Vaginal Protection and Immunity after Oral Immunization of Mice with a Novel Vaccine Strain of *Listeria monocytogenes* Expressing Human Immunodeficiency Virus Type 1 gag

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Natural transmission of human immunodeficiency virus (HIV) occurs at mucosal surfaces. During acute infection, intestinal and other mucosae are preferential sites of virus replication and rapidly become depleted of CD4+ T cells. Therefore, mucosal immunity may be critical to control both initial infection and the massive early spread of virus. An attenuated D-alanine-requiring strain of the oral intracellular microorganism *Listeria monocytogenes* expressing HIV type 1 gag was shown to induce protective cell-mediated immunity in mice against viruses that express HIV gag when immunization occurs in the presence of a transient supply of D-alanine. In this study, we examined the efficacy of new attenuated strains that are able to synthesize D-alanine from a heterologous dal gene tightly regulated by an actA-promoted resolvase recombination system. In the absence of D-alanine, Gag-specific cytotoxic T lymphocytes (CTLs) were induced systemically after intravenous immunization, and one strain, Lmdd-gag/pARS, induced strong dose-dependent Gag-specific CTLs after oral immunization. A significant level of Gag-specific CD8+ T cells was induced in the mucosal-associated lymphoid tissues (MALTs). Upon intravaginal challenge of these orally immunized mice with recombinant vaccinia virus (rVV) expressing HIV gag, gamma interferon- and tumor necrosis factor alpha-secreting Gag-specific CD8+ T cells were dramatically increased in the spleen and MALTs. Oral immunization with Lmdd-gag/pARS led to complete protection against vaginal challenge by a homologous clade B gag-expressing rVV. In addition, strong cross-clade protection was seen against clades A and C and partial protection against clade G gag-expressing rVV. These results suggest that Lmdd-gag/pARS may be considered as a novel vaccine candidate for use against HIV/AIDS.

With over 42 million people currently infected, human immunodeficiency virus (HIV)/AIDS is now the most widespread pandemic in human history (52). Although HIV spreads systemically, 90% of infections are contracted through sexual transmission, with the virus crossing the mucosal lining of the genital tract, rectum, and oropharyngeal cavities (35). HIV not only subverts the immune system by rapidly infecting and depleting CD4+ T cells, predominantly memory CD4+ T cells of mucosal surfaces and other tissues (5, 19, 28, 31, 33), but with time becomes an integral part of the genetic material of its host’s cells. Thus, there is an urgent need for rapid intervention.

Vaccinology has made a major contribution to the eradication or management of infectious diseases (21). In developing a vaccine against HIV/AIDS, it is important to induce protective local immunity that could act at mucosal surfaces as well as in the systemic compartment. While neutralization antibodies have shown the ability to block HIV/simian immunodeficiency virus (13, 51), they are difficult to elicit. However, cellular immunity plays a critical role in controlling acute infection and maintaining a low chronic viral set point. Therefore, protective antiviral cytotoxic T lymphocytes (CTLs), particularly in the mucosal compartment, should be a major component of any immune response induced by an effective vaccine against HIV/AIDS.

*Listeria monocytogenes* is a gram-positive, facultative, intracellular, food-borne bacterium that has served for decades as a model pathogen for the study of cell-mediated immunity (12, 26). Subsequent to infection and uptake by phagocytic cells, unlike other intracellular organisms such as *Salmonella* spp. and *Mycobacterium tuberculosis*, a number of bacteria escape the endocytic vacuoles and replicate in the cytoplasm. Since foreign genes can be easily and stably inserted into the *L. monocytogenes* chromosome and express at high levels, such gene products are efficiently delivered via the major histocompatibility complex (MHC) class I and MHC class II pathways and induce strong CTL and CD4 help (4, 37, 43, 47, 50). *L. monocytogenes* has been explored as a live vaccine against *M. tuberculosis* (34), model cancers (6, 7, 20, 23, 36, 39), lymphocytic choriomeningitis (17, 48), and influenza virus infection (22). Since the natural route of *L. monocytogenes* infection is through the intestinal mucosa following oral ingestion of the organism, the induction of mucosal immunity is a natural consequence of infection. In fact, mice immunized orally with recombinant *L. monocytogenes* that expressed influenza nucleoprotein showed regression of established renal and colorectal tumors that expressed the same nucleoprotein antigen (38). Also, orally administrated recombinant *L. monocytogenes* strains that express the HIV type 1 (HIV-1) gag gene result in protection against challenge by recombinant vaccinia virus (rVV) expressing HIV-1 gag (rVV-gag) (46).
Preexisting immunity against a vector could interfere with the generation of an immune response against a foreign antigen expressed by that vector. However, the annual incidence of listeriosis is very low, and few individuals are expected to have preexisting immunity. In addition, existing antilisterial immunity does not appear to inhibit the development of an *L. monocytogenes*-specific primary CTL response or diminish its therapeutically efficacious (2, 49).

