Induction of Cell-Mediated Immune Responses to Human Immunodeficiency Virus Type 1 Gag Protein by Using *Listeria monocytogenes* as a Live Vaccine Vector[1]

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Cytolytic T cells, acting through cytokines or by direct lysis of infected target cells, have been shown to play a significant role in the control of viral infections and may be responsible for the prolonged asymptomatic phase following infection by HIV. Accordingly, methods that can generate strong cell-mediated immune responses may be useful in the development of prophylactic and therapeutic vaccines against HIV. *Listeria monocytogenes* is a Gram-positive intracellular microorganism that elicits strong cell-mediated immune responses against its own secreted proteins following infection. In this study we have modified the chromosome of *L. monocytogenes* so that it stably expresses and secretes the p55 HIV *gag* gene product and examined the cell-mediated immune response of BALB/c mice to infection with this recombinant organism. Infected animals were found to mount a specific, strong, long-lasting $CD8^+$ cytolytic T cell response against a predominant epitope contained within the p24 fragment of the HIV Gag protein. This epitope previously has been shown to be recognized by CTLs obtained from some HIV-infected humans. Our results suggest that chromosomally modified strains of *L. monocytogenes* may provide valuable vaccine vectors for use against HIV. *The Journal of Immunology*, 1995, 155: 4775–4782.

Despite occasional successes of trials in animal models designed to explore protection against HIV or SIV[3] infection by immunization with inactivated whole virus, purified envelope proteins, or attenuated mutants (1–5), the prospect of achieving an effective prophylactic or therapeutic vaccine in the near future appears doubtful. This view is held because the true correlates of protection against HIV are still largely unknown. HIV-infected individuals generally express strong humoral Ab responses against the virus, as well as vigorous cytotoxic T lymphocyte responses, but these responses ultimately fail to protect against disease. CTL responses, however, are known to be important in the control of many viral infections (6–10) and to act through the release of IFN-γ and probably other uncharacterized mediators, as well as by direct lysis and elimination of virus-infected cells (6). Indeed, it has been shown that lymphocytes from HIV-infected patients often generate higher titers of HIV in vitro when $CD8^+$ T cells are first eliminated from such cultures (11–13). Furthermore, recent studies of small groups of patients have shown that CTLs directed against various HIV-1 proteins appear early after infection, coincidental with the resolution of viremia, in several cases before seroconversion, and before the appearance of assayable neutralizing Ab (14, 15). Early CTL responses have also been seen after SIV_{mac} infection of rhesus monkeys before seroconversion (16). It is possible, therefore, that CTLs are responsible for the prolonged asymptomatic phase following HIV infection, but that CTL activity eventually declines as a result of increasing destruction of the immune system.

Because CTLs can potentially eliminate cells infected by HIV and might prevent the early dissemination of infection in an infected individual, methods that are capable of generating strong CTL responses may be useful in the development of prophylactic as well as therapeutic vaccines against HIV. *Listeria monocytogenes* is a facultative intracellular bacterium that, following internalization into vacuoles in infected host cells, is released by dissolution of the vacuolar membrane to grow freely in the host cell cytoplasm (17). This cytoplasmic localization gives *L. monocytogenes* proteins direct access to the cellular arm of the immune response through the MHC class I pathway of Ag presentation, thus explaining why the immune response to this organism has been a historical paradigm for the study of T cell-mediated immunity (18–20).

In this article we describe a genetic method to stably modify the chromosome of *L. monocytogenes* so that HIV gene products can be expressed as secreted proteins under the control of a copy of the strong promoter of the hemolysin gene, which encodes listeriolysin-*O*. We demonstrate that mice immunized with one of these constructs, Lm-*gag*, mount a strong, specific, long-lasting CTL response against the HIV-1 Gag protein, and that the response is directed predominantly against an epitope present in the p24 portion of the protein.

Materials and Methods

Construction of bacterial strains

L. monocytogenes strain 10403S (21) was the wild-type organism used in these studies. It was grown in brain/heart infusion (BHI) medium (Difco Labs, Detroit, MI) and has an LD_{50} of approximately 3×10^4 when injected i.v. or i.p. into BALB/c mice (22).

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[3] Abbreviations used in this paper: SIV, simian immunodeficiency virus; BHI, brain/heart infusion (medium); BCG, bacillus Calmette-Guérin; Lm-*gag*, recombinant strain of *L. monocytogenes* carrying the HIV-1 *gag* gene.

