Induction of HIV Immunity in the Genital Tract After Intranasal Delivery of a MVA Vector: Enhanced Immunogenicity After DNA Prime-Modified Vaccinia Virus Ankara Boost Immunization Schedule

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Induction of HIV Immunity in the Genital Tract After Intranasal Delivery of a MVA Vector: Enhanced Immunogenicity After DNA Prime-Modified Vaccinia Virus Ankara Boost Immunization Schedule

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Vaccines intended to prevent mucosal transmission of HIV should be able to induce multiple immune effectors in the host including Abs and cell-mediated immune responses at mucosal sites. The aim of this study was to characterize and to enhance the immunogenicity of a recombinant modified vaccinia virus Ankara (MVA) expressing HIV-1 Env IIIb Ag (MVAenv) inoculated in BALB/c mice by mucosal routes. Intravaginal inoculation of MVAenv was not immunogenic, whereas intranasally it induced a significant immune response to the HIV Ag. Intranasal codelivery of MVAenv plus cholera toxin (CT) significantly enhanced the cellular and humoral immune response against Env in the spleen and genitorectal draining lymph nodes, respectively. Heterologous DNAenv prime-MVAenv boost by intranasal immunization, together with CT, produced a cellular immune response in the spleen 10-fold superior to that in the absence of CT. A key finding of these studies was that both MVAenv/MVAenv and DNAenv/ MVAenv schemes, plus CT, induced a specific mucosal CD8+ T cell response in genital tissue and draining lymph nodes. In addition, both immunizations also generated systemic Abs, and more importantly, mucosal IgA and IgG Abs in vaginal washings. Specific secretion of β-chemokines was also generated by both immunizations, with a stronger response in mice immunized by the DNA-CT/MVA-CT regimen. Our findings are of relevance in the area of vaccine development and support the optimization of protocols of immunization based on MVA as vaccine vectors to induce mucosal immune responses against HIV. The Journal of Immunology, 2004, 172: 6209–6220.

The stimulation of the mucosal immune response can be achieved by the administration of immunogens at mucosal inductive sites, where specialized organized lymphoepithelial follicular structures exist. The concept of a common mucosa-associated system regulating and coordinating immune responses at mucosal surfaces has been an important advance in our understanding of protection against mucosal pathogens. This system, called the mucosa-associated lymphoid tissue, is based on primed T and B lymphocytes that migrate from the site of Ag presentation via the lymphatics and blood to selectively home to lymphoid tissue at distant sites in gastrointestinal, respiratory, genitourinary, and other mucosa-associated regions. Various studies have demonstrated that both oral and intranasal administration of Ags are capable of inducing immune responses at distant effector sites. In this sense, the use of the intranasal (i.n.)3 route of immunization to stimulate inductive sites in the respiratory tract has been of considerable interest in the past years. Moreover, nasal vaccination in rodents has emerged as the optimal vaccination route for the induction of genital Ab responses. In particular, live viral vaccines administered i.n. efficiently stimulate humoral and cell-mediated immune responses in both mucosal and systemic compartments. Modified vaccinia virus Ankara (MVA) is one of the most promising live viral vectors to be applied as a recombinant vaccine, due to its safety and ability to trigger protection against a wide spectrum of pathogens (7–13). Most studies have
shown the immunogenicity of MVA recombinants when inoculated by systemic routes, but few studies have analyzed the immune response elicited after administration of MVA by mucosal routes. Indeed, i.n. delivery of MVA recombinants was effective to induce a protective response against influenza (14), parainfluenza (15), and respiratory syncytial virus (16). Moreover, we have recently described that by the i.n. route MVA produced an effective specific immune response against HIV-env Ag in mice that were previously primed i.n. with recombinant influenza virus (17). A study by Belyakov et al. (8) demonstrated that intrarectal immunization with MVA expressing gp160 of HIV-1 89.6 induced a mucosal and systemic CTL response. These previous studies indicate that MVA recombinants are activators of specific immune responses at mucosal surfaces and suggest that further exploitation should be done with MVA to further characterize and improve its immunogenicity.

DNA vaccines have most often been administered systemically, via i.m. injection or by the gene gun approach, but can also induce protection in mice when given i.n. before a lethal challenge with influenza virus (18). Limitations that may arise after mucosal DNA vaccination may be overcome, potentifying its effectiveness by adsorption of DNA into positively charged polyactide-coglycolide microparticles (19), or codelivering the DNA that encodes for the specific Ag with DNA vectors encoding cytokines (20). Also, coadministration of DNA vectors with mucosal adjuvants have been proved to be as an efficient strategy, giving protection against equine influenza in a pony model (21).