Nevertheless, *L. monocytogenes* is itself a pathogen that can cause fatal infections, particularly in immunocompromised or pregnant individuals. Therefore, we have focused on methods to attenuate the organism while still retaining its immunogenicity. We initially constructed an attenuated strain, Lmdd, by deleting the two genes, *dal* and *dat*, used for D-alanine biosynthesis and cell wall formation. A recombinant, Lmdd-gag, was shown to be a safe and promising AIDS vaccine candidate (46).

To obviate this requirement, we constructed several derivatives of Lmdd that are able to supply their own D-alanine but transiently. These strains of Lmdd, which were described previously (15, 16). Plasmids pRRR, pKRS, and pARS (see below) were individually introduced into Lmdd-gag by electroporation according to established protocols (40), and the resulting transformants, with Lmdd-gag/pRRR, Lmdd-gag/pKRS, and Lmdd-gag/pARS, were selected on brain heart infusion (BHI) agar plates containing 10 µg/ml chloramphenicol. All bacterial strains were washed extensively, and BCIP (5-bromo-4-chloro-3-indolylphosphate)–nitroblue tetrazolium chromogen was added to each well to develop color. The plates were washed, and streptavidin-conjugated alkaline phosphatase (1:100 in 1% bovine serum albumin) was added to each well for 2 h at room temperature. The plates were washed, and incubated overnight with biotinylated anti-mouse IFN-γ (R&D Systems, Minneapolis, MN), with minor modifications. Briefly, ELISPOT assay for IFN-γ-secreting cells. Enzyme-linked immunospot (ELISPOT) assays were conducted according to the manufacturer's protocol (46, 54). Briefly, tissues were digested with collagenase D and DNase I at 37°C for 30 min and then incubated in the dark, and then an equal volume of 100% FCS was added and incubated at room temperature for 1 min, followed by two washes with RPMI 1640 medium with 10% FCS. In vivo CTL assay. The in vivo CTL assay was performed essentially as described previously (6, 53). BALB/c spleen cells were labeled with either a low (0.3 µM) (CFSElow) or a high (3 µM) (CFSEhigh) concentration of CFSE, and CFSEhigh cells were further incubated with 5 µg/ml HIV-1 Gagp27–295, LLOp49–59, or lymphocytic choriomeningitis virus nucleoprotein p13,122 peptides for 1 h at 37°C in the dark. Following incubation, the cells were washed, and equal numbers (1 × 10⁷) of each population were mixed in 200 µl of PBS and injected i.v. into previously immunized naive mice. Sixteen hours later, spleens from recipients were analyzed for the presence of CFSEhigh and CFSElow populations. In vivo killing was indicated by percent specific lysis, calculated according the following formula: percent lysis = 1 – ratio unprimed/ratio primed) × 100, where the ratio is percent CFSEhigh/CFSElow.

