INFECTION AND IMMUNITY, Dec. 1997, p. 5326–5329 Vol. 65, No. 12
0019-9567/97/$04.00+0

A Nonamer Peptide Derived from *Listeria monocytogenes* Metalloprotease Is Presented to Cytolytic T Lymphocytes

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Received 10 June 1997/Returned for modification 26 August 1997/Accepted 19 September 1997

***Listeria monocytogenes* is an intracellular bacterium that secretes proteins into the cytosol of infected macrophages. Major histocompatibility complex (MHC) class I molecules bind peptides that are generated by the degradation of bacterial proteins and present them to cytolytic T lymphocytes (CTL). In this study we have investigated CTL responses in *L. monocytogenes*-immunized mice to peptides that (i) derive from the *L. monocytogenes* proteins phosphatidylinositol-specific phospholipase C, lecithinase (most active on phosphatidylcholine), metalloprotease (Mpl), PrfA, and the ORF-A product and (ii) conform to the binding motif of the H2-K^d MHC class I molecule. We identified a nonamer peptide, Mpl 84–92, that is presented to *L. monocytogenes*-specific CTL by H2-K^d MHC class I molecules. Unlike other motif-conforming peptides derived from the secreted Mpl of *L. monocytogenes*, Mpl 84–92 is bound with high affinity by H2-K^d. Mpl 84–92 is the fourth *L. monocytogenes*-derived peptide found to be presented to CTL by the H2-K^d molecule during infection and demonstrates the importance of high-affinity interactions between antigenic peptides and MHC class I molecules for CTL priming.**

Listeria monocytogenes is a gram-positive bacterium that causes disease in a broad range of mammalian hosts (9). Productive infection of mammalian hosts requires the expression of listeriolysin O (LLO) by *L. monocytogenes* (8, 15, 24). LLO lyses the phagosomal membrane surrounding engulfed bacteria, permitting access to the host cell cytosol (1, 29). While LLO is the major and essential factor required for *L. monocytogenes* virulence, several other proteins are required for full virulence. For example, to be maximally virulent, cytosolic *L. monocytogenes* must express ActA, a surface-associated protein that nucleates actin assembly and endows intracytosolic bacteria with mobility and the ability to spread to neighboring cells (6, 16). Similarly, strains of *L. monocytogenes* that lack either or both of two phospholipases, phosphatidylinositol-specific phospholipase C (PI-PLC) and lecithinase (most active on phosphatidylcholine) (PC-PLC), have diminished virulence (4, 28). An additional secreted protein, the *L. monocytogenes* metalloprotease (Mpl), participates in the activation of PC-PLC and thus appears to play an indirect role in virulence (5, 19, 25). The genes for these virulence factors are present in a small region of the *L. monocytogenes* chromosome and are all regulated by the transcription factor PrfA (23). Several additional open reading frames (ORF-X, -Y, -Z, -A, and -B) in or adjacent to this region have also been identified, but the function of their protein products has not been ascertained (31). Strains of *L. monocytogenes* deficient in PrfA have markedly diminished or no expression of these virulence factors and are avirulent (17).

In mice, infection with a sublethal dose of *L. monocytogenes* primes a vigorous, major histocompatibility complex (MHC) class I-restricted cytolytic-T-lymphocyte (CTL) response that enables the rapid systemic clearance of live bacteria (21). Several peptide targets of *L. monocytogenes*-specific CTLs have been identified. Three peptides, LLO 91–99, p60 217–225, and p60 449–457, are presented to CTLs by the H2-K^d MHC class I molecule (20, 22, 27). Two other peptides, which contain formyl-methionine at the amino terminus, are presented to CTLs by the H2-M3 MHC class Ib molecule (10, 18). A common feature of these five peptides is that they either are directly released by *L. monocytogenes* or are processed by the host cell from larger, bacterially secreted proteins. The sensible notion that secreted antigens have the greatest access to the antigen-processing pathways is supported by findings obtained by members of our laboratory (11, 20, 22) and by others (12, 13).