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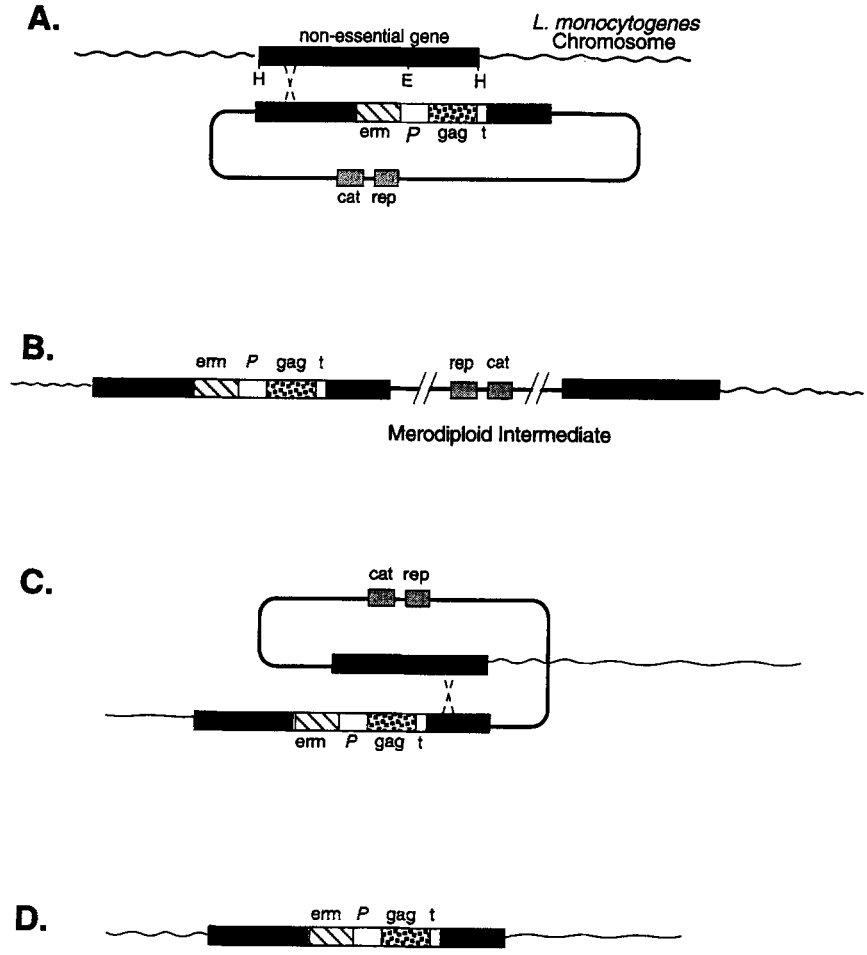

FIGURE 1. Construction by homologous chromosomal recombination of stable strains of *L. monocytogenes* that express and secrete HIV gene products. *A*, A derivative of plasmid pSKV7 was constructed to contain a copy of a nonessential region from the *L. monocytogenes* chromosome (solid black). Into the plasmid copy of this region was inserted an erythromycin resistance gene (*erm*), the promotor-signal sequence of the *L. monocytogenes* hemolysin gene (*P*), an HIV gene (*gag*), and the transcription termination sequence of the hemolysin gene (*t*). Recombinants, with the merodiploid structure shown in *B*, in which the plasmid had integrated by homologous recombination into the chromosome of *L. monocytogenes*, were selected by growth in chloramphenicol at 42°C. The designated cross-over point is arbitrary. *C*, As a result of a second random cross-over event in the second half of the nonessential region, recombinant *erm*^{R} *cm*^{S} bacteria were selected that had deleted all plasmid sequences except those contained within the homologous region, as shown in *D*. H, *Hin*dIII; E, *Eco*RI.

The chromosome of *L. monocytogenes* was modified by using the shuttle vector pKSV7 (23) and a protocol modified from Camilli et al. (24). A 2.7-kb *Hin*dIII fragment that originated from an apparently nonessential region of the *L. monocytogenes* chromosome (24) was introduced into a *Hin*dIII site on the plasmid to provide a homologous region for subsequent recombination of the plasmid into the chromosome. A multicloning sequence was inserted into an *Eco*RI site at 1.65-kb on the *Hin*dIII fragment, and the following DNA fragments were ligated sequentially into the plasmid at this site: 1) a PCR copy of the 288-nucleotide promoter sequence of the *L. monocytogenes* hemolysin gene, which contained the *prfA*-binding palindrome (25) and the first 30 amino acids (signal sequence plus 4 additional amino acids) of the gene product; PCR primers were 5′-GGGTC GACTTTTATGTGGAGGCAT-3′ (*Sal*I site underlined) and 5′-CGG GATCCGAATGCAGATGCATCCTTTG-3′ (*Bam*HI site underlined); 2) a PCR copy of the 1418-bp *gag* gene sequence of HIV-1 HXB or 89.6 (26), starting at codon 29; PCR primers were 5′-GGGGATCCTATAAATTA AAACATATAGTATG-3′ (*Bam*HI site underlined) and 5′-GGGGTAC CTCTAGATTATTGTGACGAGGGGT-3′ (*Kpn*I and *Xba*I sites, respectively, underlined); 3) a PCR copy of the A-T-rich 126-bp *L. monocytogenes* hemolysin gene transcription termination sequence; PCR primers were 5′-CCTCTAGATTGTAAAAGTAATAAAAAATTAAG-3′ (*Xba*I site underlined) and 5′-CCGGTACCTAAAAAAATTAAAAAATAAGC-3′ (*Kpn*I site underlined); this last sequence was introduced to assure the stable synthesis of transcripts of proper length; 4) a 2259-bp sequence from Tn*917* (27) that codes for erythromycin resistance, inserted at the *Sal*I site at the start of this construct; this gene was introduced to facilitate the selection of appropriate recombinants.