ELISPOT assay for IFN-γ-secreting cells. Enzyme-linked immunospot (ELISPOT) assays were conducted according to the manufacturer’s protocol (46, 54). Briefly, ELISPOT 96-well plates (Millipore, Bedford, MA) were coated with anti-mouse IFN-γ capture antibody (1:100 in normal rabbit serum and incubated overnight at 4°C. The following day, plates were washed and blocked for 2 h with 5% FCS. Suspensions containing 2 × 10⁶ spleen cells in RPMI 1640 (with 10% FCS and 1 ng/ml interleukin-2) were added to each well and stimulated in triplicate, with or without peptide (5 µg/ml) or concanavalin A (2.5 µg/ml, positive control; Sigma) at 37°C. After 18 h, plates were washed and incubated overnight with biotinylated anti-mouse IFN-γ detection antibody (1:100 in 1% bovine serum albumin) at 4°C. The following day, plates were washed extensively, and BCIP (5-bromo-5-chloro-3-indolylphosphate)–ni troblue tetrazolium chromogen was added to each well to develop color. The plates were then rinsed with distilled water and dried at room temperature. Spots were counted by use of an automated ELISPOT reader (CTL Limited).
describe here the immunogenicity and protection generated by a derivative of Lmdd-gag in which the transient supply of $\alpha$-alanine is provided by a $\alpha$-alanine-carrying plasmid from which the $\alpha$-alanine gene is excised during cytosolic growth of the bacteria through the action of the res/resolvase recombination system. As shown later, in order to achieve a sufficiently robust cellular immune response following oral vaccination, we needed to reduce the high activity of the resolvase enzyme (plasmid pRRR) by introducing into the protein a destabilizing peptide sequence encoded by ssrA (18, 25) (generating plasmid pKRS). We also transferred this recombination system onto the more stable plasmid pAM401 (generating plasmid pARS). The resulting bacteria were designated Lmdd-gag/pRRR, Lmdd-gag/pKRS, and Lmdd-gag/pARS. All three recombinant L. monocytogenes strains contain the same clade B HIV-1 gag gene in their chromosomes, express a Bacillus subtilis $\alpha$-alanine gene, and synthesize $\alpha$-alanine under the stringent regulation of an actA-promoted resolvase recombination system (55).

Figure 1A shows that all of the new strains grew in bacteriological media in the absence of $\alpha$-alanine at only slightly lower rates than wild-type Lm-gag (15) or Lmdd-gag plus $\alpha$-alanine (16, 46). Nevertheless, these organisms were attenuated in mice, as shown in Fig. 1B, which indicates that they survived for only 3 days following iv. infection. Thus, while the wild-type Lm-gag recombinant bacteria were increasing in number in spleen at days 2 and 3 after infection, the viability of the attenuated strains had fallen 3 to 5 log$_{10}$. As anticipated, the presence of the destabilizing ssrA peptide sequence on the resolvase enzyme of Lmdd-gag/pARS allowed these bacteria to survive slightly longer in mice than Lmdd-gag/pRRR. Additional characterization of these strains revealed that whereas they could replicate for 10 h or longer within the cytoplasm of macrophage-like J774 cells, they did not survive sufficiently long (3 to 4 days) to form plaques (55) on monolayers of mouse L2 fibroblasts (data not shown).

Induction of antigen-specific CTLs in the spleen after systemic immunization with Lmdd-gag derivative strains. The
50% lethal dose of Lmdd-gag as well as that of the three new derivative strains was approximately 1 \times 10^9 to 2 \times 10^9 CFU/mouse (data not shown). To determine whether the new strains, like Lmdd plus d-alanine, could induce protective CD8⁺ T cells in the absence of d-alanine, we first examined CD8⁺ T-cell activation in the spleens of systemically immunized mice. Groups of mice were immunized through the tail vein with a sublethal dose (10⁸) of recombinant bacteria and boosted with 10⁸ of the same bacteria. Six days later, splenocytes were isolated and stained for the presence of antigen-specific CD8⁺ T cells by use of Gag 197–205 or LLO91–99 H-2Kd tetramers to detect the dominant CD8⁺ T cell response to each of these antigens in BALB/c mice (8, 30). As shown in Fig. 2A, robust expansion of antigen-specific CD8⁺ T cells was seen. Most of these cells showed high expression of CD11a, a β2 integrin up-regulated upon activation of CD8⁺ T cells and present at high levels on memory CD8⁺ T cells (27). In the presence of d-alanine, Lmdd-gag induced 1.12% Gag-specific and 10.2% LLO-specific CD8⁺ T cells in the spleen, while in the absence of d-alanine, Lmdd-gag/pRRR and Lmdd-gag/pKRS induced comparable levels of GAG-specific (1.09% and 1.35%) and LLO-specific (10.1% and 12.1%) CD8⁺ T cells. Lmdd-gag/pARS induced the highest percentages of antigen-specific (1.55% Gag-specific and 13.1% LLO-specific) CD8⁺ T cells. In summary, like Lmdd-gag with exogenous d-alanine, Lmdd-gag derivative strains were all able to stimulate substantial antigen-specific CD8⁺ T-cell responses without exogenous d-alanine, despite showing a 50% lethal dose similar to that of Lmdd-gag.