Because the H2-K^d molecule has been shown to play a major role in the presentation of *L. monocytogenes* antigens to CTLs

TABLE 1. Sequences of H2-K^d motif-conforming *L. monocytogenes* peptides

Peptide	Sequence[a]
PrfA 61–69	Gln-Tyr-Tyr-Lys-Gly-Ala-Phe-Val-Ile
PrfA 94–102	Ala-Tyr-Val-Ile-Lys-Ile-Asn-Glu-Leu
PrfA 206–214	Phe-Tyr-Val-Gln-Asn-Leu-Asp-Tyr-Leu
OrfA 34–42	Leu-Tyr-Gly-Leu-Lys-Ile-Gly-Asp-Leu
OrfA 137–145	Ile-Tyr-Ser-Glu-His-Ile-Asn-Asn-Leu
OrfA 167–175	Arg-Tyr-Ser-Met-Asn-Gly-Phe-Ile-Ile
PlcA 4–12	Asn-Tyr-Leu-Gln-Arg-Thr-Leu-Val-Leu
PlcA 92–100	Leu-Tyr-Gln-Gln-Leu-Glu-Ala-Gly-Ile
PlcB 110–118	Pro-Tyr-Tyr-Asp-Thr-Ser-Thr-Phe-Leu
PlcB 195–203	Ala-Tyr-Glu-Asn-Tyr-Val-Asp-Thr-Ile
Mpl 84–92	Gly-Tyr-Leu-Thr-Asp-Asn-Asp-Glu-Ile
Mpl 289–297	Glu-Tyr-Tyr-Lys-Asn-Val-His-Gln-Leu
Mpl 363–371	Glu-Tyr-Glu-Gly-Gln-Ser-Gly-Ala-Leu

[a] These peptide sequences are derived from the deduced amino acid sequences of PrfA (17), OrfA and PlcB (31), PlcA (3), and Mpl (5).

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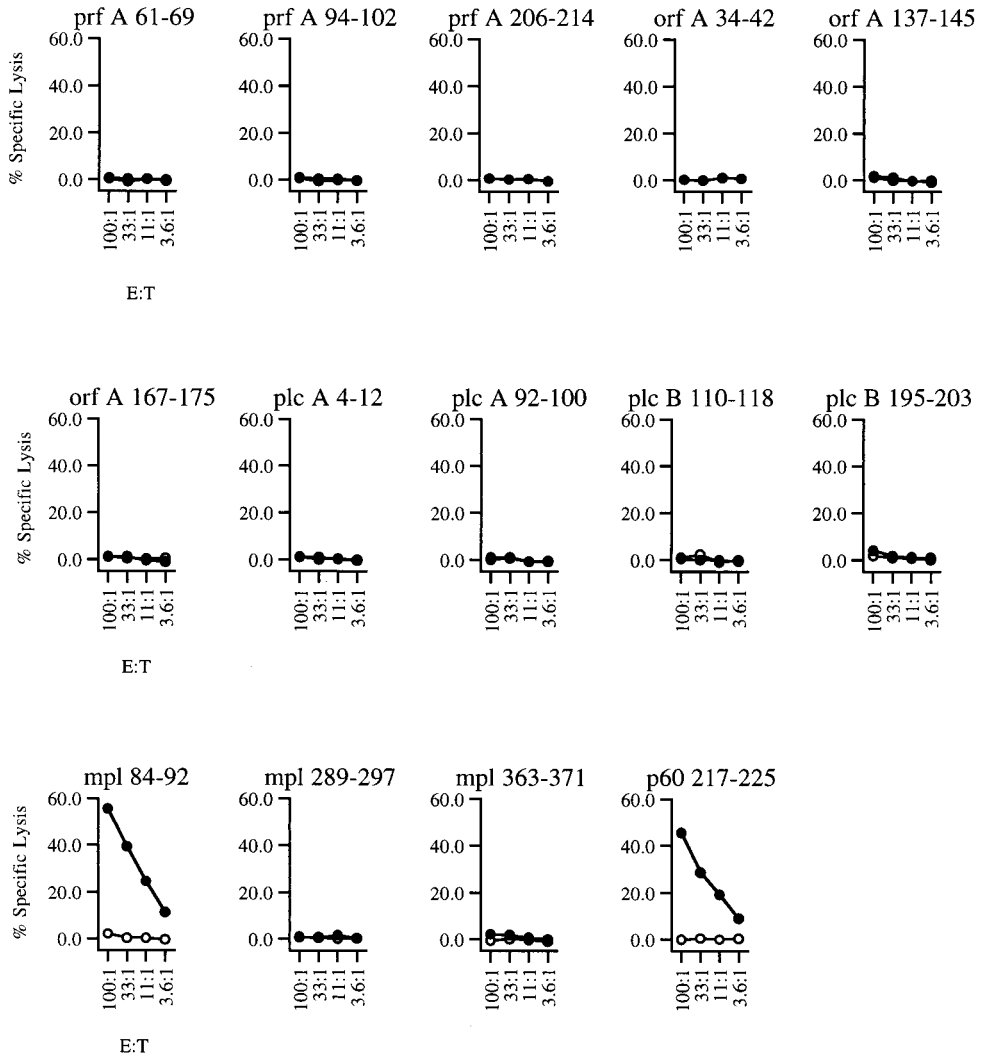