The resulting plasmid was introduced into *L. monocytogenes* strain 10403S by electroporation of penicillin-treated bacteria (28) and allowed to participate in the two-step allelic exchange reaction shown in Figure 1. Integrants into the chromosome were selected by growth at 40°C in BHI medium containing chloramphenicol (10 μg/ml). Excision of vector sequences occurred during subsequent growth at 30°C in BHI medium containing erythromycin (1 μg/ml). Useful recombinants were identified as *cm*^{S} *erm*^{R} cells that, by Southern analysis, had a bifurcated nonessential region containing added genetic material of the appropriate length. The recombinant strain of *L. monocytogenes* carrying the HIV-1 *gag* gene is hereafter referred to as Lm-*gag*. It has an LD_{50} of 5×10^7 in BALB/c mice.

Western immunoblotting

TCA precipitation of culture supernatant fluids and subsequent SDS-polyacrylamide gel electrophoresis was performed as described (29). Proteins were transferred to Immobilon P supports (Millipore, Bedford, MA), using a Transphor electroblotter (Hoefer Scientific, San Francisco, CA). Blots were stained with a 1:1000 dilution of anti-HIV-1 p25/24 Gag antiserum, generously provided by K. Steimer through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, National Institutes of Health, Bethesda, MD) and counter-stained with alkaline phosphatase-labeled anti-rabbit goat Ab (Kirkegaard and Perry, Gaithersburg, MD).

Generation and expansion of Gag-specific CTLs

Six- to eight-week old BALB/c mice (H-2^d) (Charles River Laboratories, Raleigh, NC) were immunized by i.p. inoculation with 10^7 live Lm-*gag* (about 0.2 LD_{50}), the recombinant *L. monocytogenes* that expressed HIV-1 Gag protein, either once or a second time 10 or more days later. Splenocytes obtained from such mice at 20 days or later were purified over Lympholyte M (Cedarlane, Hornby, Ontario, Canada). Approximately 6×10^7 splenocytes were cocultured with 3×10^7 splenocytes that had been infected with five plaque-forming units per cell of Vac-*gag* (vVK1, vac/gag) (30) for 7 days at 37°C in 40 ml of Iscove's modified DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Some in vitro stimulations were extended 4 to 7 days following resuspension of splenocytes in fresh medium and addition of murine IL-2 on day 3. This second incubation had the effect of significantly reducing the number of nonspecific cytolytic cells.

^{51}Cr release assays

Target cells for cytotoxicity assays were P815 mouse mastocytoma cells (H-2^d), washed twice with PBS, resuspended at a concentration of 10^7 cells per ml in Eagle's minimal essential medium supplemented with 2.5% fetal bovine serum and 100 mM HEPES, and infected with 20 plaque-forming units of vaccinia virus per cell. The vaccinia viruses used were VSC8 (Vac-*gal*), vVK1 (Vac-*gag*), vPE16 (Vac-*env*), and vTFNef (Vac-*nef*), all kindly provided by B. Moss, and NYCBH (Vac^+), vAbT141 (p55), vAbT228 (p17), and vAbT286 (p24), kindly provided by D. Panicali (31). After adsorption for 1 to 2 h at 37°C, the infected cells were resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics, and incubated at 32°C for 14 to 16 h. The cells were then pelleted, resuspended in 100 μl medium containing 85 uCi $Na^{51}CrO_4$ per 3 to 5 × 10^5 cells, and incubated at 37°C for 1 h.

In vitro-stimulated splenocytes in 100 μl of RPMI 1640 medium were incubated at various ratios with 2 × 10^4 washed, ^{51}Cr-labeled target cells suspended in 100 μl RPMI 1640 medium in round-bottom, 96-well plates for 4 to 6 h at 37°C. For peptide-targeting experiments, uninfected P815 cells were labeled with $Na^{51}CrO_4$, washed, and then incubated in 100 μl for 1 to 2 h at 37°C with peptide, before addition of splenocytes.

Released ^{51}Cr was determined in 100 μl of supernatant and the percentage of specific lysis was calculated as (experimental cpm − spontaneous cpm)/(total cpm − spontaneous cpm) × 100. All assays were performed in triplicate or quadruplicate.

Depletion of $CD4^+$ and $CD8^+$ T cells

10^6 in vitro-stimulated splenocytes in 100 μl were incubated 30 min at 0°C with an equal volume containing either 300 ng of purified anti-CD4 rat mAb GK1.5 or a culture supernatant containing anti-CD8 rat mAb 3.155, followed by a second incubation at 37°C in the presence or absence of a 1:10 dilution of rabbit serum complement.

Overlapping peptides of Gag p24

Twenty-two overlapping 20-mer peptides with 10-amino acid overlaps, spanning residues 133 to 362 of the HIV-1_{SF2} Gag p24 protein, were provided by the MRC AIDS Reagent Project. These peptides had been HPLC-purified and were provided at greater than 80% (most greater than 95%) purity as determined by fast atom bombardment mass spectroscopy. Following our initial screening (see Fig. 6), the active peptides were subjected to further analysis in our laboratory by matrix-assisted laser desorption ionization (MALDI) mass spectral analysis. They were found to contain a major peak of the appropriate m.w., and no evidence of shorter contaminating peptides.