To determine whether the induced Gag-specific CD8⁺ T cells retained effector functions, we measured the in vivo epitope-specific cytolytic activities of CD8⁺ T cells in mice immunized systemically with Lmdd-gag/pRRR as described above. Splenocytes labeled with a high concentration of CFSE were coated with either Gag₁₉⁷–₂₀₅ peptide or a control peptide, LCMV-NP₁₁₈–₁₂₆, and coinjected with equal numbers of non-peptide-coated splenocytes labeled with a lower concentration of CFSE into immunized mice at day 6 after the booster infection. After 16 h, the ratio of CFSE<sup>high</sup> (peptide-coated): CFSE<sup>low</sup> (no peptide) cells served as an indication of CTL activity. As shown in Fig. 2B, robust Gag-specific killing occurred in both Lmdd-gag (79% lysis of targets)- and Lmdd-gag/pRRR (79.5% lysis of targets)-immunized mice, while no killing was detected in unimmunized mice. As expected, we detected little lysis of LCMV-NP₁₁₈–₁₂₆-coated splenocytes. These results suggested that systemic immunization with Lmdd-gag derivatives in the absence of d-alanine could generate functional CTLs.

**Induction of antigen-specific CTLs in the spleen and MALTs after oral immunization with Lmdd-gag/pARS.** To examine the induction of antigen-specific CD8⁺ T-cell responses in the spleens of orally immunized mice, groups of mice were immunized by gavage to deliver a single oral dose of 2 \times 10⁸ CFU of recombinant L. monocytogenes. Six days later, as shown in Fig. 3A, both Lmdd-gag/pKRS and Lmdd-gag/pARS, but not Lmdd-gag/pRRR, induced significant levels of Gag-specific (0.27% and 0.33%) CD8⁺ T cells. Levels of LLO-specific CD8⁺ T cells were high in all immunized mice, but again Lmdd-gag/pRRR showed the lowest response. The response produced by Lmdd-gag/pARS was comparable to that produced by Lmdd-gag plus d-alanine.

Therefore, subsequent experiments focused on Lmdd-gag/pARS. Figure 3B illustrates the relationship between the immunization dose and the magnitude of antigen-specific CD8⁺ T cells induced in the spleen and shows that increased numbers of recombinant Lmdd-gag/pARS delivered orally resulted in increased numbers of antigen-specific CD8⁺ T cells. With the lowest dose (2 \times 10⁹ CFU), although LLO-specific CD8⁺ T cells were readily detected, Gag-specific CD8⁺ T cells scarcely exceeded background levels. The highest dose (10¹⁰ CFU)
induced 0.61% Gag-specific and 1.06% LLO-specific CD8+ T cells. Based on these results, in subsequent oral immunization experiments we used \(2 \times 10^9\) CFU of Lmdd-gag/pARS for primary immunizations and \(10^{10}\) CFU for boosts.

Since protection against HIV infection is likely to require immunity at mucosal sites, we further examined whether antigen-specific CD8+ T cells could be induced in MALTs, such as mesenteric lymph nodes (MLN) and PP. Groups of mice were fed \(2 \times 10^9\) CFU bacteria for a primary immunization and \(10^{10}\) CFU bacteria for a boost. Six days later, lymphocytes were isolated from MLN, PP, and spleens and analyzed for antigen-specific CD8+ T cells by use of tetramer staining. As shown in Fig. 3C, all tissues of immunized mice contained significant numbers of Gag-specific and LLO-specific CD8+ T cells, with spleens containing the highest percentages. In MLN and PP, antigen-specific CD8+ T cells were detectable only after the boost despite the fact that large numbers of Lmdd-gag/pARS were found in these tissues at days 1 and 3 after initial infection (data not shown). Unlike results for spleen, the numbers of Gag- and LLO-specific CD8+ T cells were equivalent in MLN and PP. In unimmunized mice, only background levels of tetramer-positive cells were detected in any tissue.