Although the immunological correlates of protection against HIV in humans are yet to be defined, several studies in nonhuman primates support the correlation between vaccine-induced HIV-specific cellular immunity and protection against subsequent challenge with pathogenic simian/human immunodeficiency virus (7, 22). In the same way, the importance of CTLs in protection was demonstrated in seronegative individuals at risk of HIV infection and slow or nonprogressors (23, 24). Therefore, most vaccines in preclinical experiments in macaques and phase II/II clinical trials in humans are based on eliciting an effective CTL response against HIV. However, a series of recent reports raised challenging questions about a vaccine strategy based only on inducing a CTL response, implying that vaccine candidates must consider multiple important aspects of vaccination against HIV, as targeting the mucosal immune system and associated regional lymph nodes (LN), as well as enhancing innate immunity and stimulating broadly based adaptive immune responses (1, 25).

Systemic vaccine strategies based on prime-boost regimens that imply a DNA prime immunization followed by a booster with recombinant poxvirus have largely proved that this approach elicits potent cellular immune responses to SIV and HIV Ags (7, 26, 27). With the exception of a few studies (28–30), these immunization strategies have been principally performed after delivery of the vectors by systemic routes, and the measurements of immune parameters have focused on systemic immunity (7, 26, 27). To our knowledge, there are no reports characterizing the efficacy of rMVA inoculated by a mucosal route in a DNA prime/MVA boost protocol against HIV Ags.

The aim of this study was to analyze different protocols of immunization to enhance the immunogenicity of rMVA expressing the HIV-1 Env IIIB Ag delivered by different mucosal routes. After inoculation of MVA i.n., a significant immune response was induced, which was considerably incremented by its codelivery with the mucosal adjuvant cholera toxin (CT). Also, an i.n. DNA prime-rMVA boost immunization scheme, in the presence of CT, induced a potent specific cellular immune response in the spleen and at mucosal effector sites of the urogenital tract and genital draining LN. Moreover, both rMVA + CT/rMVA + CT and rDNA + CT/rMVA + CT schemes induced mucosa-specific Abs to Env in vaginal washings. These findings are important in vaccination because of the global need to design immunization strategies that can induce mucosal immunity against HIV.

Materials and Methods

Viruses and cells

The rVEnv (Western Reserve (WR) strain) and rMVAenv viruses used in this study have been previously described (31, 32). Both recombinant viruses have the inserted foreign gene in the thymidine kinase viral locus and express the complete IIIB HIV-1 gp160 under the synthetic p7.5 early late promoter. WR recombinants were grown in human HeLa cells, whereas MVA derivatives were grown in primary chicken embryo fibroblasts. Sucrose cushion-purified viral stocks of rMVA and rWR were titered in baby hamster kidney BHK21 cells or African green monkey kidney BSC-40 cells by immunostaining of fixed infected cultures with polyclonal serum reactive against vaccinia virus (VX) proteins or by plaque assays, respectively (30).

Plasmid for DNA immunization

DNA immunization was done with a DNA plasmid encoding the HIV-1 strain IIIB Env gene (pEnv), which was expressed under the control of the CMV IE promoter. The DNA vector penv was a gift of A. Bülthoff (Max von Pettenkofer Institut, Genzentrum, Ludwig Maximilians Universität München, Munich, Germany) and contain gp120 modified for optimized codon usage (syncp120) cloned in pCR3 (33). DNA plasmid was purified on Qiagen columns (Qiagen, Chatsworth, CA) using pyrogen-free material and eluted in pyrogen-free deionized water.

Immunization protocols

BALB/c mice (H-2D^b) (6–8 wk old) (Harlan Orlac, Blackthorn U.K.) were used in these studies. Parenteral immunizations were given by the s.c. route (in the base of the tail) (100 μl), by i.p. route (200 μl), or in the trigeminal muscle (100 μl; i.m. route). Mucosal immunizations were performed under light anesthesia (halothane), by intranasal route (25 μl; i.n. route) or intravaginal route (25 μl; i.vag. route). In all the experiments described, the viral dose applied was 10^7 PFU/mouse, and the quantity of DNA used in the immunizations was 50 μg/mouse. Prime and boost immunizations doses were spaced by 14 days. CT from Vibrio cholerae (Sigma-Aldrich, St. Louis, MO) was administered in a dose of 10 μg/mouse mixed with the viral vector dose (MVAenv, 10^7 PFU/mouse) or with DNA vector.

Sample collection and processing

Serum was obtained by retro-orbital bleedings 10–14 days after the last immunization and was allowed to clot 30 min at 37°C; after leaving samples at 4°C overnight, they were spun down in a microcentrifuge, and sera were removed and stored at −20°C. Vaginal wash samples were obtained by flushing several times 50 μl of PBS into the vaginal canals; then samples were spun down to remove cellular debris and frozen at −70°C until quantification of Abs by ELISA.

