P815 cells), gentamicin was added to a final concentration of 5 μg/ml in order to kill the extracellular bacteria. The total time of infection was 6 h.

For infection with heat-killed *L. monocytogenes*, a similar protocol was used; however, the multiplicity of infection was increased to 100:1 and no gentamicin was added. For supernatant-pulsed cells, 1 ml of supernatant was added to $10^6$ cells suspended in 5 ml of MEM supplemented with 7.5% fetal calf serum. These cells were incubated for 3 h and then added to a cytotoxicity assay.

**Cytotoxicity assay.** The p60-specific CD8$^+$ T-cell line L9.6 was obtained from Eric Pamer (Yale University School of Medicine, New Haven, Conn.). This clone recognizes the peptide KYGVSVQDI in the context of the MHC class I H-2K$^d$ molecule, as described by Pamer (11). Infected and uninfected APCs were labeled with $^{51}$Cr, and a standard 4-h cytolysis assay was performed.

**p60-specific cytolytic T cells kill macrophage-like lines and primary macrophages infected with wild-type or LLO$^-$ *L. monocytogenes*.** *L. monocytogenes* p60 is a 60-kDa protein that is present on the surface of the bacterium and is also secreted by viable bacteria (8). A CD8$^+$ CTL recognizing an epitope derived from the p60 molecule presented by the MHC class I molecule H-2K$^d$ has been derived (11). This peptide epitope can be recovered from H-2K$^d$ molecules 6 h after infection with wild-type *L. monocytogenes* (11). We examined whether

* Corresponding author. Mailing address: Division of Basic Immunology, Department of Medicine, National Jewish Center for Immunology and Respiratory Medicine, 1400 Jackson St., Denver, CO 80206-2761.

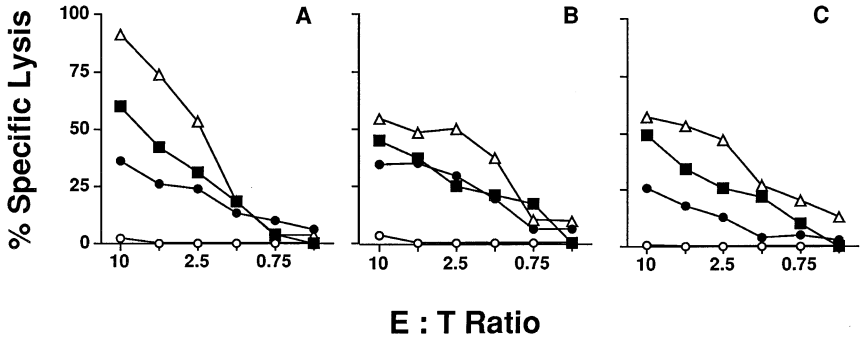


FIG. 1. Cytotoxic reactivity of p60-specific CTL clone for APCs infected with *L. monocytogenes* as described in the text. The target cells are denoted by the following symbols: open circles, uninfected cells; filled squares, cells infected with wild-type *L. monocytogenes*; filled circles, cells infected with LLO$^-$ *L. monocytogenes*; open triangles, cells pulsed with the p60 peptide from residues 217 to 225. The following APCs were used: P815 cells (A), J774A.1 macrophages (B), and bone marrow macrophages (C). E:T ratio, ratio of effector cells to target cells.