FIG. 1. *L. monocytogenes*-specific CTLs recognize Mpl 84–92. CB6 mice were immunized with a sublethal dose of *L. monocytogenes*, and 7 days later splenocytes were restimulated in vitro with each of the H2-K^d motif-conforming peptides. Five days after restimulation, peptide-specific CTL activity was tested with ^{51}Cr-labeled P815 cells in a standard ^{51}Cr release assay. The percent specific lysis is plotted for four different dilutions of CTLs in the presence (closed circles) and absence (open circles) of the respective targeting peptide. E:T, effector/target ratio.

(2, 20, 22, 27), we decided to scan several other known *L. monocytogenes* proteins for nonamer peptide sequences that conform with the H2-K^d peptide binding motif. Since H2-K^d binds peptides that contain tyrosine in the second position from the amino terminus and either leucine or isoleucine in the ninth, carboxy-terminal position (7), peptides that conform to this sequence were synthesized. Because PrfA-regulated proteins are associated with virulence, we reasoned that these proteins might be targets for *L. monocytogenes*-specific CTLs. Thirteen peptides derived from PrfA, PlcA, PlcB, the Orf-A product, and Mpl were identified (Table 1) and tested for the ability to stimulate *L. monocytogenes*-immune murine splenocytes. C57BL/6 × BALB/c F_1 (*H2^b* × *H2^d*) (CB6) mice were infected intravenously with a sublethal dose (5,000 organisms) of *L. monocytogenes* ATCC 43251, and 7 days later immune splenocytes were isolated. Immune splenocytes were restimulated in vitro with peptide-coated (10^{-6} M), irradiated (3,000 rads), naive CB6 splenocytes as previously described (20, 27). Five days later, restimulated splenocytes were assayed for peptide-specific cytolytic activity, using ^{51}Cr-labeled P815 (H2^d) target cells in the presence of the respective stimulating peptide. A known target of *L. monocytogenes*-specific CTLs, p60 217–225, was included as a positive control (20). Indeed, immune splenocytes restimulated with p60 217–225 lysed peptide-coated target cells (Fig. 1). Immune splenocytes did not respond to any of the other peptides except one (Fig. 1), Mpl 84–92, corresponding to amino acids 84 to 92 of the secreted *L. monocytogenes* Mpl. The response to Mpl 84–92 5 days after in vitro peptide restimulation is quantitatively similar to the response to p60 217–225. Of note, splenocytes from unimmunized mice did not respond to in vitro restimulation with either p60 217–225 or Mpl 84–92 (results not shown). Staining with anti-CD8 and anti-CD4 antibodies revealed that Mpl 84–92-specific CTLs are exclusively CD8$^+$ CD4$^-$ (results not shown). Thus, Mpl 84–92 is the fourth identified peptide that is presented to CTLs by the H2-K^d MHC class I molecule during *L. monocytogenes* infection. In common with LLO 91–99, p60 217–225, and p60 449–457, Mpl 84–92 derives from a secreted protein. Interestingly, Mpl 84–92 derives from the predicted pro-region of Mpl (6), suggesting that entry of Mpl into the