Results

Construction of chromosomal recombinants of L. monocytogenes *that express and secrete HIV-1 Gag protein*

Stable chromosomal recombinants of *L. monocytogenes* that synthesize and secrete Gag protein were constructed following a protocol adapted from Camilli et al. (24). The procedure required a site on the *L. monocytogenes* chromosome into which foreign genetic material could be inserted without disrupting bacterial genes necessary for the growth and spread of the organism. Such a nonessential region had previously been identified (24), and a copy of the site was inserted into the *rep*^{ts}*cm*^{R} plasmid, pKSV7, to act as a region of homology for subsequent recombination with the chromosome. The promoter and signal sequence of the *L. monocytogenes* hemolysin gene, *hly*, which encodes listeriolysin-*O*, an abundantly synthesized and secreted protein that facilitates release of the organism from phagocytic vacuoles into the cytoplasm of infected host cells, was inserted near the center of this region of homology and used to drive the expression and secretion of downstream HIV-1 genes. The HIV-1 *gag* gene constructs examined in this paper began at *gag* codon 29 in order to eliminate three contiguous lysine residues which formed part of a sequence of six positively-charged amino acids near the N terminus of the Gag protein. Such a highly charged region following a signal sequence has been shown to prevent secretion of proteins across the bacterial cytoplasmic membrane (32). Participation of the completed plasmid construct in the two-step allelic exchange reaction shown in Figure 1 produced the stable chromosomal recombinants used in these studies. This cassette can be used to introduce any foreign DNA sequence into the *L. monocytogenes* chromosome.

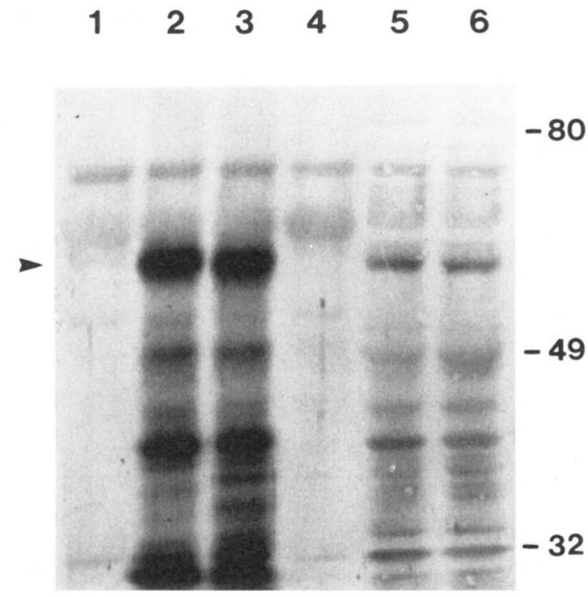

FIGURE 2. Synthesis and secretion of HIV *gag* gene product by recombinant *L. monocytogenes*, Lm-*gag*. Proteins present in bacterial cells and culture fluids were collected after 6 h growth at 35°C. Western blot was stained with anti-HIV-1 Gag antiserum. *Lanes 1–4*, Secreted proteins from: *lane 1*, wild-type *L. monocytogenes*; *lane 2*, *L. monocytogenes* recombinant expressing HIV-1_{HXB} *gag* gene; *lane 3*, *L. monocytogenes* recombinant expressing HIV-$1_{89.6}$ *gag* gene; and *lane 4*, *L. monocytogenes* recombinant expressing HIV-1_{NL43} *nef* gene. *Lanes 5* and *6*, Total cell-associated proteins from: *lane 5*, *L. monocytogenes* recombinant expressing HIV-1_{HXB} *gag* gene; and *lane 6*, *L. monocytogenes* recombinant expressing HIV-$1_{89.6}$ *gag* gene. Position of marker proteins is shown on right in kDa. The major product in *lanes 2, 3, 5,* and *6* is approximately 55 kDa (arrowhead). Products smaller than 55 kDa were seen both in the culture fluid and intracellularly.

Western blot analysis of the proteins synthesized and secreted by these recombinant organisms verified that the foreign genetic material functioned properly in its new genetic context. Figure 2 shows that the secreted proteins from the culture medium of two strains of Lm-*gag* (*lanes 2* and *3*) contained 55-kDa proteins as well as various smaller cleavage products, detectable by anti-HIV-1 Gag protein antiserum. However, the medium from two *L. monocytogenes* strains included as controls, the wild-type strain (*lane 1*) and an *L. monocytogenes* recombinant that expressed a different HIV protein (*lane 4*), were devoid of this material. Intracellular proteins from the Lm-*gag* strains included similar proteins in equivalent or lower amounts (*lanes 5* and *6*; these lanes represent 20% of the amount of culture examined in *lanes 1–4*). These results indicate that at least half the HIV-1 Gag protein synthesized by the recombinant organisms was secreted into the medium in which the cells were grown.

