Human Immunodeficiency Virus Type 1 Gag-Specific Mucosal Immunity after Oral Immunization with Papillomavirus Pseudoviruses Encoding Gag

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Mucosal surfaces are the primary portals for human immunodeficiency virus (HIV) transmission. Because systemic immunization, in general, does not induce effective mucosal immune responses, a mucosal HIV vaccine is urgently needed. For this study, we developed papillomavirus pseudoviruses that express HIV-1 Gag. The pseudoviruses are synthetic, nonreplicating viruses, yet they can produce antigens for a long time in the immune system. Here we show that oral immunization of mice by the use of papillomavirus pseudoviruses encoding Gag generated mucosal and systemic Gag-specific cytotoxic T lymphocytes that effectively lysed Gag-expressing target cells. Furthermore, the pseudoviruses generated Gag-specific gamma interferon-producing T cells and serum immunoglobulin G (IgG) and mucosal IgA. In contrast, oral immunization with plasmid DNA encoding HIV-1 Gag did not induce specific immune responses. Importantly, oral immunization with the pseudoviruses induced Gag-specific memory cytotoxic T lymphocytes and protected mice against a rectal mucosal challenge with a recombinant vaccinia virus expressing HIV-1 Gag. Thus, papillomavirus pseudoviruses encoding Gag are a promising mucosal vaccine against AIDS.

One of the most important primary portals for human immunodeficiency virus (HIV) transmission is the mucosal surface. Mucosal membranes, especially intestinal mucosa, contain lymphocytes, macrophages, and dendritic cells. By in situ hybridization, HIV RNA has been found in intestinal lamina propria cells (18), and by immunohistochemical analysis, HIV proteins were detected in lamina propria T cells and macrophages in HIV-infected individuals (74, 78). Also, CD68+ macrophages and follicular dendritic cells in the rectal mucosa contain HIV proteins (8). Importantly, infectious HIV can be isolated from the rectal mucosa of seropositive individuals (44, 51). HIV antigens have also been found in cells of cervical biopsy specimens from HIV-infected women (57). Furthermore, it has been shown that there is an ongoing simian immunodeficiency virus (SIV) propagation in intestines of macaques infected with a pathogenic SIV, which is a model for HIV (11). These studies provide evidence that mucosal cells harbor HIV during the course of AIDS. Therefore, it is important to generate not only systemic but also mucosal HIV-specific immune responses to prevent the entry of HIV into the mucosa, to inhibit HIV replication, and to clear HIV during and after transmission.

Virus-specific cytotoxic T lymphocytes (CTLs) have been implicated in controlling HIV infection (7, 10, 21, 22, 32, 36, 49, 56, 66, 77, 83, 84). CTLs inhibit viral replication in vitro (77, 84). The early containment of HIV replication upon infection appears to coincide with the development of HIV-specific CTL responses against multiple viral proteins (7, 49, 66). Cytotoxic responses were detected during the asymptomatic phase in the peripheral blood of HIV-infected individuals in the absence of in vitro stimulation (22, 32) because of the high frequency of activated HIV-specific CTLs (33, 34, 39, 41, 60). The progression to AIDS is marked by an increase in viral replication associated with a decline in CTL activity (10, 21, 56). Furthermore, a depletion of CD8+ CTLs in SIV-infected macaques resulted in a dramatic rise in plasma viremia (25). These studies show that CTLs play an important role in the control of HIV and SIV replication.

The HIV type 1 (HIV-1) Gag protein is one of the most conserved viral proteins; thus, it is a suitable target antigen for the development of an HIV vaccine. Several CTL epitopes in the Gag protein have been identified (52). Broad, cross-clade CTL responses against conserved epitopes of Gag have been detected in HIV-1-infected individuals (6, 12, 15, 17, 41, 45). CTLs that are specific for Gag play an important role in clearing primary viremia and in controlling later viral replication, resulting in the slow progression of the disease (23, 31, 33, 46, 53, 59, 62, 63, 64, 82). Furthermore, the presence of mucosal HIV-1-specific CTLs in the cervix is associated with an absence of detectable HIV-1 infection in the genital mucosa (27), and SIV-specific CTLs in the intestinal lamina propria are associated with protection against a colonic SIV challenge (37, 48). These results strongly support the important role of mucosal HIV-1-specific CTLs in protecting the host against HIV-1 infection.