Splenocytes and lymphocytes from the genitourinary-associated lymphoid tissue (1) (iliac and inguinal LN), and nasal associated lymphoid tissue (cervical LN; CLN) were isolated with routine methods. Lymphocytes from the urogenital tract were obtained as previously described (34). Briefly, the urogenital tracts (vagina, cervix, and urethra horns) were aseptically dissected, and tissue was longitudinally split, cut in very small pieces, and washed with RPMI 1640 (incomplete medium). Then, tissue segments were dissociated enzymatically with a mixture of collagenase type VIII (Sigma-Aldrich; 1 mg/ml) and dispase (1 mg/ml; (Roche Diagnostics, Mannheim, Germany) in RPMI (5% FCS), with agitation for 20 min. Collagenase was removed, and tissue was longitudinally spliced, cut in very small pieces, and washed with RPMI 1640 (incomplete medium). Then, tissue segments were dissociated enzymatically with a mixture of collagenase type VIII (Sigma-Aldrich; 1 mg/ml) and dispase (1 mg/ml; (Roche Diagnostics, Mannheim, Germany) in RPMI (5% FCS), with agitation for 20 min. After three digestion cycles, cells from the different digestions were pooled, and RBC were lysed in 0.1 M NH4Cl and washed. Lymphocytes were then enriched by placing the cell suspension on a discontinuous gradient containing 75% and 40% (2-ml fractions) Percoll (Amersham Pharmacia Biotech, Bucks, U.K.). After centrifugation (600 × g, 20 min) the interface layer was harvested, washed, and finally resuspended in RPMI-10% FCS.

Evaluation of CD8^+ T cells by ELISPOT

ELISPOT to detect Ag-specific CD8^+ T cells was performed as previously described (35). Briefly, 96-well nitrocellulose plates were coated with 8 μg/ml anti-mouse IFN-γ mAb R4-6A2 (BD PharMingen, San Diego, CA),

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or 5 µg/ml anti-mouse macrophage-inflammatory protein (MIP)-1α AF-450-NA (R&D Systems, Abingdon, U.K.) in 100 µl of PBS. After overnight incubation at room temperature, wells were washed three times with RPMI 1640, and 200 µl of complete medium supplemented with 10% FCS were added to each well. Afterward, the plate was incubated at 37°C for 1 h. Splenocytes cells from LNs and from genital tissue of mice were added in triplicate of 2-fold dilutions. P815 cells (a mast cytoma cell line that expresses only MHC class I molecules) were used as APCs. When the number of specific CD8+ T cell anti-VV Ags was evaluated, P815 cells (105 cells/well) were infected at a multiplicity of infection of 5 PFU/cell of WRlac virus, and at 4.5 h postinfection, cells were washed and treated with mitomycin C (30 µg/ml; Sigma-Aldrich). To evaluate the number of CD8+ IFN-γ or MIP-1α secreting cells specific for the V3 loop epitope of the HIV-1 env protein, P815 cells were pulsed with 10-6 M of the synthetic peptide p13IIIB-I10 (RGPGRAFVTI), which was found to be more active than the full P18 peptide to sensitize DR-expressing target cells (36, 37). Afterward, P815 target cells were treated with Mitomycin C (30 µg/ml) during 20 min. After several washes with culture medium, 105 P815 cells were added to each well. As control, P815 cells not pulsed with the peptide nor infected with virus, but treated under similar conditions were used. Plates were incubated for 24 h in a 37°C incubator with a 5% CO2 atmosphere, washed extensively with PBS plus 0.05% Tween 20 (PBS-T) and incubated during 2 h with a solution of 2 µg/ml biotinylated anti-IFN-γ (BD PharMingen) in PBS-T or MIP-1α biotinylated anti-mouse MIP-1α BAF450 (R&D Systems U.K.). Thereafter, plates were washed with PBS-T, and 100 µl of peroxidase-labeled streptavidin-avidin (BD PharMingen, San Diego, CA) was incubated at 1/800 dilution in PBS-T. Plates were coated with 1 µg/ml of complete medium supplemented with 10% FCS and incubated at 37°C for 1–2 h. Splenocytes cells (depleted of RBC) and lymphocytes from LNs and vaginal wash samples. Purified gp160 (Intracel) was suspended in carbonate buffer, pH 9.6, plated at 1 µg/ml; Sigma-Aldrich) or 1 µg/ml BSA (Sigma-Aldrich) (as a negative controls wells) in 100 µl of complete medium. After overnight incubation at 4°C, wells were washed three times with RPMI 1640 and blotted with 200 µl of complete medium supplemented with 10% FCS and incubated at 37°C for 2 h at room temperature with biotinylated goat anti-mouse IgG or IgA (Sigma-Aldrich); then wells were incubated during 1 h with peroxidase-labeled streptavidin-avidin (BD PharMingen) at a 1/800 dilution in PBS-T and spots were developed by adding 1 µg/ml amounts of the substrate 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) in 50 mM Tris-HCl, pH 7.5, containing 0.015% hydrogen peroxide. Spots were counted with the AID ELISPOT reader system.