To test whether the antigen-specific CD8+ T cells induced by oral immunization with Lmdd-gag/pARS possess effector functions, we measured the in vivo cytolytic activities of these animals. Groups of mice were fed \(2 \times 10^9\) CFU bacteria and boosted twice with \(10^{10}\) CFU Lmdd-gag/pARS. At day 6 after the last immunization, a total of \(2 \times 10^7\) cells containing equal numbers (#) of peptide-coated CFSEhigh and peptide-uncoated CFSELow spleen cells were coinjected into unimmunized or immunized BALB/c mice, and in vivo cytolysis of CFSE-labeled donor cells was assessed 16 h later. The percent lysis value was calculated as described in Materials and Methods. Individual percentages of CFSEhigh and CFSELow splenocytes in the recipient mice are shown (small font). Peptides used to coat spleen cells were Gag197–205, LLO91–99, and LCMV-NP118–126.

**FIG. 3.** Antigen (Ag)-specific CD8+ T cells are generated in the spleens and MALTs of mice orally immunized with Lmdd-gag/pARS. BALB/c mice were orally immunized with \(2 \times 10^9\) CFU of various bacterial strains (A) or with the indicated doses of Lmdd-gag/pARS (B). The percentages of Ag-specific CD8+ T cells in the spleens were determined by Ag-specific tetramer staining 9 days after immunization. (C) BALB/c mice were orally immunized with \(2 \times 10^9\) CFU and boosted once with \(10^{10}\) CFU Lmdd-gag/pARS. Six days after the boost, the frequencies of Ag-specific CD8+ T cells in the spleens (SP), mesenteric lymph nodes (MLN), and Peyer’s patches (PP) were determined by Ag-specific tetramer staining. Open bars, Gag-specific CD8+ T cells; filled bars, LLO-specific CD8+ T cells.

**FIG. 4.** In vivo CTL activity is generated in mice orally immunized with Lmdd-gag/pARS. BALB/c mice were orally immunized with \(2 \times 10^9\) CFU and boosted twice with \(10^{10}\) CFU Lmdd-gag/pARS. At day 6 after the last immunization, a total of \(2 \times 10^7\) cells containing equal numbers (#) of peptide-coated CFSEhigh and peptide-uncoated CFSELow spleen cells were coinjected into unimmunized or immunized BALB/c mice, and in vivo cytolysis of CFSE-labeled donor cells was assessed 16 h later. The percent lysis value was calculated as described in Materials and Methods. Individual percentages of CFSEhigh and CFSELow splenocytes in the recipient mice are shown (small font). Peptides used to coat spleen cells were Gag197–205, LLO91–99, and LCMV-NP118–126.
lated spleen cells from mice that had been immunized orally three times as described above and rested for 8 weeks after the last vaccination. As shown in Fig. 5, substantial and comparable numbers of Gag-specific and LLO-specific IFN-γ-secreting cells were detected in the Lmdd-gag/pARS (without D-alanine)- and Lmdd-gag (with D-alanine)-immunized mice, whereas only a few nonspecific spots were detected in response to the unrelated LCMV-NP peptide. Depletion of CD8+ T cells from the splenocytes before antigen-specific stimulation resulted in complete loss of IFN-γ secretion. These data indicate that oral immunization with Lmdd-gag/pARS in the absence of D-alanine induced a pool of systemic antigen-specific memory CD8+ T cells.

We subsequently determined whether these memory CD8+ T cells could mount a recall response following challenge with an HIV-1 gag-expressing virus. Since mice are not susceptible to HIV infection, we used a surrogate virus—recombinant vaccinia virus expressing HIV-1 gag from clade B (rVV-gag, VVK1). Mice that received three oral doses of Lmdd-gag/pARS were challenged with VVK1 either systemically (i.p., 1.2 × 10^6 PFU), intravaginally (IVAG, 8 × 10^7 PFU), or intranasally (i.n., 1 × 10^7 PFU) with vaccinia virus expressing HIV-1 gag from clade B (VVK1). Controls were unimmunized (Naıve) or age-matched animals that had been infected only with rVV-gag (VVK1). Six days after challenge, splenocytes from unchallenged (A) or challenged (B) mice were isolated and measured for Gag-specific CD8+ T cells by tetramer staining. (C) Splenocytes from IVAG-challenged mice were assayed for IFN-γ and TNF-α production following Gag 197–205 peptide stimulation using intracellular staining. Plots shown in panels B and C are of gated CD8 cells and are representative of two to three mice per group.