APCs infected with either wild-type or LLO$^-$ *L. monocytogenes* would be recognized and killed by the p60-specific CTL clone. The p60-specific CTL clone lysed J774 and P815 target cells infected with wild-type *L. monocytogenes*, verifying that the p60 epitope is presented by H-2K$^d$ on the surface of these target cell lines (Fig. 1A and B). The same target cells infected with the mutant strain 162/85 were also lysed by the p60-specific CTL (Fig. 1A and B). The killing of APCs infected with 162/85 by the p60-specific CTL suggests that even though these bacteria did not escape the phagosome and enter the cytosol, the p60 molecule that they produced reached the class I processing pathway. To determine that this mode of presentation was not confined to these immortalized cell lines and was also a property of normal macrophages, we examined whether bone marrow-derived macrophages behaved similarly and were able to present the p60 epitope from both wild-type and LLO$^-$ *L. monocytogenes*. The bone marrow macrophages infected with both wild-type and LLO$^-$ strains were lysed by the p60-specific CTL (Fig. 1C). The killing was not due to extracellular loading of p60-derived epitopes from p60 secreted by extracellular bacteria, since APCs pulsed with bacterial culture supernatant could not be lysed by the p60-specific CTL (Fig. 2A). Similarly, supernatant from washes of *L. monocytogenes* were unable to sensitize APCs for lysis by the p60-specific CTL (data not shown). A previous study which demonstrated class I presentation by LLO$^-$ *L. monocytogenes* (20) used T cells elicited by APCs infected with heat-killed bacteria. We therefore examined whether heat-killed LLO$^+$ would sensitize the APCs for lysis by the p60-specific CTL clone. In contrast to cells infected with live bacteria, cells infected with heat-killed wild-type *L. monocytogenes* were not killed by the p60-specific CTL (Fig. 2B), suggesting that either p60 is not produced by heat-killed bacteria or it is unable to reach the class I processing pathway in sufficient quantities to sensitize the APCs for lysis by the CTL.

**p60-specific CTL recognize J774 macrophages infected with *L. innocua*.** We examined whether p60 from other *Listeria* species which do not escape the phagosome can be processed and presented by the class I pathway. Similar to the 162/85 mutant *L. monocytogenes*, *L. innocua* does not produce a lysin which allows for escape from the phagosome. Consequently, *L. innocua* resides in the phagosome of infected cells and is considered avirulent. *L. innocua* secretes a p60-related protein which is highly conserved between *L. innocua* and *L. monocytogenes* (3), and the epitope (residues 217 to 225) recognized by the CTL clone is identical for the two species. As shown in Fig. 3, APCs infected with *L. innocua* are lysed by the p60-specific CTL, and the level of killing was similar to that of cells infected with wild-type *L. monocytogenes*. Thus, p60 produced by other *Listeria* species which do not escape the phagosome can be processed and presented by MHC class I molecules.

Our observation that cytosolic location is not required for class I presentation is in contrast with the results of previous studies that did not demonstrate CTL-mediated lysis of macrophages infected with LLO$^-$ *L. monocytogenes* (2). The difference between those observations and our results could be due to the difference in the effector T-cell populations used. The bulk T-cell cultures used by Bouwer et al. were of unknown specificity, whereas the T-cell clone we used is known to recognize an epitope from the p60 protein. Our experiments are in agreement with the experiments of Szalay and colleagues, who demonstrated that *Listeria*-derived proteins from LLO$^+$ and LLO$^-$ *L. monocytogenes* could be presented to *Listeria*-specific T-cell lines (20). Our system is, however, distinct from their study, as Szalay et al. utilized T-cell lines that were raised against cells infected with heat-killed *Listeria* species, whereas the p60-specific T-cell clone used in the present study was raised against live bacteria and was unable to lyse cells infected with heat-killed *L. monocytogenes*. It is conceivable that the presentation pathways for live and dead bacteria are different.

The finding that *L. monocytogenes* bacteria do not need to reside in the cytosol for their antigens to be presented by class I molecules is consistent with the finding that CD8$^+$ CTL

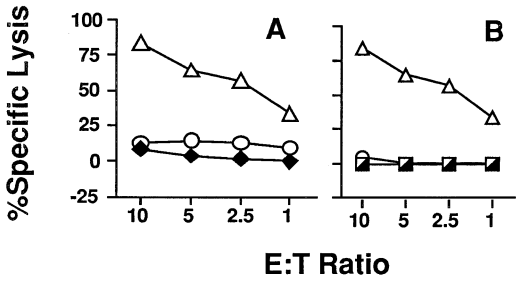


FIG. 2. p60-specific CTL do not recognize J774A.1 cells infected with heat-killed *L. monocytogenes* (A) or pulsed with supernatant from a log-phase *L. monocytogenes* culture ($10^9$ bacteria) (B). The target cells are denoted by the following symbols: open circles, uninfected cells; open triangles, cells pulsed with the p60 peptide from residues 217 to 225; half-filled squares, cells infected with heat-killed *L. monocytogenes*; filled diamonds, cells pulsed with supernatant from log-phase culture. APCs were infected as described in the text. E:T ratio, ratio of effector cells to target cells.