**Evaluation of Ab-secreting cells (ASC) by the ELISPOT assay**

The numbers of gp160-specific ASCs (IgA and IgG) were determined following methods previously described (38). Briefly, 96-well nitrocellulose plates were coated with 1 µg/ml gp160 from HIV-1 (Intracel, Cambridge, MA) or 1 µg/ml BSA (Sigma-Aldrich) (as a negative controls wells) in 100 µl of carbonate buffer, pH 9.6. After overnight incubation at 4°C, wells were washed three times with RPMI 1640 and blotted with 200 µl of complete medium supplemented with 10% FCS and incubated at 37°C for 2 h at room temperature with biotinylated goat anti-mouse IgG or IgA (Sigma-Aldrich); then wells were incubated during 1 h with peroxidase-labeled streptavidin-avidin (BD PharMingen) at a 1/800 dilution in PBS-T and spots were developed by adding 1 µg/ml amounts of the substrate 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) in 50 mM Tris-HCl, pH 7.5, containing 0.015% hydrogen peroxide. Spots were counted with the AID ELISPOT reader system.

**T cell-specific cytokine and chemokine production**

Lymphocytes from spleen and LNs were suspended in complete medium (RPMI 1640) supplemented with 10% FCS, 2 mM L-glutamine, and 10 mM 2-ME and cultured in triplicate (106 cells/well) into 96-well microtiter flat-bottom plates and stimulated with purified gp160 (1 µg/ml; Intracel) or Con A (1 µg/ml; Sigma-Aldrich); P815 cells pulsed with the p13IIIB-I10 peptide; or medium alone. After 72 h of incubation at 37°C in 5% CO2, culture supernatants were harvested and analyzed by ELISA for specific cytokines (IFN-γ and IL-4; BD PharMingen) and chemokine contents (MIP-1α, MIP-1β, and RANTES; R&D Systems, U.K.) following the manufacturers’ instructions.

**Ab measurements by ELISA**

ELISA was used to determine the presence of Abs against gp160 in serum and vaginal wash samples. Purified gp160 (Intracel) was suspended in carbonate buffer, pH 9.6, plated at 1 µg/ml and incubated overnight at 4°C. After three washes with PBS-T, plates were blocked (PBS-10% FCS) and incubated during 1 h at 37°C. The plates were washed once with PBS-T, and samples diluted in blocking buffer were added and incubated for 1 h at 37°C. Then, plates were washed three times before the detection Ab was added. Peroxidase-conjugated goat anti-mouse IgG, IgG2a (Southern Biotechnology Associates, Birmingham, AL), IgG, or IgA (Sigma-Aldrich) Abs were diluted in blocking buffer and incubated 1 h at 37°C. After the plates were washed three times with PBS-T, the wells were reacted with the peroxidase substrate o-phenylenediamine dihydrochloride (Sigma-Aldrich). After 10–15 min of incubation at room temperature, the addition of 2 N H2SO4 stopped the reaction, and OD values were measured at 450 nm on a Labsystems Multiskan Plus plate reader.

**Results**

Differences in immunogenicity between replicating and nonreplicating VV recombinants expressing HIV-1 Env (WRenv and MVAenv) after mucosal inoculation in mice

A large number of studies have analyzed the immune capacities of poxvirus vectors, but fewer studies have characterized their potential efficacy or benefit as mucosal vectors. In a previous work from our laboratory, we showed that after oral inoculation, WR-based vectors induced mucosal immune responses against the vector and against recombinant Ags (31). To extend those studies, here we have compared different mucosal routes of inoculation for two vectors: WRenv (derivative from the WR strain); and MVAenv (derivative from the modified VV Ankara), both expressing the complete gp160 Ag of HIV-1 env IIIB. To this aim, mice were inoculated with WRenv or MVAenv recombinants (105 PFU/mouse) by i.vag., i.n., or s.c. (inoculated in the base of tail) routes. Ten days after the immunization, the cellular immune response induced in the spleen and genitourinary-associated LN (GRLNs; iliac and inguinal) (1) against VV Ags or against the V3 loop peptide (p18IIIB-I10) of gp160 Ag was evaluated. In Fig. 1, it can be seen that the WRenv vector induced a strong CD8+ T cell response against VV-Ags (evaluated by an ELISPOT assay) independently of the inoculation route used. Thus, the polyclonal anti-VV response ranged from 7629 (s.c) to 13,148 (i.vag.) spots/106 cells in the spleen (Fig. 1A, left) and from 378 (i.n.) to 2417 (i.vag.) spots/106 cells in the LNs (Fig 1A, right). When the specific anti-Env response was evaluated (Fig. 1B), the number of IFN-γ-secreting CD8+ T cells detected ranged from 1565 (i.n.) to 2780 (i.vag.) spots in the spleen; and in the GRLNs the quantities varied from 186 (s.c) to 362 (i.vag.) spots/106 cells. Thus, the magnitude of the Env-specific response induced by WRenv was nearly 3- to 4-fold less with respect to the response mounted against the viral vector Ags. The cellular immune response found in mice mucosally immunized with the nonreplicating MVAenv vector was minor compared with that generated by WRenv vector. A striking finding was that by the i.vag. route, poor immunogenicity was detected after MVA inoculation, contrasting with the strong immune response that WRenv induced by this inoculation route. In a recent study from our laboratory (39), we found that after i.vag. inoculation with rMVA no significant expression of the luciferase reporter gene was detected in the urogenital tract and in the rest of target organs, perhaps due to a particular lability of MVA to the acidic environment of the vagina or possibly that particular genes that are necessary to infect the vaginal epithelium cells are lost in MVA. However, as shown here, mice inoculated i.n. or s.c. (base of tail) showed a significant cellular immune response against VV Ags and against the recombinant Env Ag (Fig. 1). Approximately 290 and 1350 spots/106 cells of anti-VV were detected in the spleen after i.n. and s.c inoculation, respectively, whereas a significant response in the GRLNs (630 spots) was detected only after s.c inoculation of rMVA. MVA does not produce progeny virus in mouse tissues, but after a single mucosal administration (i.n. inoculation), it induces a systemic immune response against the recombinant Ag (116 spots in the spleen). After s.c. (base of tail) inoculation, a strong response against the Env Ag was found in the draining LN (inguinal and iliac LNs). This response was nearly 2.8 times higher in GRLN of rMVA- vs rWR-inoculated mice (p < 0.01), contrasting with the anti-VV response which was in GRLN.
slightly superior in rWR- vs rMVA-inoculated mice (782 vs 630 spots, respectively; Fig. 1).