Papillomaviruses (PVs) are a group of small DNA viruses that naturally infect skin and mucosal surfaces. The PV major protein L1 can be assembled spontaneously into virus-like particles (VLPs) when it is expressed in insect cells, yeast, and even bacteria (19, 29, 50, 61, 69, 80). Furthermore, VLPs can be used to package unrelated plasmids to form PV pseudoviruses (28, 72, 76). PV pseudoviruses are mucosa tropic and induce mucosal cellular immune responses (72). Because these
pseudoviruses are synthetic, nonreplicating viruses, they are safe vaccines for exposing an antigen to the immune system for a long time. To develop a mucosal vaccine that can induce HIV-1 Gag-specific CTLs at mucosal surfaces, we constructed human PV (HPV) and bovine PV (BPV) pseudoviruses encoding HIV-1 Gag. We report here for the first time that oral immunization with the PV pseudoviruses expressing HIV-1 Gag induced mucosal and systemic CTL responses that protected mice against a rectal mucosal challenge with a recombinant vaccinia virus expressing HIV-1 Gag.

MATERIALS AND METHODS

Construction of a plasmid encoding HIV-1 Gag. The gag gene used for this study was the full-length wild-type gag gene of HIV-1 subtype B. The following Gag-1 and Gag-2 primer pair was used to amplify the full-length gag gene: Gag-1, 5′-GAT ACT CTA GAA TTC GCC GCC ACC ATC GTT GCG AGA CGA GCG TCA (5′ to 3′); and Gag-2, 5′-GCT CTA GAT CGT CCA GAG TGT TAG GTC CTA CAG C (5′ to 3′). The plasmid DNA was transformed into Escherichia coli DH5α bacteria that were grown in Luria-Bertani (LB) medium in the presence of ampicillin. Plasmids were extracted by the alkaline lysis method followed by purification on cesium chloride density gradients. The DNA concentration was determined by measuring the optical density at 260 nm. The integrity of the plasmids and the absence of contaminating E. coli DNA or RNA were checked by agarose gel electrophoresis. The DNA was stored at −20°C in Tris-EDTA buffer.

Production of PV pseudovirus encoding HIV-1 Gag and evaluation of efficiency of plasmid DNA encapsidation in VLPs. Briefly, 100 μg of purified HPV-16 VLPs or BPV-1 VLPs was incubated in 25 mM Tris-HCl buffer (pH 8.0) containing 15 mM NaCl, 10 mM EGTA, and 20 mM dithiothreitol in a final volume of 200 μl at room temperature for 60 min. At this step, 20 μg of pCI-Gag was added to the disrupted VLPs. The preparation was then incubated with CaCl2 (25 mM) and 20% dimethyl sulfoxide in an equal volume at 20°C for 1 h to form a BPV pseudovirus encoding Gag (BPVpsv-Gag) or an HPV pseudovirus expression Gag (HPVpsv-Gag). For evaluations of the efficiency of plasmid DNA encapsidation in the VLPs, the preparation was separated into two fractions of equal volumes, and one was treated with 80 U of Benzonase (Bz) (Sigma, St. Louis, Mo.) for 1 h at 20°C while the other was not treated. Both fractions were then heated at 100°C for 10 min and digested with proteinase K at 5°C (50 μg/ml) and 5% CO2 for 7 days in complete cell culture medium with 10 U of interleukin-2 (IL-2)/ml and 5 μg of HPV-1 Gag peptide (AMQMLKETI [H-2Dd-restricted epitope])/ml. Gag-specific cytolytic activity was measured with a 51Cr release assay, and Gag-specific gamma interferon (IFN-γ)-producing cells was determined with an ELISPOT assay (see below).