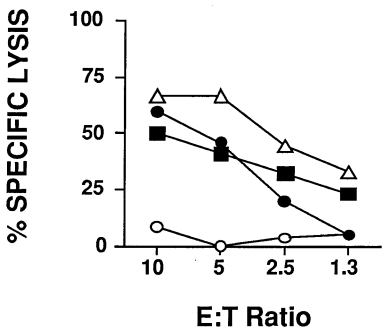


FIG. 3. Cytotoxic reactivity of p60-specific CTL clone for J774A.1 cells infected with *L. monocytogenes* or *L. innocua*. The target cells are denoted by the following symbols: open circles, uninfected cells; filled squares, cells infected with wild-type *L. monocytogenes*; filled circles, cells infected with *L. innocua*; open triangles, cells pulsed with the p60 peptide from residues 217 to 225. E:T ratio, ratio of effector cells to target cells.

killing can be generated in response to infection with other organisms, such as *Chlamydia trachomatis* (1), *Yersinia enterocolitica* (19), and *Mycobacterium tuberculosis* (4, 14, 17), which do not reach the cytosol of the infected cell.

Several mechanisms have been proposed to explain the presentation of noncytosolic antigens. Harding et al. have demonstrated that macrophages which phagocytose particulate antigens can "regurgitate" peptides that are subsequently loaded on adjacent cells (6, 7). This process is quick, requiring only 45 min to occur, and is not blocked by brefeldin A, suggesting that the processing of new class I molecules and intracellular loading is not required. A similar mechanism may account for class I presentation of antigens from *Y. enterocolitica* (19). Other researchers have obtained evidence that components of the classical class I processing pathway are required for processing antigens derived from the phagosome (10, 15, 18). In these systems, antigenic proteins phagocytosed together with beads are processed by the classical class I pathway. Although the beads are retained in the phagosome, the integrity of the phagosomal membrane may be compromised, enabling entry of the antigenic protein into the cytosol.

Several groups have used *L. monocytogenes* as a recombinant vaccine vector in experimental systems (9, 12, 16). In these systems, the *L. monocytogenes* used is $LLO^+$ and potentially virulent. If other proteins share the properties of p60 in that they are able to be processed and presented by the class I presentation pathway even if they are produced by $LLO^-$ *L. monocytogenes* strains that remain in the phagosome, then it may be plausible to utilize avirulent *L. monocytogenes* to target recombinant vaccines to the class I pathway.

In conclusion, we have demonstrated that it is possible for antigens from an intracellular pathogen to be presented by class I molecules even if the organism remains in the phagosome. Whether this is a general phenomenon or is a characteristic influenced by the nature of the protein is unknown at this stage.

This research was supported by grants AI28115 and AI37905 from the National Institutes of Health. T.A.P. is a scholar of the Leukemia Society of America.

*Editor:* S. H. E. Kaufmann

We thank Eric Pamer for supplying the p60-specific cytotoxic T-cell line.