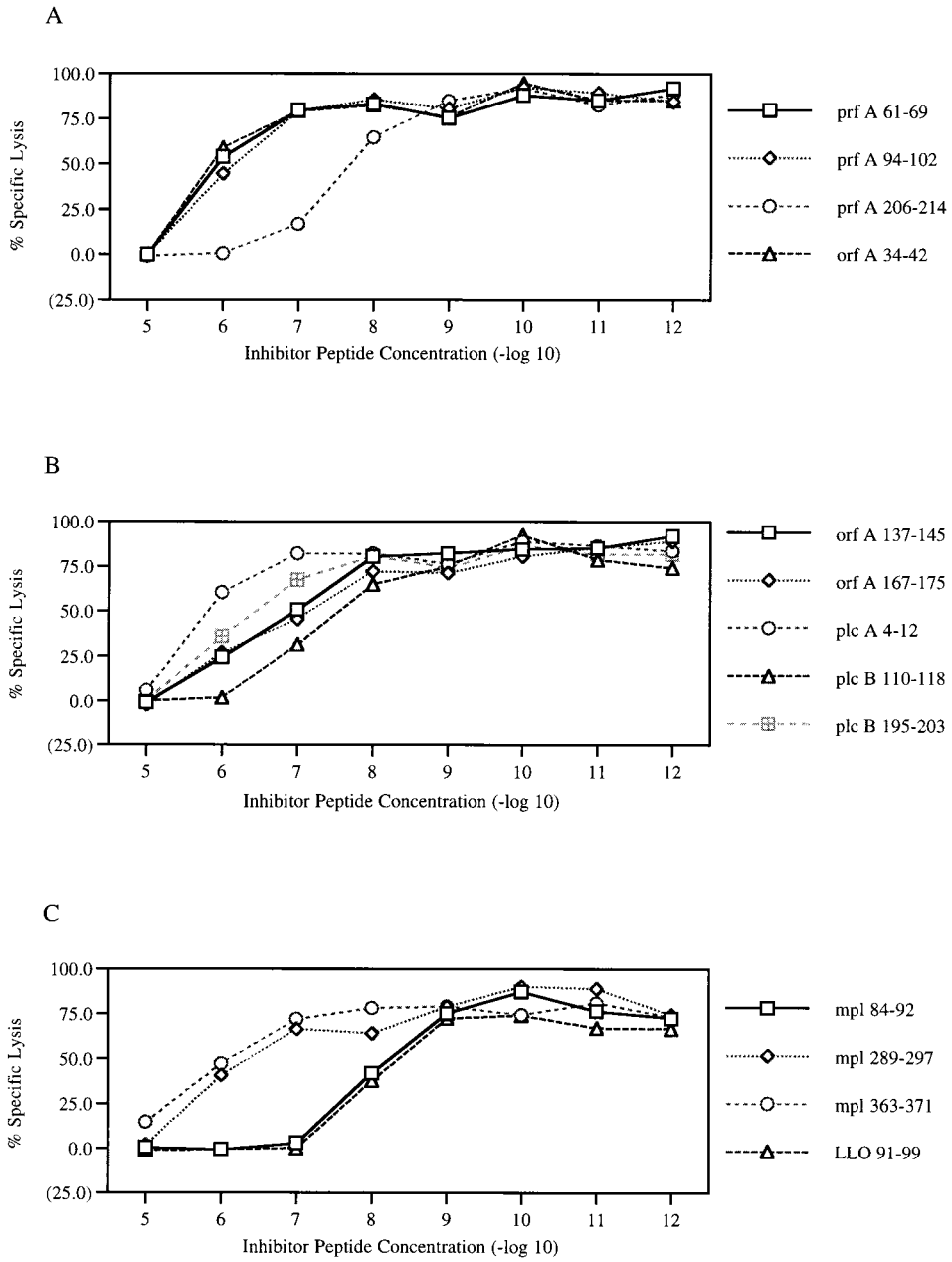

FIG. 2. Mpl 84–92 binds to H2-K^d with high affinity. ^{51}Cr-labeled P815 target cells were incubated in the presence of 5×10^{-11} M p60 217–225 and a range of concentrations of each of the H2-K^d motif-conforming peptides. The percent specific lysis of target cells obtained with CTL clone L9.6 (specific for p60 217–225) was determined and plotted.

MHC class I antigen processing pathway may be linked to activation of the proenzyme.

Many of the H2-K^d motif-conforming peptides were not detected by immune splenocytes (Fig. 1). Similarly, several H2-K^d motif-conforming peptides derived from LLO and p60 were also not detected by immune splenocytes (27, 32). In the case of p60- and most LLO-derived peptides, the nonantigenic peptides were bound by H2-K^d with significantly lower affinity than the antigenic peptides (27, 32). To determine if the nonantigenic peptides described in this report might similarly be of lower affinity, we performed a peptide competition experiment (32). ^{51}Cr-labeled P815 target cells were incubated in the presence of 5×10^{-11} M p60 217–225 and various concentrations of each of the H2-K^d motif-conforming peptides. CTL clone L9.6, which is specific for p60 217–225 (20), was added to the target cells and the percent specific lysis was determined. Peptides that bind H2-K^d with high affinity would be expected to block p60 217–225-specific lysis at relatively low concentrations because of effective competition for peptide-receptive H2-K^d molecules. In contrast, lower-affinity peptides must be present at higher concentrations to block lysis. LLO 91–99, a peptide that associates with H2-K^d with high affinity (32), and Mpl 84–92 were able to block p60 217–225-specific lysis at lower concentrations than any of the other peptides (Fig. 2). Only PrfA 206–214 (Fig. 2A) and PlcB 110–118 (Fig. 2B) came close to competing as effectively as LLO 91–99 and Mpl 84–92 for