For the detection of mucosal CTLs, small intestines were flushed with cold PBS to remove the fecal contents. After the Peyer’s patches (PP) were excised and the mesenteric lymph nodes (MLN) were removed, the intestines were opened longitudinally, minced into 5- to 10-mm-long pieces, and washed extensively with cold PBS. Mucosal pieces were then treated twice for 30 min each time at 37°C with 5 mM EDTA (Sigma, St. Louis, Mo.) and 10% FCS in PBS. After each treatment with EDTA, the mucosal pieces were washed with cold PBS and the supernatants were discarded. The remaining tissues were then digested for 30 min in Dulbecco’s modified Eagle’s medium (DMEM) containing 100 U of collagenase (Sigma)/ml, 25 mM HEPES, 7 mM CaCl2, and 20% FCS. The digestion was repeated four times. After each 30-min digestion, the released cells were centrifuged, washed, and stored in DMEM with 10% FCS on ice. Large debris were eliminated from the cell suspension by a passage through nylon wool columns at room temperature. The column eluant was diluted in DMEM with 5% FCS containing 0.3 mg of dithiothreitol (Life Technologies, Grand Island, N.Y.)/ml, and viable cells were isolated by centrifugation over Lymphohyte-M (Cedarlane, Ontario, Canada). After centrifugation, the cells were collected from the interface and washed. PP and MLN lymphocytes were isolated by mechanical dissociation in complete cell culture medium. Gag-specific cytolytic activity was measured with a 51Cr release assay, and Gag-specific IFN-γ-producing cells were determined with an ELISPOT assay (see below).

For the detection of Gag-specific antibodies, blood was withdrawn from the heart, and centrifuged and the serum was collected. In addition, the small intestinal contents were flushed with cold PBS (5 ml) containing a protease inhibitor set (Roche Applied Science, Indianapolis, Ind.). The intestinal contents were mixed on a vortex machine for 30 s and centrifuged for 10 min to remove insoluble material. The resulting extract was passed through a 0.45-μm-pore-size filter (Millipore, Bedford, Mass.), and aliquots were stored at −20°C. Gag-specific IgG in sera and IgA in the mucosal washings was measured with an enzyme-linked immunosorbent assay (ELISA) (see below).

35S Cr release assay. Target cells (106 p815 cells) were labeled with 35S Cr (100 μCi) for 1 h at 37°C and 5% CO2 and were then washed three times. The cells were then loaded with peptides by directly adding an HIV-1 Gag peptide (AMQMLKETI) to the cells at 5 μg/ml. Target cells (2,000 cells per well) were then incubated with effector cells at different effector/target ratios in V-bottomed 96-well microtiter plates for 6 h at 37°C. The supernatants were collected, and 35S Cr release was quantified by use of a γ counter (ICN Biomedical Inc., Huntsville, Ala.). Specific lysis was calculated according to the following formula: specific lysis (%) = [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. Spontaneous release was determined in control microwells containing 35S-labeled target cells in culture medium with no effector cells. Maximum release was determined by lysing 35S-labeled target cells with 0.5% (vol/vol) NP-40.

ELISPOT assay. The ELISPOT assay described by Taguchi et al. (75) was modified to detect specific CD8 T lymphocytes. First, 96-well filtration plates (Millipore) were coated with a rat anti-mouse IFN-γ antibody (2 μg/ml; Pharmingen, San Diego, Calif.). The wells were kept under pathogen-free conditions. The protocol for mouse was approved by the Institutional Animal Care and Use Committee.

Immunization and detection of Gag-specific T cells and antibodies. Mice (four per group) were given 2.66 × 1011 PBV pseudovirions encoding Gag by gastric intubation, the same amount of BPV VLPs used for the preparation of BPVpsv-Gag, or 10 μg of plasmid DNA (pCI-Gag) (the same amount of plasmid DNA used for the preparation of the pseudovirus) in 100 μl of PBS for 3 h. Some groups of mice were given a boost of HPVpsv-Gag (2.66 × 1011 pseudovirions), HPV VLPs, or 10 μg of pCI-Gag. Fourteen days after the boost, the mice were used for the detection of HIV-1 Gag-specific immune responses (see below). For the determination of Gag-specific memory responses and protection against a mucosal challenge, 5 weeks after the boost the mice were challenged intrarectally with the recombinant vaccinia virus expressing HIV-1 Gag (5 × 106 PFU) on day 8 after the challenge, the mice were used for the detection of HIV-1 Gag-specific immune responses and vaccinia virus titers. Some mice were used to detect HIV-1 Gag-specific immune responses 5 weeks after the boost without a challenge.