Strain Lm-*gag* grew well in bacteriologic media, but its ability to spread from cell to cell (21, 33) through a monolayer of the murine macrophage-like cell line, J774, was severely inhibited when compared with wild-type *L. monocytogenes* (data not shown). Furthermore, when mice were infected with this organism, its virulence was attenuated by three logs in comparison to that of

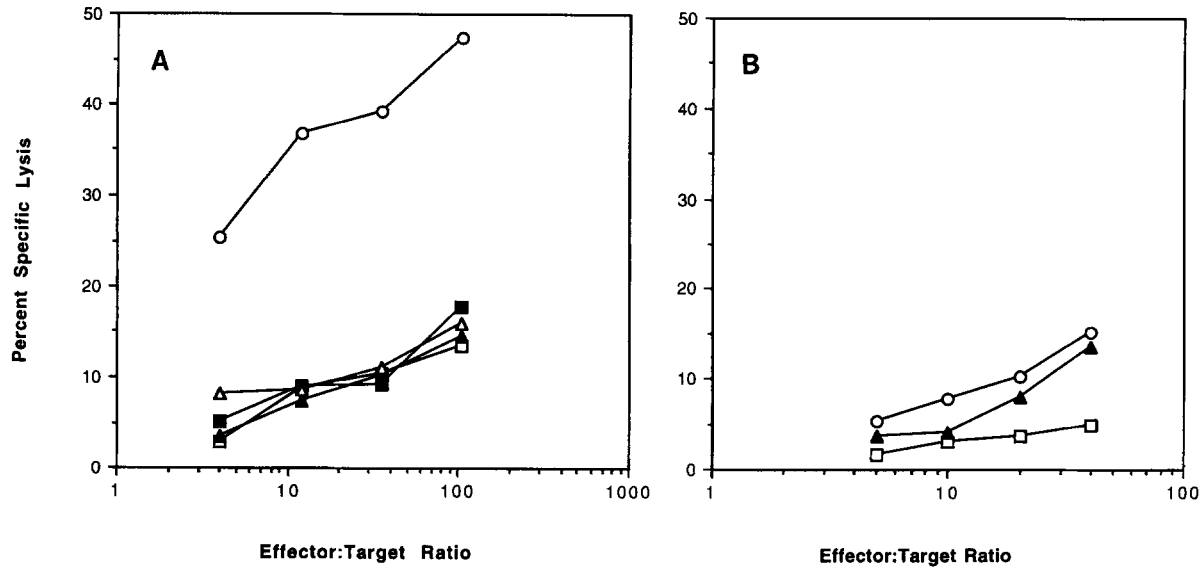

FIGURE 3. Activity of splenocytes from mice 20 days after infection with Lm-*gag*, a recombinant *L. monocytogenes* expressing the HIV-1_{HXB} Gag protein, or from mice after infection with wild-type *L. monocytogenes*. A standard ^{51}Cr release assay showing specific cytolytic activity of spleen cells against target cells expressing different proteins. *A*, Spleen cells isolated from a mouse infected with Lm-*gag*. *B*, Spleen cells isolated from a mouse infected with wild-type *L. monocytogenes*. Target cells were mouse P815 cells infected with vaccinia vectors carrying an intact HIV-1_{BH10} *gag* gene (○), HIV-1_{NL43} *nef* gene (■), HIV-1_{BH8} *env* gene (△), *E. coli* β-galactosidase gene (▲), or were uninfected (□).

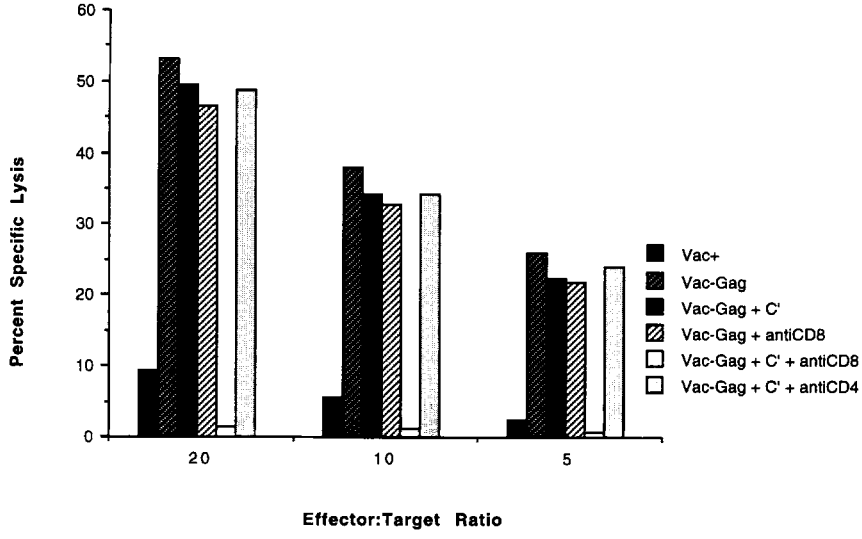

FIGURE 4. Characterization of the effector population from Lm-*gag*-infected mice at 15 wk postinfection as CD8$^+$ T cells. ^{51}Cr release assay showing cytolytic activity of spleen cells against Gag-expressing target cells following depletion of either CD4$^+$ or CD8$^+$ T cells from the splenocyte population by incubation with anti-CD4 rat mAb GK1.5 or anti-CD8 rat mAb 3.155, followed by incubation with rabbit complement.

wild-type *L. monocytogenes*. While reduced virulence would appear to be useful in a vaccine, the exact mechanism of this attenuation of pathogenesis is not known and requires further study.