Next, we evaluated whether the Th type of immune response induced was affected by either the vector or the route used. Thus, we quantified the levels of type 1 (IFN-γ/H9253) and type 2 (IL-10) cytokines in cell culture supernatants, restimulated with specific Ags.

Table I describes the levels of cytokines after restimulation with VV Ags or gp160. We found that a higher anti-VV Th1:Th2 ratio (indirectly measured as IFN-γ/H9253:IL-10 ratio) was induced after WRenv mucosal administration (Th1:Th2, 52–54), compared with that produced after s.c. administration of the vector (Th1:Th2, 9). The same picture was observed for lymphocytes from WRenv-immunized mice after restimulation with gp160 Ag. After mucosal administration of rMVA, cytokine quantities in cell supernatants were low, in concordance with the ELISPOT results. However, by i.n. inoculation, rMVA induced significant levels of cytokines in cell supernatants of splenocytes, and a comparison of both vectors by this inoculation route revealed a higher anti-VV Th1:Th2 ratio for rWR- than for rMVA-inoculated mice. After s.c. immunization, a higher anti-Env Th1:Th2 ratio was found in the GRLN for rMVA vs WR (12.6 vs 2.5), in accordance with a superior specific CD8+ T cell response (Fig. 1B).

The experiments shown in Fig. 1 and Table I revealed that the replicating rWR is highly immunogenic after mucosal (i.n. and i.vag.) or systemic (s.c.) administration. In the case of rMVA given s.c., the vector was equally or even more active than rWR (in regional LN) to induce a cellular immune response against the recombinant Ag. rMVA was able to induce a significant immune response against the Env Ag after a single i.n. inoculation, but it was poorly immunogenic after i.vag. administration.

**Codelivery i.n. of a mucosal adjuvant with MVA expressing HIV-1 Env enhanced the specific cellular and humoral immune responses against the Ag**

The experiments described above demonstrated that rMVA was able to induce a cellular immune response against HIV-1 Env Ag after i.n. delivery. To determine whether it was possible to improve the immunogenicity of MVAenv after i.n. delivery, we codelivered the vector with a well-characterized mucosal adjuvant like CT (40). Thus, 10^7 PFU of rMVA were coadministered with 10^6 CT per mouse. The CT dose was selected based on previous observations (41, 42). Mice were immunized according to the schemes depicted in Fig. 2, and 10 days later the specific immune response induced by the different immunization regimens was assessed. As shown in Fig. 2A, the number of anti-p18IIIB-I10 IFN-γ-secreting cells in the spleen following two i.n. doses of MVAenv (MVA/MVA) was 2-fold higher than that induced after a single dose of rMVA (p < 0.05). The application of the adjuvant was
highly effective, given that in mice primed with rMVA + CT (MVA-CT group), the response was 8-fold higher \((p < 0.01)\) with respect to that observed in mice primed only with MVAenv (MVA group). When two immunization doses were applied, the administration of CT together with MVAenv during prime/booster (MVA-CT/MVA-CT group) improved the response nearly 4-fold compared with mice that received two rMVA doses without the adjuvant (MVA/MVA group) \((p < 0.01; \text{Fig. 2A})\). Surprisingly, in the presence of CT two doses of rMVA (MVA-CT/MVA-CT group) did not improve the response produced after one dose (MVA-CT). Because CT is a potent