For the detection of systemic CTLs, spleen cells were isolated from each mouse. After incubation in nylon wool columns for 1 h at 37°C in 5% CO2, enriched T cells were washed through the column with complete cell culture medium. The cells were cultured at 37°C in 5% CO2 for 7 days in complete cell culture medium with 10 U of interleukin-2 (IL-2)/ml and 5 μg of HIV-1 Gag peptide (AMQMLKETI [H-2Dd-restricted epitope])/ml. Gag-specific cytolytic activity was measured with a 51Cr release assay, and Gag-specific gamma interferon (IFN-γ)-producing cells was determined with an ELISPOT assay (see below).
cells per well and 10 μl of recombinant human IL-2 (PharMingen)/ml. The cells were stimulated for 48 h with the HIV-1 Gag peptide, the plates were washed, and then the cells were incubated with a biotinylated anti-mouse IFN-γ antibody (2 μg/ml; PharMingen). The plates were washed, and the cells were incubated with streptavidin-horseradish peroxidase (1 μg/ml; PharMingen). Spots were developed with freshly prepared substrate buffer (0.33 mg of 3-amino-9-ethylcarbazole/ml and 0.015% H2O2 in 0.1 M sodium acetate, pH 5).

ELISA. Ninety-six-well plates were coated with Gag protein (Research Diagnostics, Inc., Flanders, N.J.) (200 ng/100 μl/well) or PBS at room temperature overnight. Blocking solution (PBST with 1% bovine serum albumin) (200 μl/well) was incubated at room temperature for an hour, and then serum or mucosal washings were diluted in blocking solution and incubated in the wells (100 μl/well) for 2 h. The wells were washed, and 50 μl of biotinylated anti-mouse IgG or IgA (Sigma) (diluted 1:1,000 in 1% bovine serum albumin-PBST) (2 μg/ml) was added to the wells and incubated for 1 to 2 h at room temperature. After washing, streptavidin-horseradish peroxidase (DAKO Corporation, Carpinteria, Calif.) was incubated in a volume of 100 μl/well (diluted 1:5,000 in PBS) for 30 min at room temperature. After washing, the substrate 3,3′,5,5′-tetramethylbenzidine (Sigma) was added (100 μl/well), and the reaction was stopped by the addition of 100 μl of 2 N H4SO4 per well. The optical densities at 450 nm were measured with an ELISA reader.

Recombinant vaccinia virus expressing HIV-1 Gag. The HIV-1 gag gene was amplified by PCR and cloned into the Smal site of the plasmid pSC11 by blunt-end ligation. The resulting pSC11 plasmid containing gag in the correct orientation was introduced into the WR strain of vaccinia virus by homologous recombination as previously described (54). The recombinant vaccinia virus encoding gag (rVVgag) was plaque purified four times, and the identity of the gag gene in the recombinant virus was confirmed by DNA sequencing.

Vaccinia virus protection assay. Mice were challenged by intraocular inoculation with 5 × 104 PFU of rVVgag. Six days after challenge with the virus, the mice were sacrificed, and both ovaries of each mouse were removed, homogenized, and resuspended in minimum essential medium supplemented with 5% FCS at a concentration of one ovary per milliliter. Individual samples from each mouse were kept at ~70°C. For measurements of virus titers, the samples were thawed, sonicated, and assayed by plating serial 10-fold dilutions on CV-1 cells in 12- or 6-well plates. The plates were incubated for 48 h and then stained with crystal violet (0.1% [wt/vol] crystal violet in 20% [vol/vol] ethanol). Individual plaques at each dilution were counted. The limit of detection of virus plaques by this assay was 10 PFU.