Mice immunized with live Lm-gag mount a specific, strong, long-lasting CD8$^+$ CTL response against target cells that express the HIV-1 Gag protein

Splenocytes were isolated from BALB/c mice that had been immunized once or boosted 10 or more days later by i.p. injection(s) of Lm-*gag*. After 7 days in coculture with Gag-expressing splenocytes (infected with Vac-*gag*) to expand the number of potential Gag-specific CTL cells and reduce nonspecific background, the bulk splenocyte population was assayed for its ability to lyse ^{51}Cr-labeled P815 target cells that expressed the Gag protein. Figure 3*A* shows the results of such an assay. The Lm-*gag*-induced splenocytes showed at least a three- to fivefold greater ability to lyse target cells expressing HIV-1 Gag protein than targets expressing β-galactosidase, HIV-1 Env, or Nef, or uninfected targets. In contrast, mice immunized with wild-type *L. monocytogenes* (Fig. 3*B*) or different *L. monocytogenes* recombinants (data not shown) failed to produce splenocytes that recognized and lysed Gag-expressing targets.

To examine the nature of T cells that mediated the cytolytic activity, stimulated splenocytes were treated with anti-CD4 or anti-CD8 mAbs in the presence or absence of rabbit complement. Abrogation of cytolytic activity of anti-CD8-treated, but not anti-CD4-treated, splenocytes indicated that cytolytic activity was mediated by CD8$^+$ T cells (Fig. 4).

The cytolytic activity induced by Lm-*gag* infection of BALB/c mice was a long-lived response. High levels of activity were seen as early as 20 days postinfection, and were maintained at this level for at least 15 wk (see Fig. 4) following a single immunization with

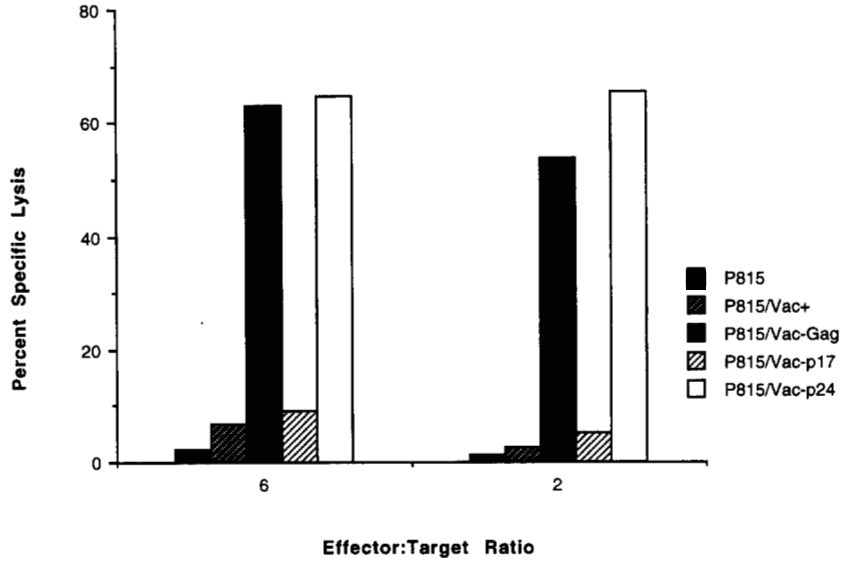

FIGURE 5. Lm-*gag*-induced splenocytes recognize immunodominant epitope(s) present in the p24 region of the HIV-1 Gag protein. Spleen cells were isolated 6 wk postinfection. ^{51}Cr release assay showing specific cytolytic activity of spleen cells against target cells infected with vaccinia vectors containing the intact HIV-1_{BH10} *gag* gene or with two different truncations of the *gag* gene.

Lm-*gag*. By 36 wk, the CTL activity of such mice had declined substantially, and showed between 5 and 10% of the maximum activity. However, mice that had been boosted once 13 days after initial infection continued to show 50% of the maximum CTL activity at 6 mo (data not shown).

Lm-gag *induces a CTL response in BALB/c mice directed against an epitope that is present in the p24 region of HIV-1 Gag protein*

The specificity of the response of BALB/c mice to immunization with Lm-*gag* was further examined by the use of target cells infected with recombinant vaccinia virus expressing truncated portions of the HIV-1 Gag protein. The results, shown in Figure 5, indicate that only the p24 region of the Gag protein stimulated Lm-*gag*-reactive splenocytes, while the p17 region at the N terminus of the Gag protein and the p15 region at the C terminus (p55−[p17+p24]) contained no epitopes recognized by T cells from these mice.

Twenty-two overlapping peptides that span the entire p24 region of the Gag protein were individually adsorbed to P815 cells. These cells were then used as targets in cytotoxicity assays. The results of one of these assays is shown in the *inset* of Figure 6. P815 cells incubated with Gag peptides 193–212 (GHQAAMQMLKETI NEEAAEW) and 233–252 (GSDIAGTTSTLQEQIGWMTN) were recognized as targets by the Lm-*gag*-induced splenocytes. When the concentration dependence of the targeting activity of these two peptides was examined, however, peptide sequence 193–212 was found to be approximately 500-fold more effective than sequence 233–252 for the induction of specific cell lysis (Fig. 6). Gag peptide 193–212, therefore, clearly contains the immunodominant epitope for the Gag-specific $CD8^+$ T cell response.