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<th>Immunization Route</th>
<th>Ag</th>
<th>WR IFN-(\gamma) (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
<th>IFN-(\gamma)/IL-10</th>
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**FIGURE 2.** Delivery i.n. of MVA expressing HIV-1 Env III B in combination with a mucosal adjuvant induces both cellular and humoral immune responses. Groups of four BALB/c mice were inoculated i.n. with MVAenv \((10^7\text{ PFU})\) alone or in combination with 10 \(\mu\)g of CT per mouse. Mice received one or two immunization doses separated by 14 days, as described in the immunization schemes. \(A\), After 10 days the specific CD8\(^+\) T cell response against the p18IIIB-I10 peptide induced in spleen was evaluated by means of ELISPOT. Bars represent the average number of spots ± SD for triplicate wells of pooled splenocytes from four mice. \(B\), Ten days after the immunization, ASC (IgA and IgG) against gp160 Ag were evaluated by ELISPOT in lymphocytes from genitoreal LN, as described in Materials and Methods. Bars represent the average number of spots ± SD for triplicate wells of pooled splenocytes from four mice. Average background spots found in control (Ctrl) negative wells have been subtracted. **, \(p < 0.01\); arrows, statistical differences between the groups. Data are representative of two experiments with comparable results.
adjuvant to increment Ab responses in mucosal compartments, we also evaluated the humoral arm of the specific immune response. Thus, ASC present in GRLNs specific for gp160 Ag were quantified by ELISPOT as described in Materials and Methods. Although no significant differences with respect to the control group were found in mice from groups immunized with one or two doses of rMVA, co-immunization with the adjuvant induced anti-Env IgA and IgG secreting cells (ASC) in the genitorectal draining LN (Fig. 2B). Moreover, two rMVA immunization doses in the presence of CT (MVA-CT/MVA-CT) incremented the specific IgA and IgG ASC responses 4- and 3-fold, respectively ($p < 0.01$), compared with a single dose. We also evaluated serum anti-Env IgG subtypes induced in the different groups (Fig. 3). Significant titers of serum Abs (above those found in the control group) were found only in mice groups that received two immunization doses. Therefore, two rMVA doses in the absence of CT induced higher IgG2a than IgG1 titers, whereas the codelivery of CT reverses the ratio.

Thus, these experiments showed that the immunogenicity of rMVA after i.n. inoculation can be incremented by its codelivery with CT. Moreover, the mucosal codelivery of the adjuvant with the viral vector induced specific IgA and IgG ASC in GRLNs.

Mucosal DNA-prime/MVA-boost immunization in the presence of CT induced a potent cellular immune response in the spleen against the recombinant HIV-1 Env Ag

In the previous experiment, we demonstrated a way to improve the immunogenicity of MVA delivered i.n. through the application of a mucosal adjuvant. To further improve the response obtained, we decided to establish whether administration of a DNA/MVA immunization regimen by the i.n. route together with a mucosal adjuvant can be an efficient strategy to induce both systemic and mucosal immune responses against the HIV Env Ag.

Evaluation of the systemic cellular immune response

BALB/c mice were immunized as depicted in Fig. 4, with groups I to IV inoculated i.n., whereas the systemic immunization DNA/MVA scheme of group V, previously evaluated in our laboratory (43), was included as a positive control group. Twelve days after the boost, the specific CD8$^+$ T cell immune response induced against the recombinant Env Ag was determined. By quantifying the number of specific anti-p18IIIB-I10 IFN-γ-secreting cells in the spleen (Fig. 4A), we found that administration of CT during the DNA prime and MVA boost (group IV) enhanced the response nearly 15-fold compared with the DNA/MVA immunization scheme without CT (group II; $p < 0.01$) (Fig. 4A). Moreover, the response found in group IV was even superior (2.5-fold higher) to that induced in the positive control (group V), systemically immunized ($p < 0.01$). In contrast, only in the presence of CT did the heterologous DNA/MVA regimen give a response 2-fold superior to that obtained with the homologous MVA + CT/MVA + CT scheme ($p < 0.01$), whereas in the absence of the adjuvant by the i.n. route the DNA/MVA and MVA/MVA immunizations produced similar responses. To further analyze the CD8$^+$ T cell effector response induced, we investigated whether splenocytes from the different immunization groups can elicit the Ag-specific production of β-chemokines. CCR5 acts as a coreceptor for HIV entry into cells, and competitive inhibition of this virus-coreceptor interaction by CCR5-specific β-chemokines such as MIP-1α, MIP-1β, or RANTES may inhibit the intercellular spread of HIV and the natural progression of the infection to AIDS (44). The specific production of the chemokine MIP-1α was analyzed by means of a fresh ELISPOT (Fig. 4B). Mice from group IV have the highest number of cells producing the MIP-1α chemokine, and in this group the response was nearly 2.4-fold superior with respect to that of group III ($p < 0.01$). We also quantified the production of the chemokines MIP-1β and RANTES in cell culture supernatants after 3 days of incubation of lymphocytes in the presence of APCs coated with the MHC class I-restricted p18IIIB-I10 peptide (Fig. 4C). The levels of MIP-1α in supernatants of cultured splenocytes paralleled the finding of the ELISPOT (Fig. 4; compare B and C). When the levels of RANTES and MIP-1β were evaluated, the higher levels of these two β-chemokines were found in groups III and IV (Fig. 4C).