Statistical analysis. The data were analyzed with statistical software (SPSS 8.0; SPSS Science, Chicago, Ill.) and also by one-way analysis of variance followed by a least significance difference test. A P value of <0.05 was considered statistically significant.

RESULTS

Development of PV pseudoviruses encoding HIV-1 Gag. HPV-16 L1 or BPV-1 L1 VLPs were produced in Spodoptera frugiperda (Sf9) insect cells by use of a recombinant baculovirus encoding either HPV-16 L1 or BPV-1 L1. Twenty-five amino acids from the C terminus of BPV-1 L1 and 34 amino acids from the C terminus of HPV-16 L1 were deleted to enhance the production of the VLPs (47, 55). Plasmid DNA encoding HIV-1 subtype B Gag (pCI-Gag) was packaged into the VLPs to form HIV-PV pseudoviruses. To further determine the efficiency of plasmid DNA encapsidation in the VLPs, we used Bz after the package to digest DNAs on the surfaces of the VLPs. The Bz-treated pseudoviruses were then digested with proteinase K to disrupt VLPs, and the amount of plasmid DNA inside the VLPs was determined by ethidium bromide fluorescence quantitation and spectrophotometry. As a control, a portion of the pseudoviruses were only treated with proteinase K and the amount of plasmid DNA on and inside the VLPs was determined in the same way. As shown in Fig. 1A, about one-fifth of the pCI-Gag was packaged inside the VLPs.

Expression of HIV-1 Gag protein in cells that are pseudoinfected by pseudoviruses. To determine whether PV pseudoviruses encoding Gag were able to pseudoinfect cells so that HIV-1 Gag could be expressed, we first incubated CV-1 cells with BPVpsv-Gag, HPVpsv-Gag, or control VLPs and
then infected the cells with the recombinant vaccinia virus VTF 7-3 to provide T7 RNA polymerase to increase Gag expression (19, 68). The expression of Gag by the pseudovirus-pseudoinfected cells was then determined by indirect immunofluorescence. As shown in Fig. 1B, Gag was expressed in cells that were pseudoinfected by the pseudoviruses. The expression was localized to surface membranes, which is consistent with previous reports (70, 81). In contrast, HIV-1 Gag was not expressed in cells that were incubated with control VLPs (data not shown). Our data demonstrated that PV pseudoviruses encoding Gag were able to pseudoinfect cells, leading to HIV-1 Gag expression.

Oral immunization with PV pseudoviruses encoding HIV-1 Gag induced Gag-specific mucosal and systemic CTLs and antibodies. To determine whether oral immunization with PV pseudoviruses encoding Gag induced both mucosal and systemic HIV-1 Gag-specific CTLs and antibody responses, we immunized mice (BALB/c females) by gavage with BPVpsv-Gag, BPV VLPs, or plasmid DNA encoding HIV-1 Gag (pCI-Gag). Fourteen days later, the mice were given a boost with HPVpsv-Gag, pCI-Gag, or BPV VLPs. Two weeks after the boost, the mice were sacrificed. PP and MLN lymphocytes (A and B) and lamina propria (LP) lymphocytes (C) were isolated and used immediately to detect Gag-specific cytolytic activity. (D and E) Splenic lymphocytes were isolated and stimulated in vitro with a Gag peptide (AMQMLKETI) for 7 days before being tested in a \[^{51}Cr\] release assay. The target cells were p815 cells pulsed with or without the Gag peptide (AMQMLKETI). The effector-to-target (E:T) ratios were 100:1, 30:1, 10:1, and 3:1 for each group. The lymphocytes lysed p815 pulsed with the Gag peptide significantly more than the target cells without the peptide in panels A, B, C, and E (P < 0.04), but not in panel D. **+, P < 0.002 versus +; §, P < 0.02 versus ¶.