Discussion

It is currently believed that cellular immunity and $CD8^+$ cytolytic T cells play a significant role in controlling viral infections and that such immunity is best induced by use of live organisms (9, 34). This result can now be understood in the context of differences between Ag-processing pathways and their respective requirements for Ag localization (35). Ags delivered by killed organisms are taken up into endosomal compartments for presentation by MHC class II molecules with the resulting generation of CD4-mediated responses and humoral immunity. For MHC class I-restricted recognition and the generation of $CD8^+$ CTL, however, it is necessary for the Ag to be delivered to the cytosol of the cells, usually in the form of newly synthesized proteins, for intracytoplasmic processing (36–38). Because of the cytoplasmic localization of live *L. monocytogenes*, proteins synthesized and secreted by this organism can target the class I pathway for the production of CD8-mediated immune responses (39, 40).

We have been developing *L. monocytogenes* as a live vaccine vector to target foreign proteins to the immune system (41), and have shown that recombinants of *L. monocytogenes* that express foreign bacterial or viral Ags can elicit in vivo CTL responses to those Ags (22, 40). However, these earlier *L. monocytogenes* vaccines were not ideal, either because the recombinant protein was not secreted outside the bacterial cell (22) or because of the potential instability of plasmid transformations (40). In this paper, we have constructed stable chromosomal recombinants of *L. monocytogenes* by integrating a foreign gene sequence, regulated by the *L. monocytogenes hly* promoter and signal sequence, into the bacterial chromosome through a double allelic exchange. Recombinant organisms that contained the HIV-1 *gag* gene expressed and secreted the Gag protein and, when used to infect mice, produced a strong, specific, long-lasting CTL response directed against this protein. As many as 60% of vaccinia-infected target cells expressing HIV-1 Gag protein were lysed by splenocytes isolated from Lm-*gag*-infected mice, even at E:T ratios as low as one or two. Over 90% of cells targeted by a particular Gag peptide were lysed. Neither vaccinia-infected target cells expressing other HIV proteins or β-galactosidase nor target cells treated with other Gag peptides were lysed. Similarly, target cells incubated with splenocytes derived from mice infected with wild-type *L. monocytogenes* or with other *L. monocytogenes* recombinants were not lysed. Gag-specific $CD8^+$ T cells continued to be present 4 mo or more after Lm-*gag* infection, indicating that the vaccine induced good memory CTL responses.

The Gag-specific CTL response in BALB/c ($H\text{-}2^d$) mice induced by Lm-*gag* was directed, as in many human responses to HIV-1