Delivery i.n. of rMVA plus CT in MVA/MVA and DNA/MVA schemes induced mucosal and systemic Abs against the recombinant HIV-1 Env Ag

Mucosal immunity against HIV at mucosal sites is an aspect of high importance for protection against the viral infection, because the virus spreads principally by sexual transmission. Although cellular immunity plays an important role in protection, Abs, especially those associated with mucosal secretions, have also been implicated as important arms to prevent or diminish the infection loads in the first stages of virus infection (45, 46). Therefore, we evaluated the presence of specific anti-gp160 Abs in vaginal washings of mice from groups depicted in Fig. 4. Twelve days after the boost dose, we found that mice from groups III and IV (those that received the adjuvant) had the highest levels of specific IgG and IgA (Fig. 5A); however, the levels of specific Abs detected were too low to evaluate its neutralizing capacity. Moreover, determination of serum IgG subtypes showed that in both MVA/MVA and DNA/MVA schemes, the coadministration of CT induced the production of serum IgG anti-gp160, in accordance with the humoral mucosal immune response (associated with a Th2 response).

Immunization i.n. schemes of MVA/MVA and DNA/MVA in combination with CT induced specific IFN-γ-secreting cells in the mucosal female genital tract and in the genitorectal draining LN

The experiments described above show that i.n. administration of CT in both MVA/MVA and DNA/MVA immunization schemes induced specific IgA and IgG mucosal Abs in vaginal washings,
associated with an elevated specific serum IgG1 response. Thus, to analyze in more depth the specific cellular mucosal immune response induced by those immunization schemes, we performed another experiment in which six female mice per group were immunized with the MVA-CT/MVA-CT and DNA-CT/MVA-CT regimens, as for groups III and IV of Fig. 4. Fig. 6 shows the specific CD8⁺ T cell response (IFN-γ-secreting cells) induced in different tissue compartments. We evaluated the specific cellular immune response induced locally in the regional CLNs draining the nasal-associated lymphoid tissue (Fig. 6A), and at distal places from the immunization site as in lymphocytes from the iliac LN (ILN) draining the urogenital tract (Fig. 6B), and in lymphocytes from the urogenital tract (Fig. 6, A–C). In the regional CLNs and also in the ILNs the specific response was 2-fold higher in the DNA-CT/MVA-CT-immunized group (p < 0.05), with respect to that of the MVA-CT/MVA-CT group. We found that the quantity of specific IFN-γ-secreting cells found in the LNs (CLNs and ILNs) was noticeably lower than in the genital tissue; this may be due to the propensity of effector T cells to migrate out of LN and preferentially reside in nonlymphoid organs (47, 48). The key important finding of this experiment was the induction of a significant specific cellular immune response in lymphocytes from the urogenital tract (Fig. 6C) and in lymphocytes from the ILNs that drain the urogenital tract.

Discussion
In this study, we have conducted a detailed analysis of the immunogenicity of the attenuated MVA virus vector expressing the
HIV-1 Env IIIB Ag after inoculation by different mucosal routes in BALB/c mice. We found that i.n. inoculation of rMVA induced a significant immune response against the recombinant Ag and that the immunogenicity can be incremented by codelivery of rMVA with the mucosal adjuvant CT. The DNA prime-MVA boost i.n. immunization scheme together with CT induced a potent specific cellular immune response in the spleen, in the genital-draining LN, and more importantly at mucosal effector sites of the genital tract. Moreover, both MVA-MVA and DNA-MVA schemes coadministered with CT induced mucosa-specific Abs in vaginal washings.