FIG. 2. Induction of HIV-1 Gag-specific mucosal and systemic CTL responses by PV pseudoviruses encoding Gag. Groups of BALB/c mice were orally immunized with BPVpsv-Gag, plasmid DNA encoding HIV-1 Gag (pCI-Gag), or BPV VLPs and then were boosted with HPVpsv-Gag, pCI-Gag, or BPV VLPs. Two weeks after the boost, the mice were sacrificed. PP and MLN lymphocytes (A and B) and lamina propria (LP) lymphocytes (C) were isolated and used immediately to detect Gag-specific cytolytic activity. (D and E) Splenic lymphocytes were isolated and stimulated in vitro with a Gag peptide (AMQMLKETI) for 7 days before being tested in a \[^{51}Cr\] release assay. The target cells were p815 cells pulsed with or without the Gag peptide (AMQMLKETI). The effector-to-target (E:T) ratios were 100:1, 30:1, 10:1, and 3:1 for each group. The lymphocytes lysed p815 pulsed with the Gag peptide significantly more than the target cells without the peptide in panels A, B, C, and E (P < 0.04), but not in panel D. **+, P < 0.002 versus +; §, P < 0.02 versus ¶.

As shown in Fig. 2, lymphocytes from PP, MLN, lamina propria, or spleens of mice immunized with BPVpsv-Gag and boosted with HPVpsv-Gag significantly lysed the HIV-1 Gag peptide-pulsed target cells. The cytolytic activity of this group was higher than that of mice immunized only with BPVpsv-Gag without a boost (P < 0.02 for spleen cells and P < 0.002 for PP and MLN). Furthermore, more specific cytolytic activity was detected in PP and MLN lymphocytes than in splenic lymphocytes in the mice that were primed and boosted (P < 0.031). In contrast, the lymphocytes from mice immunized with control VLPs or pCI-Gag did not kill the target cells pulsed with the Gag peptide. Figure 3 shows the numbers of IFN-γ-producing cells in response to stimulation with the Gag peptide in the different groups of mice. Similar to the cytolytic activity, priming with BPVpsv-Gag and a boost with HPVpsv-Gag induced more PP, MLN, and spleen T cells to produce IFN-γ in response to the HIV-1 Gag peptide than did one immunization with BPVpsv-Gag (P < 0.003 for PP and MLN and P < 0.02 for spleen cells). Similarly, oral immunization with pCI-Gag or control VLPs did not induce any Gag-specific T cells.

As shown in Fig. 4, oral immunization with PV pseudoviruses encoding Gag induced Gag-specific mucosal IgA and serum IgG. It was interesting that one immunization with BPVpsv-Gag induced a very strong Gag-specific serum IgG response, similar to that induced by priming and booster immunizations. In contrast, priming and booster immunizations...
induced a stronger mucosal Gag-specific IgA response than one immunization ($P < 0.019$). Mice immunized with control VLPs or pCI-Gag did not develop Gag-specific antibodies.

Oral immunization with PV pseudoviruses encoding Gag induced a memory CTL response and protected mice against rectal challenge with a recombinant vaccinia virus expressing HIV-1 Gag. To test whether PV pseudoviruses encoding Gag induced memory T cells specific for HIV-1 Gag, we orally immunized mice with BPVpsv-Gag and gave them a booster with HPVpsv-Gag. Some naive mice were also challenged as controls. On day 6 after the challenge, the spleen, PP, MLN, and ovaries were removed. Lymphocytes from spleens, PP, and MLN were used to measure memory T cells that were specific for HIV-1 Gag by a $^{51}$Cr release assay and an ELISPOT assay. The ovaries were used to determine the titers of the recombinant vaccinia virus. Mucosal washings and serum were used to determine Gag-specific antibody responses. We also determined the immune responses in mice who were immunized similarly but did not receive the vaccinia virus challenge. As shown in Fig. 5A, Gag-specific cytolytic activity was found among freshly isolated PP and MLN lymphocytes in mice who were immunized but not challenged. However, no Gag-specific CTLs could be found among splenic lymphocytes without stimulation with a Gag peptide in vitro (data not shown). After in vitro stimulation for a week, Gag-specific CTLs were detectable among splenic lymphocytes (Fig. 5B). The Gag-specific cytolytic activity was higher among lym-
phocytes from mice receiving the challenge than from those from unchallenged mice ($P < 0.001$ for PP and MLN and $P < 0.03$ for spleen cells). The presence of Gag-specific memory T cells in PP, MLN, and spleens from mice with and without the challenge was further demonstrated with an ELISPOT assay (Fig. 5C and D). In a group of naive mice that had been challenged with rVVgag, no Gag-specific CTLs were detected on day 6 after the challenge. These data indicate that PV pseudoviruses encoding Gag induced a memory CTL response against HIV-1 Gag. The mucosal IgA response specific for Gag 5 weeks after the boost appeared to be less than that 2 weeks after the boost. It increased after the challenge with the vaccinia virus. In contrast, the serum IgG response specific for Gag 5 weeks after the challenge was similar to that 2 weeks after the boost. The vaccinia challenge did not further increase the IgG level in serum.