H2-K^d binding. Since Mpl 84–92 competes for H2-K^d binding as effectively as the other three known peptides presented by H2-K^d, our findings confirm the importance of MHC/peptide affinity in determining antigenicity. Why some peptides, such as PrfA 206–214, do not prime specific CTLs following *L. monocytogenes* infection remains unknown, but the reason may relate to a lack of PrfA access to the MHC class I antigen processing pathway. Alternatively, peptides such as PrfA 206–214 may not be generated by host cell proteasomes or they may not be transported by the transporter associated with antigen processing. Yet another possibility is that these peptides are processed and presented by infected cells but that the T-cell repertoire of infected mice does not detect them. Clarifying these issues will require further investigations.

LLO 91–99, p60 217–225, p60 449–457, and Mpl 84–92 all derive from secreted *L. monocytogenes* proteins and associate with H2-K^d with high affinity. It is tempting to speculate at this point that both antigen secretion and high-affinity peptide binding are essential for the priming of a potent CTL response. While evidence for the importance of peptide binding affinity is compelling (14, 26, 27, 30, 32), the requirement for protein secretion remains undetermined. In this study, the peptides derived from PrfA, a nonsecreted transcription factor, all associated with H2-K^d with lower affinity than the antigenic peptides. Thus, determining the importance of protein secretion by intracellular *L. monocytogenes* for CTL priming will require further experiments.

This work was supported by Public Health Service grant AI-33143. E.G.P. is a Scholar of the Pew Charitable Trust. D.H.B. is supported by the Deutsche Forschungsgemeinschaft (DFG).