When the CD8+ T cells were restimulated with Gag 197–205 peptide and analyzed for IFN-γ and TNF-α production, we found that the majority of activated CD8+ T cells produced both of these inflammatory cytokines in response to peptide (Fig. 6C). There was no response in the absence of peptide or response to the unrelated LCMV peptide (data not shown). Consistent with the tetramer staining results, cells from mice infected only with VVK1 produced only low levels of cytokine.
We examined the presence of Gag-specific CD8$^+$ T-cell responses in various tissues (PP, MLN, and ILN). Moreover, intravaginal challenge induced much higher responses in all other tissues examined. The response in CLN was higher after intranasal challenge. The majority of Gag-specific CD8$^+$ T cells in the ILN, which enlarged dramatically after virus challenge, showed that both intravaginal and intranasal challenges induced significant levels of Gag-specific CD8$^+$ T cells that were only slightly higher than the background staining levels for the naïve animals (Table 1). Taken together, these data indicate that mucosal challenge of mice immunized orally with Lmdd-gag/pARS induced strong Gag-specific CD8$^+$ T-cell recall responses in the MALTs.

**Protection against systemic and mucosal virus challenges.** Finally, we determined the efficacy of the immunity induced by the oral Lmdd-gag/pARS vaccine to protect against a mucosal challenge by rVV-gag by either the intravaginal or intranasal route. Mice were orally immunized three times and then challenged with rVV-gag (VVK1 or VP1287) expressing a homologous clade B gag gene. Six days after the challenge, mice were sacrificed and tissues were assayed for virus content.

In naïve, nonimmune mice, high titers of virus were consistently found in ovaries after i.p. infection (Fig. 7A) and intravaginal infection (Fig. 7B), in agreement with previous reports that the virus has a tropism for the ovary (1). However, following intranasal infection, no virus was found in ovaries. Rather, a modest level of virus (4 log$\text{_{10}}$ PFU) was seen in the lung (Fig. 7A) and occasionally in the draining cervical lymph node (data not shown). Surprisingly, we found that intravaginal infection, but not i.p. infection, resulted in a log$\text{_{10}}$-higher titer of virus in the oviduct than in the ovaries (Fig. 7B), indicating that this tissue is an even more preferred site for virus replication following vaginal infection. Little virus was detected in the oviduct after i.p. infection (Fig. 7C).

Figure 7A compares the effects of oral and systemic immunization with Lmdd-gag/pARS on the level of virus in ovaries after a systemic virus challenge. Although mice immunized by the i.v. route were completely protected against i.p. challenge, orally immunized mice showed only a 2- to 3-log$\text{_{10}}$ reduction in virus titer in the ovaries. This is consistent with previous observations that oral immunization with recombinant *L. monocytogenes* expressing HIV-1 gag produced complete protection against systemic or mucosal rVV-gag challenge after oral immunization with attenuated Lmdd-gag/pARS. Naïve BALB/c mice were unimmunized (open bars), immunized orally (filled bars) as described in the legend for Fig. 5, or immunized i.v. (hatched bar) as described in the legend for Fig. 2. Four weeks after their final immunizations, mice were challenged with rVV-gag expressing the homologous HIV gag from clade B (VVK1 or VP1287) by various routes: systemically (i.p.) (A, left columns [L], and C), intranasally (i.n.) (A, right columns [R]), or intravaginally (B). Six days after challenge, rVV-gag titers in various tissues (indicated above the columns) were assayed by infection of BSC-1 cells. Data shown are mean virus titers ± standard deviations for groups of three to five mice. The detection limit was 50 PFU. The dose of VVK1 was 10$^7$ PFU for i.n., 1.2 × 10$^9$ PFU for i.p., and 8 × 10$^9$ PFU for IVAG challenge. For VP1287, 6 × 10$^7$ PFU was used for IVAG challenge and 1 × 10$^6$ PFU for i.p. challenge.
against mucosal but not systemic challenge by rVV-gag (46). Figure 7A also shows that oral immunization led to partial protection against virus replication in the lung, the apparent target organ after nasal infection by the virus. Not discernible from the mean value shown in the figure is that four of the five intranasally challenged mice in this experiment were fully protected.

As shown in Fig. 7B, oral immunization with Lmdd-gag/pARS completely protected mice against an intravaginal challenge, as indicated by the 6-log10 reduction in virus titer in oviduct and the 4- to 5-log10 reduction in titer in ovaries, compared with titers in unimmunized control animals. As expected, no protection against challenge with vaccinia virus expressing a nef gene was observed in immune mice (data not shown). Figure 7C further confirms that virus replication in ovaries following systemic virus challenge (using two different clade B gag-expressing viruses) is only partially inhibited after oral immunization. Figure 7C also shows that there was little replication of virus in oviduct following this i.p. route of challenge.