Figure 6 shows that $3.1 \times 10^7$ PFU (mean) of rVVgag were detected in every mouse from the control group, whereas no vaccinia virus was found in mice immunized with PV pseudoviruses encoding Gag ($P < 0.001$). This finding indicates that PV pseudoviruses encoding Gag protected mice against the mucosal challenge with a recombinant vaccinia virus expressing Gag.

**DISCUSSION**

Our data suggest (i) that PV VLPs delivered HIV-1 gag DNA to mucosal and systemic lymphoid tissues and (ii) that the DNA encoding HIV-1 Gag was expressed, resulting in the induction of mucosal and systemic HIV-1 Gag-specific CTLs and antibodies. We also demonstrated that oral immunization with a plasmid DNA encoding Gag (pCI-Gag) did not induce Gag-specific CTLs. This was most likely because the plasmid DNA could not reach mucosal lymphoid tissues. Thus, PV VLPs are excellent vehicles for the delivery of plasmid DNA to mucosal lymphoid tissues. In addition, it has been shown that PV VLPs can activate dendritic cells so that the activated cells

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

![Graph D](image4)

**FIG. 5.** Gag-specific T-cell responses with and without mucosal challenge with rVVgag. (A and B) Groups of mice were orally primed and boosted as described in the legend for Fig. 2. Five weeks after the boost, the mice were sacrificed. Other groups of mice were primed, boosted, and then challenged intrarectally with rVVgag 5 weeks after the boost. On day 6 after the challenge, the mice were sacrificed. PP, MLN, and splenic lymphocytes were isolated and used to detect Gag-specific CTLs in $^{51}$Cr release and ELISPOT assays. Splenic lymphocytes were stimulated in vitro with a Gag peptide (AMQMLKETI) for 7 days before being tested in the $^{51}$Cr release assay. The effector-to-target ratios were 100:1, 30:1, 10:1, and 3:1. $*P < 0.001$ versus (A), $P < 0.03$ versus (B), $P < 0.004$ versus (C), and $P = 0.058$ versus (D). A group of naive mice who were infected only with the vaccinia virus for 5 days did not show any CTL response specific for HIV-1 Gag (data not shown).

**FIG. 6.** Oral immunization with PV pseudoviruses encoding Gag protected mice against a rectal mucosal challenge with recombinant vaccinia virus expressing HIV-1 Gag. Female BALB/c mice were orally immunized and boosted with PV pseudoviruses encoding Gag or PBS (control). Five weeks after the boost, the mice were challenged intrarectally with $5 \times 10^8$ PFU of recombinant vaccinia virus expressing HIV-1 Gag. On day 6 after the challenge, the ovaries were obtained from each mouse and homogenized, and then viral titers were measured. The data are means of vaccinia virus titers for each group (four mice per group). Error bars represent standard deviations. $*, P < 0.001$ versus the control group.
express more costimulatory molecules, such as CD80 and CD86, and produce more proinflammatory cytokines, such as IL-6 and tumor necrosis factor alpha, than resting dendritic cells (38, 65). We believe that activated antigen-presenting cells may have enhanced the capacity for the activation of HIV-1 Gag-specific CTL precursors because of their augmented expression of costimulatory molecules and proinflammatory cytokines. In addition, PV VLPs have been shown to induce T-helper type 1 cells (13, 14, 38, 42), which may also increase the expansion of Gag-specific CTLs and help to produce Gag-specific antibodies. Thus, PV VLPs might contribute to the generation of Gag-specific immune responses in addition to serving as a delivery vector.