## REFERENCES

1. **Beatty, P. R., and R. S. Stephens.** 1994. CD8+ T lymphocyte-mediated lysis of *Chlamydia*-infected L cells using an endogenous antigen pathway. J. Immunol. **153:**4588–4595.
2. **Bouwer, H. G. A., C. S. Nelson, B. L. Gibbins, D. A. Portnoy, and D. J. Hinrichs.** 1992. Listeriolysin O is a target of the immune response to *Listeria monocytogenes*. J. Exp. Med. **175:**1467–1471.
3. **Bubert, A., M. Kuhn, W. Goebel, and S. Köhler.** 1992. Structural and functional properties of the p60 proteins from different *Listeria* species. J. Bacteriol. **174:**8166–8171.
4. **De Libero, G., I. Flesch, and S. H. E. Kaufmann.** 1988. Mycobacteria-reactive Lyt-2+ T cell lines. Eur. J. Immunol. **18:**59–66.
5. **Gaillard, J. L., P. Berche, and P. Sansonetti.** 1986. Transposon mutagenesis as a tool to study the role of hemolysin in the virulence of *Listeria monocytogenes*. Infect. Immun. **52:**50–55.
6. **Harding, C. V., and R. Song.** 1994. Phagocytic processing of exogenous particulate antigens by macrophages for presentation by class I MHC molecules. J. Immunol. **153:**4925–4933.
7. **Harding, C. V., R. Song, J. Griffin, J. France, M. J. Wick, J. D. Pfeifer, and H. J. Geuze.** 1995. Processing of bacterial antigens for presentation to class I and II MHC-restricted T lymphocytes. Infect. Agents Dis. **4:**1–12.
8. **Hess, J., I. Gentschev, G. Szalay, C. Ladel, A. Bubert, W. Goebel, and S. H. E. Kaufmann.** 1995. *Listeria monocytogenes* p60 supports host cell invasion by and in vivo survival of attenuated *Salmonella typhimurium*. Infect. Immun. **63:**2047–2053.
9. **Ikonomidis, G., Y. Paterson, F. J. Kos, and D. A. Portnoy.** 1994. Delivery of a viral antigen to the class I processing and presentation pathway by *Listeria monocytogenes*. J. Exp. Med. **180:**2209–2218.
10. **Kovacsovics-Bankowski, M., and K. L. Rock.** 1995. A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. Science **267:**243–246.
11. **Pamer, E. G.** 1994. Direct sequence identification and kinetic analysis of an MHC class I-restricted *Listeria monocytogenes* CTL epitope. J. Immunol. **152:**686–694.
12. **Pan, Z.-K., G. Ikonomidis, A. Lazenby, D. Pardoll, and Y. Paterson.** 1995. A recombinant listeria monocytogenes vaccine expressing a model tumour antigen protects mice against lethal tumour cell challenge and causes regression of established tumours. Nat. Med. **1:**471–477.
13. **Portnoy, D. A., P. S. Jacks, and D. J. Hinrichs.** 1988. Role of hemolysin for the intracellular growth of *Listeria monocytogenes*. J. Exp. Med. **167:**1459–1471.
14. **Rees, A., A. Scoging, A. Mehlert, D. B. Young, and J. Ivanyi.** 1988. Specificity of proliferative response of human CD8 clones to mycobacterial antigens. Eur. J. Immunol. **18:**1881–1887.
15. **Rock, K. L., C. Gramm, L. Rothstein, K. Clark, R. Stein, L. Dick, D. Hwang, and A. L. Goldberg.** 1994. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell **78:**761–771.
16. **Shen, H., M. K. Slifka, M. Matloubain, E. R. Jensen, R. Ahmed, and J. F. Miller.** 1995. Recombinant *Listeria monocytogenes* as a live vaccine vehicle for the induction of protective anti-viral cell-mediated immunity. Proc. Natl. Acad. Sci. USA **92:**3987–3991.
17. **Silva, C. L., M. F. Silva, R. C. Pietro, and D. B. Lowrie.** 1994. Protection against tuberculosis by passive transfer with T-cell clones recognizing mycobacterial heat-shock protein 65. Immunology **83:**341–346.
18. **Sousa, C. S., and R. N. Germain.** 1995. Major histocompatibility complex class I presentation of peptides derived from soluble exogenous antigen by a subset of cells engaged in phagocytosis. J. Exp. Med. **182:**841–851.
19. **Starnbach, M. N., and M. J. Bevan.** 1994. Cells infected with Yersinia present an epitope to class I MHC-restricted CTL. J. Immunol. **153:**1603–1612.
20. **Szalay, G., J. Hess, and S. H. E. Kaufmann.** 1994. Presentation of *Listeria monocytogenes* antigens by major histocompatibility complex class I molecules to CD8 cytotoxic T lymphocytes independent of listeriolysin secretion and virulence. Eur. J. Immunol. **24:**1471–1477.