Finally, to determine whether the oral immunization with Lmdd-gag/pARS could induce cross-clade protection, orally immunized mice were challenged intravaginally with rVV expressing HIV gag from different viral clades (Fig. 8). As shown in Fig. 8, the clade B Gag of Lmdd-gag/pARS generated strong cross-clade protection against both clades A and C but weaker protection against a clade G rVV-gag. Incomplete intravaginal protection against VT196, one of the two clade C gag-expressing rVVs used in this study, was observed, possibly due to a greater virulence of VT196 than VT369. Indeed, systemic infection with VT196 resulted in a 2-log10 greater virus titer in ovaries than infection with VT369 (Fig. 8B). Curiously, the replication of the clade A gag-expressing rVV, but not the other viruses, was almost completely blocked during a systemic i.p. challenge.

DISCUSSION

HIV transmission occurs predominantly through sexual exposure of mucosal surfaces of the genital tract and rectum to virus and virus-infected cells. Recent studies have shown that the mucosal system, particularly the gastrointestinal immune system, is the major target for HIV infection and replication at early stages, with more than 50% of memory T cells destroyed at this site within the first 2 weeks of infection (5, 19, 28, 31, 33). These findings imply that the induction of strong virus-specific immunity at mucosal sites may be crucial for an effective AIDS vaccine. Consequently, while the challenge remains to induce neutralizing antibodies, there is now a major effort to develop vaccines that maximize cell-mediated mucosal immunity, especially in the intestine and genital tract. Although mucosal immunity can be induced by many parenterally administered vaccines, a more robust response is usually elicited by vaccines that act at the oral, nasal, vaginal, or rectal mucosa.

L. monocytogenes is therefore an attractive vaccine vehicle since it naturally infects after oral ingestion and induces strong innate immune responses and potent mucosal and systemic cell-mediated adaptive immunity. In the present study, mice orally immunized with attenuated recombinant L. monocytogenes bacteria responded with functional Gag-specific CD8+ T-cell responses following mucosal and systemic immunization (Fig. 2...
alone induced very low levels of antigen-specific CD8+ T-cell responses in the spleen, comparable to that induced by the original d-alanine-dependent strain, Lmdd-gag. However, after mucosal immunization, only Lmdd-gag/pARS induced substantial CD8+ T-cell responses in the spleen and MALTs. Therefore, the largest difference in immunogenicity among the three vaccines was detected after oral immunization, whereas after systemic infection, this difference was not significant, suggesting that the gastric route presents a more demanding environment for the bacteria. Furthermore, we showed that induction of antigen-specific CD8+ T cells in the spleen is dose dependent after oral immunization: high bacterial doses resulted in consistent induction of a higher percentage of antigen-specific CD8+ T cells, while low doses resulted in lower and more-varied levels of responses (Fig. 3B). Therefore, most oral immunization experiments were initiated with a moderate dose of bacteria (2 × 107 CFU) and then boosted with a fivefold-higher dose (1010 CFU). This regimen induced substantial antigen-specific CD8+ T cells in Peyer’s patches, mesenteric lymph nodes, and spleens (Fig. 3C), whereas priming alone generated barely detectable responses in these tissues, consistent with others’ findings (42). The responses were strongly boosted by intranasal or intravaginal virus challenge with vaccinia virus expressing a homologous gag gene (Fig. 6B and Table 1). Viral infection alone induced very low levels of antigen-specific CD8+ T cells.

We and others (15, 41, 45, 46) have shown that antigen-specific CD8+ T cells are induced by wild-type and attenuated L. monocytogenes strains by use of 51Cr release in vitro CTL measurements, Gag/LLO tetramer binding assays, and peptide-stimulated IFN-γ intracellular staining. In this study, we also sought to determine whether the new constructs could elicit a CTL response detectable in vivo, an activity likely to be necessary in the control of an HIV infection. This study is the first to use this more sensitive assay to evaluate the endogenous Gag-specific CTL activity induced by an oral L. monocytogenes vaccine. In fact, in vivo lysis of antigen-labeled target cells was detected after oral immunization with both Lmdd-gag and Lmdg-gag/pARS (Fig. 4). Interestingly, unlike a previously reported study (41), we detected similar levels of Gag-specific and LLO-specific CD8+ T cells in MLN and PP (Fig. 3C), whereas a higher level of LLO-specific CD8+ T cells was consistently observed in the spleen after oral immunization (Fig. 3A to C). This suggested that Gag and LLO antigen presentation might be organ specific after oral immunization and that an attenuated strain of L. monocytogenes expressing HIV-1 Gag might disseminate systemically differently from wild-type Lm-gag.