One immunization with BPVpsv-Gag was able to induce a Gag-specific mucosal CTL response but a weak systemic CTL response. A boost with HPVpsv-Gag greatly enhanced the mucosal and systemic CTL responses. In contrast, one immunization induced high levels of Gag-specific serum IgG, and a booster immunization did not further enhance the antibody level. However, a booster immunization did augment the level of Gag-specific mucosal IgA. These data suggest that a booster immunization is required to induce an effective CTL response and a mucosal IgA response but not a systemic IgG response.

Because the intestinal mucosa is one of the primary portals for HIV infection and because HIV-infected blood cells can circulate to and reside in the mucosa, the induction of intestinal mucosal HIV-specific CTL responses is extremely important for controlling the disease. Oral immunization with PV pseudoviruses encoding Gag induced intestinal mucosal HIV-1 Gag-specific CTL responses. We further tested whether the vaccine could provide mucosal protection against a challenge with rVVgag. Because mice cannot be infected with HIV-1, we used rVVgag as a substitute, as originally done by Belyakov et al. (1, 3). We showed that the vaccine provided protection against the mucosal challenge, although protection against the vaccinia virus was not a perfect model for protection against HIV-1. We believe that the protection was due to mucosal Gag-specific CTL responses rather than Gag-specific antibody responses because Gag was expressed by the recombinant vaccinia virus after it had infected cells. Gag was not part of the vaccinia virion. Thus, the protection provided by PV pseudoviruses encoding Gag was mediated by CTL lysis of virally infected cells expressing HIV-1 Gag. In fact, there was a strong cytolytic activity against target cells that were pulsed with an HIV-1 Gag peptide among lymphocytes that were freshly isolated from PP in mice immunized with PV pseudoviruses encoding Gag and challenged with rVVgag. This finding strongly suggests that mucosal CTLs specific for HIV-1 Gag mediated anti-HIV-1 Gag activity when the mice were challenged with rVVgag.

PV pseudoviruses encoding Gag are synthetic pseudoviruses that contains two major components, namely, plasmid DNA and PV VLPs. Both have been safe for humans in clinical trials (9, 20, 24). There is no possibility for PV pseudoviruses encoding Gag to regain virulence as either PV or HIV; thus, they are an extremely safe mucosal vaccine. In addition, oral vaccines eliminate the unnecessary stress associated with systemic vaccination. Because HIV-1 also infects vaginal mucosa, it would be interesting to test whether oral immunization with this vaccine will induce vaginal mucosal HIV-specific CTL responses, and this is currently being investigated by our group.

Several mucosal vaccines have been developed against HIV-1 by the use of different delivery vector systems, such as Listeria monocytogenes, a Salmonella type III antigen delivery system, a Shigella DNA vaccine vector, Sindbis virus-based replication particles or poly(lactide-co-glycolide) microparticles, and modified vaccinia virus Ankara and Ty VLPs or hemagglutinating virus of Japan liposome, and by the use of mucosal adjuvants, such as cholera toxin and E. coli labile toxin, with or without cytokines such as IL-12 (1–5, 16, 26, 30, 35, 40, 43, 58, 67, 71, 73, 79). These vaccines have been shown to induce mucosal immune responses to HIV-1 or SIV proteins. Our study demonstrates that PV pseudoviruses encoding Gag may serve as another novel mucosal vaccine against HIV-1 because they induce a strong HIV-1 Gag-specific memory CTL response in mucosal and systemic lymphoid tissues and mucosal protection through oral immunization. These studies provide promising candidate vaccines for future clinical trials.

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REFERENCES


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