REFERENCES

1. **Bielecki, J., P. Youngman, P. Connelly, and D. A. Portnoy.** 1990. Bacillus subtilis expressing a haemolysin gene from Listeria monocytogenes can grow in mammalian cells. Nature **345:**175–176.
2. **Bouwer, H. G., K. F. Lindahl, J. R. Baldridge, C. R. Wagner, R. A. Barry, and D. J. Hinrichs.** 1994. An H2-T MHC class Ib molecule presents Listeria monocytogenes-derived antigen to immune CD8+ cytotoxic T cells. J. Immunol. **152:**5352–5360.
3. **Camilli, A., H. Goldfine, and D. A. Portnoy.** 1991. Listeria monocytogenes mutants lacking phosphatidylinositol-specific phospholipase C are avirulent. J. Exp. Med. **173:**751–754.
4. **Camilli, A., L. G. Tilney, and D. A. Portnoy.** 1993. Dual roles of plcA in Listeria monocytogenes pathogenesis. Mol. Microbiol. **8:**143–157.
5. **Domann, E., M. Leimeister-Wächter, W. Goebel, and T. Chakraborty.** 1991. Molecular cloning, sequencing, and identification of a metalloprotease gene from *Listeria monocytogenes* that is species specific and physically linked to the listeriolysin gene. Infect. Immun. **59:**65–72.
6. **Domann, E., J. Wehland, M. Rohde, S. Pistor, M. Hartl, W. Goebel, M. Leimeister-Wachter, and T. Chakraborty.** 1992. A novel bacterial virulence gene in Listeria monocytogenes required for host cell microfilament interaction with homology to the proline-rich region of vinculin. EMBO J. **11:** 1981–1990.
7. **Falk, K., O. Roetzschke, S. Stevanovic, G. Jung, and H.-G. Rammensee.** 1991. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature **351:**290–296.
8. **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.
9. **Gellin, B. G., and C. V. Broome.** 1989. Listeriosis. JAMA **261:**1313–1320.
10. **Gulden, P. H., P. Fischer, N. E. Sherman, W. Wang, V. H. Engelhard, J. Shabanowitz, D. H. Hunt, and E. G. Pamer.** 1996. A Listeria monocytogenes pentapeptide is presented to cytolytic T lymphocytes by the H2-M3 MHC class Ib molecule. Immunity **5:**73–79.
11. **Harty, J. T., and M. J. Bevan.** 1992. CD8 T cells specific for a single nonamer epitope of Listeria monocytogenes are protective in vivo. J. Exp. Med. **175:**1531–1540.
12. **Hess, J., I. Gentschev, D. Miko, M. Welzel, C. Ladel, W. Goebel, and S. H. E. Kaufmann.** 1996. Superior efficacy of secreted over somatic antigen display in recombinant Salmonella vaccine induced protection against listeriosis. Proc. Natl. Acad. Sci. USA **93:**1458–1463.
13. **Horwitz, M. A., B. W. Lee, B. J. Dillon, and G. Harth.** 1995. Protective immunity against tuberculosis induced by vaccination with major extracellular proteins of Mycobacterium tuberculosis. Proc. Natl. Acad. Sci. USA **92:**1530–1534.
14. **Jameson, S., and M. J. Bevan.** 1992. Dissection of major histocompatibility complex and T cell receptor contact residues in a Kb-restricted ovalbumin peptide and an assessment of the predictive power of MHC-binding motifs. Eur. J. Immunol. **22:**2263–2269.
15. **Kathariou, S., P. Metz, H. Hof, and W. Goebel.** 1987. Tn*916*-induced mutations in the hemolysin determinant affecting virulence of *Listeria monocytogenes*. J. Bacteriol. **169:**1291–1297.
16. **Kocks, C., E. Gouin, M. Tabouret, P. Berche, H. Ohayon, and P. Cossart.** 1992. L. monocytogenes-induced actin assembly requires the actA gene product, a surface protein. Cell **68:**521–531.