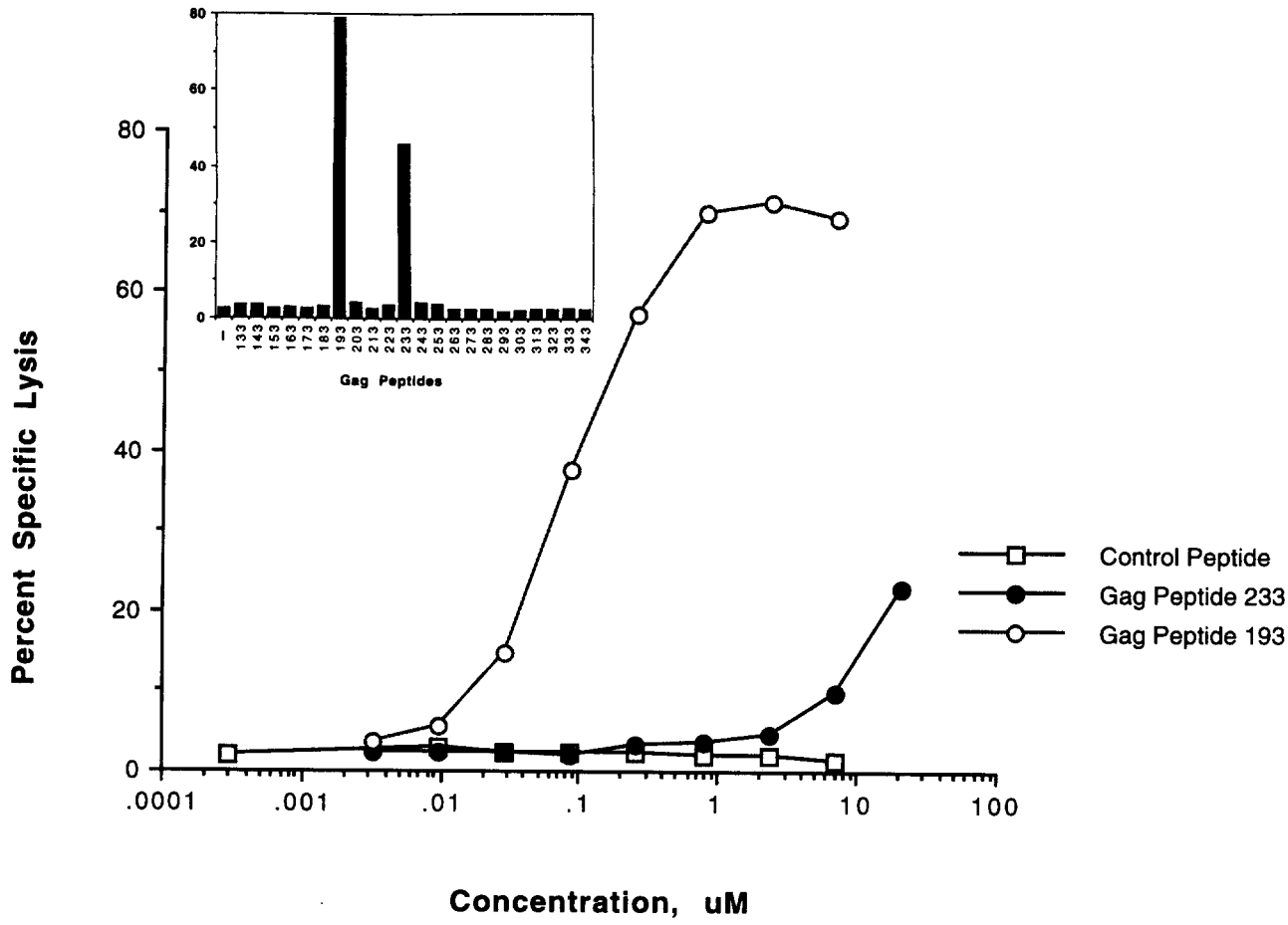

FIGURE 6. Epitope mapping (*inset*) and concentration-dependence of peptide targeting (*main figure*) by Lm-*gag*-induced splenocytes. Target cells were incubated 2 h with members of an overlapping set of 20-mer peptides at 6 μM that spanned the entire p24 region of the HIV-1_{SF2} gag protein (*inset*) or with the indicated concentrations of Gag peptides 193 or 233 (*main figure*). Each Gag peptide is identified with the number of its first amino acid. Different preparations of peptides were used in the two experiments shown in this figure. The control peptide is CTRRTRLS GLSWGNDTSKS.

infection, to a peptide sequence within the p24 segment of the Gag protein. Curiously, this peptide, Gag 193–212, did not display the normal motifs expected of peptides previously shown to target the H-2^d MHC class I molecules of BALB/c mice, and was longer than is usual for strong MHC class I binding (42, 43). Naturally processed peptides associated with the three H-2^d MHC class I molecules (K^d, L^d, D^d) are usually 8 to 11 amino acids in length and have a hydrophobic amino acid residue at the carboxy terminus and at least one other dominant anchor residue. Not all known MHC class I-restricted peptides, however, have a classical peptide binding motif (44–46) and peptides that appear to contain only one defined anchor residue have been previously reported (42, 44). It is assumed that when long peptides are effective in CTL assays, they either contain contaminating truncated peptides of appropriate length (47, 48) or are cleaved into shorter peptides by extracellular proteases present in culture media (49, 50). While peptide 193–212 did contain a number of potential H-2^d carboxy-terminal anchor residues, none was positioned at an appropriate distance from other required dominant anchor residues usually associated with H-2^d binding. It is of interest, therefore, that a peptide within this 20-mer is recognized despite the presence of many classical H-2^d binding motifs within the remainder of the Gag sequence.

The strong and persistent CD8$^+$ T cell response to Lm-*gag* differs in several ways from the results of other studies of HIV-*gag* immunization in BALB/c mice and may reflect differences in the mode of delivery of the Lm-*gag* Ag. Earlier studies showed that BCG-*gag* recombinant bacteria induced a detectable CD8$^+$ cytolytic response against mixed Gag peptides (51). Vac-gag_{BRU} infection of BALB/c mice (52) induced class II-restricted CD4$^+$ T cells directed against Gag epitope 262–280. In another study, immunization with the 23-mer Gag peptide 287–309 (53) resulted in a class I-restricted CTL response against that peptide. In humans, CTL responses against the Gag protein, which is highly conserved among sequenced HIV-1 isolates, have been demonstrated in a majority of HIV-1-seropositive subjects (54). Interestingly, CD8$^+$ CTLs from several patients have been found to recognize sequences present in the same Gag peptide targeted by Lm-*gag*-induced splenocytes from BALB/c mice (55, 56).

The ability of Lm-*gag* to effectively induce cell-mediated immune responses in this mouse model suggests that *L. monocytogenes* might usefully be explored as a vaccine vector for HIV infection, perhaps with multiple HIV genes or variants of genes in one or multiple recombinant organisms. Although several other bacterial species have been considered as potential Ag carriers for HIV, including BCG (51, 57) and *Salmonella* (58), *L. monocytogenes* is especially promising for this purpose. Recent experiments indicate that PBMC from HIV-1-infected patients show impaired IL-12 production (59) and that, with progression of disease, there may be a depression of the ratio of Th1/Th2 CD4$^+$ T cells in such

individuals (60–62). *L. monocytogenes* has been shown to promote IL-12 production by murine macrophages in vitro (63, 64), which may account for the Th1-type $CD4^+$ T cell responses that are thought to occur in vivo in *L. monocytogenes* infections in mice (65). This attribute makes *L. monocytogenes* not only an effective inducer of cell-mediated immunity in general, but a particularly interesting candidate vaccine vector for HIV. Plasmid-based, as well as chromosomal recombinant strains of *L. monocytogenes* constructed by a procedure analogous to that used in this paper, have recently been shown to provide protection against infection of mice with lymphocytic choriomeningitis virus (66, 67).

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