Although L. monocytogenes has been explored as an oral vaccine against several infectious diseases, it is not known whether it induces effective immune responses at mucosal surfaces other than the gastrointestinal tract, particularly since the respiratory and genital tracts are major routes of entry of many human pathogens. Since CD8+ T-cell responses in mucosal tissues are difficult to assess, such responses are often measured in the lymph nodes draining mucosal tissues. Expansion of Gag-specific CD8+ T cells in the spleen and lymph nodes draining the respiratory tract (cervical lymph node) and the genital tract (iliac lymph node) was observed following oral immunization with Lmdd-gag/pARS (Table 1). These responses were strikingly augmented following infection with an rVV expressing a homologous gag gene (VVK1 or VP1287), administered through either the intranasal or the intravaginal route. This suggests that effective mucosal immune responses can be induced at those sites after oral immunization. Importantly, the dramatically increased frequency of Gag-specific CD8+ T cells in the iliac lymph node would be expected to translate into protection against intravaginal virus challenge. Our preliminary studies have shown that the nasal mucosa is more sensitive than the gut to infection and translocation by L. monocytogenes (unpublished data). It is therefore of interest to determine whether nasal infection with L. monocytogenes induces vaginal and intestinal responses in mice.

HIV genetic diversity has been a major challenge to the development and evaluation of effective HIV vaccines, since HIV exhibits a high frequency of mutation and leads to recombiant formation following superinfection. The three major groups of HIV have been subdivided into 11 clades. In this study, we showed that immunization with Lmdd-gag/pARS expressing HIV-1 clade B gag induced cross-clade protection against intravaginal challenge with rVV expressing HIV-1 gag from clades A and C but only weak protection against clade G (Fig. 8). In addition, when orally immunized mice were systemically challenged with virus, a 4-log10 reduction in virus titer of clade A gag-expressing rVV was observed, comparable to that of clade B gag-expressing rVV, whereas only a minor reduction in virus titer occurred in mice challenged with clade C gag- or clade G gag-expressing virus. The cross-protection is most likely mediated by CTLs recognizing closely similar H2-Kd-restricted Gag-specific CTL epitopes shared between clades A, B, and C but presented less efficiently by clade G. Our data support but are slightly different from a previously published report that a clade G Gag-specific cell-mediated immunity was found to be cross-protective with clades A and C but not clade B (24). Nevertheless, a clade B-based HIV-1 vaccine was shown to be capable of inducing cross-clade immunity in uninfected volunteers (14). Cross-clade immunity to Gag, Nef, and Env has also been reported to occur in HIV-1-infected individuals (10, 32).

In conclusion, this mouse study makes the novel observation that a new oral L. monocytogenes-based vaccine expressing the HIV gag gene induced strong antigen-specific protective CTL responses in the mucosal immune system and cross-clade protection against intravaginal virus challenge. Recent studies have already revealed that oral immunization of rhesus macaques with recombinant strains of L. monocytogenes can be effective inducers of cellular immune responses in this nonhuman primate model (3; S. Jiang, R. Rasmussen, K. McGeethan, F. Frankel, J. Lieberman, H. McClure, K. Williams, U. Babu, R. Raybourne, E. Strobert, and R. Ruprecht, submitted for publication). While the mouse model has served to prove our concept, in general it is unreliable for assessing immunogenicity in nonhuman primates and humans. We therefore look forward to further evaluation of Lmdd-gag/pARS in monkeys, where it may prove to be a particularly safe and effective mucosal vaccine against HIV/AIDS. These results may also have important implications in the development and evaluation of mucosal vaccines against other sexually transmitted diseases, such as herpes simplex virus.
VAGINAL PROTECTION AFTER ORAL LISTERIA IMMUNIZATION

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