17. **Leimeister-Wachter, M., C. Haffner, E. Domann, W. Goebel, and T. Chakraborty.** 1990. Identification of a gene that positively regulates expression of listeriolysin, the major virulence factor of Listeria monocytogenes. Proc. Natl. Acad. Sci. USA **87:**8336–8340.
18. **Lenz, L. L., B. Dere, and M. J. Bevan.** 1996. Identification of an H2-M3-restricted Listeria epitope: implications for antigen presentation by M3. Immunity **5:**63–72.
19. **Marquis, H., V. Doshi, and D. A. Portnoy.** 1995. The broad-range phospholipase C and a metalloprotease mediate listeriolysin O-independent escape of *Listeria monocytogenes* from a primary vacuole in human epithelial cells. Infect. Immun. **63:**4531–4534.
20. **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.
21. **Pamer, E. G.** 1997. Immune response to Listeria monocytogenes, p. 131–142. *In* S. H. E. Kaufmann (ed.), Host response to intracellular pathogens. R. G. Landes Company Biomedical Publishers, Austin, Tex.
22. **Pamer, E. G., J. T. Harty, and M. J. Bevan.** 1991. Precise prediction of a dominant class I MHC-restricted epitope of Listeria monocytogenes. Nature **353:**852–855.
23. **Portnoy, D. A., T. Chakraborty, W. Goebel, and P. Cossart.** 1992. Molecular determinants of *Listeria monocytogenes* pathogenesis. Infect. Immun. **60:** 1263–1267.
24. **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.
25. **Poyart, C., E. Abachin, I. Razafimanantsoa, and P. Berche.** 1993. The zinc metalloprotease of *Listeria monocytogenes* is required for maturation of phosphatidylcholine phospholipase C: direct evidence obtained by gene complementation. Infect. Immun. **61:**1576–1580.
26. **Sette, A., A. Vitiello, B. Reherman, P. Fowler, R. Nayersina, W. M. Kast, C. J. Melief, C. Oseroff, L. Yuan, J. Ruppert, J. Sidney, M.-F. del Guerico, S. Southwood, R. T. Kubo, R. W. Chestnut, H. M. Grey, and F. V. Chisari.** 1994. The relationship between class I binding affinity and immunogenicity of potential cytotoxic T cell epitopes. J. Immunol. **153:**5586–5592.
27. **Sijts, A. J. A. M., A. Neisig, J. Neefjes, and E. G. Pamer.** 1996. Two Listeria monocytogenes CTL epitopes are processed from the same antigen with different efficiencies. J. Immunol. **156:**685–692.
28. **Smith, G. A., H. Marquis, S. Jones, N. C. Johnston, D. A. Portnoy, and H. Goldfine.** 1995. The two distinct phospholipases C of *Listeria monocytogenes* have overlapping roles in escape from a vacuole and cell-to-cell spread. Infect. Immun. **63:**4231–4237.
29. **Tilney, L. G., and D. A. Portnoy.** 1989. Actin filaments and the growth, movement, and spread of the intracellular parasite, Listeria monocytogenes. J. Cell Biol. **109:**1597–1608.
30. **van den Burg, S. H., M. J. W. Visseren, R. M. P. Brandt, W. M. Kast, and C. J. M. Melief.** 1996. Immunogenicity of peptides bound to MHC class I molecules depends on the MHC-peptide complex stability. J. Immunol. **156:**3308–3314.
31. **Vazquez-Boland, J.-A., C. Kocks, S. Dramsi, H. Ohayon, C. Geoffroy, J. Mengaud, and P. Cossart.** 1992. Nucleotide sequence of the lecithinase operon of *Listeria monocytogenes* and possible role of lecithinase in cell-to-cell spread. Infect. Immun. **60:**219–230.
32. **Wipke, B. T., S. C. Jameson, M. J. Bevan, and E. G. Pamer.** 1993. Variable binding affinities of listeriolysin O peptides for the H2-Kd class I molecule. Eur. J. Immunol. **23:**2005–2010.

Editor: S. H. E. Kaufmann