The most important reason for using mucosal routes of vaccination instead of parenteral ones is that the majority of the infections occur or take their departure from a mucosal surface, like the HIV infection that is a sexually transmitted virus. Other reasons for applying mucosal vaccines are the greater level of general acceptability of needle-free methods for the delivery of a vaccine, as it could be an oral or an aerosol (intranasal) administration, and their potential to overcome the known barriers of parenteral vaccination caused either by pre-existing systemic immunity as a result of previous vaccination (49) or by selective systemic immunodepression such as that caused by HIV infection (50). Although studies related to mucosal vaccines are of great importance, there are few studies analyzing the mucosal immunogenicity of candidate vaccines based on poxvirus vectors such as MVA. Different phase I clinical trials in which MVA vectors are used are under way (www.hvtn.org). Thus, the optimal use of rMVA as a mucosal immunogen is highly desirable. In this work, comparison of the immune response induced by the replicating WRenv vector vs the highly attenuated vector MVAenv showed that WRenv was equally highly immunogenic after mucosal (i.n. and i.vag.) or systemic (s.c.) administration. After s.c. (base of tail) inoculation, rMVA induced a strong response against the Env Ag in the local LNs (inguinal and iliac LNs). Moreover, this response was nearly 3 times higher in rMVA- vs rWR-inoculated mice ($p < 0.01$), contrasting with the anti-VV response which was superior in rWR- vs rMVA-inoculated mice. We have previously described similar findings analyzing the CD8$^+$ T cell response in the spleen after i.p. inoculation with the same recombinants (32) and also for recombinants expressing the β-gal Ag (51). MVAenv resulted poorly immunogenic.
after i.vag. inoculation, while a significant immune response against the recombinant Ag was obtained after a single MVAenv i.n. immunization. Thus, although MVA does not produce progeny virus in mouse tissues, a single mucosal administration (i.n. inoculation) induced a significant systemic immune response against Env Ag. In a recent study of our laboratory (39), we demonstrated that MVA inoculation by the i.n. route, targeted the virus to the nasal associated lymphoid tissue and the lungs, whereas the i.vag., intrarectal, and intragastric inoculations failed to induce efficient infection. Thus, perhaps due to the highly attenuation of the MVA vector, not all the mucosal routes are equally efficient for the viral infection and expression of recombinant Ags. In this sense, in a recent study performed in macaques (52), a recombinant attenuated NYVAC virus for SIV Ags induced mucosal CD8⁺ T cell responses after intrarectal, i.n., or even systemic immunization, implying that depending on the characteristics of the live virus vector used, the effectiveness of the mucosal route applied may differ.

Despite the interesting characteristics of mucosal vaccination, it has often been difficult to induce strong mucosal immune responses. Thus current efforts are directed to finding more efficient means of delivering Ags and developing mucosal adjuvants that must be effective but also safe. CT, the cholera-inducing enterotoxin produced by *V. cholerae* O1 and O139, is one of the most potent mucosal immunogens and adjuvants known (5). The principal effects that caused its potent adjuvanticity are those leading to enhanced Ag presentation by APC, in that it has been found to directly affect both the cytokine profile and the cell surface phenotype of APC (53, 54).

Experiments performed in this study showed that the immunogenicity of a rMVA vector expressing HIV-1 Env III B after intranasal inoculation can be incremented by the administration of CT. The codelivery of the adjuvant plus the viral vector increased the cellular immune response in the spleen and also induced specific IgA and IgG ASC in GRLNs. Moreover, after two rMVA immunization doses plus CT, we observed that the humoral immune response was incremented compared with a single immunization, whereas the specific cellular immune response was maintained. Those mice in which a specific IgG and IgA vaginal response was found also showed a correlating significant IgG1 (Th2) serum response. Hence, our results indicate that the codelivery of rMVA + CT induced a combined Th1-Th2 response.

Different studies have previously shown that CT by itself primes for an exhaustive Th2 response inhibiting IL-12 production (53) but that when it is mucosally administered as an adjuvant it induces a mixed Th1-Th2 response (55–57). Indeed, it has been previously reported that by the i.n. route CT is a useful adjuvant for CTL responses.

**FIGURE 6.** Intranasal immunization schemes. MVA/MVA and DNA/MVA in combination with CT induced specific IFN-γ-secreting cells in the female genital tract and in the genitorectal draining LN. Two groups of six female BALB/c mice received the same immunization schemes of groups III and IV of Fig. 4. Fourteen days after boost, ELISPOTs (performed in triplicate) were conducted to evaluate the specific IFN-γ-secreting CD8⁺ T cell response induced locally: in the regional LN (CLNs) (A); and at distal places: in the LN draining the genital tract (ILNs; B), and in the female genital tract (vagina and uterus; C). Spots representing IFN-γ-secreting CD8⁺ T cells specific for the p18IIIB-I10 peptide of HIV-1 III B Env were visualized (top panels). The spots were counted, and the mean values + SD are shown (bottom panels). Similar results were obtained in two different experiments.
Mechanisms underlying the mixed Th1-Th2 response observed may be CT induction of levels of costimulatory molecules on DC that affect both Th1 and Th2 commitments. Thus, a recent study described that conjugation of Ag to CTB preferentially induces Th2 responses after in vitro treated Ag-pulsed DC, whereas CT primed for a Th1-Th2 response (57). This mixed CT induced response was associated with the up-regulation of costimulatory molecules as CD80 and CD86, and a IL-1β response. Therefore, the increase in CD80/CD86 expression may contribute to the magnitude of the T cell activation (60), and in contrast, IL-1 expression can be responsible for a substantial part of the adjuvant activity of CT, given that it can act as an efficient mucosal adjuvant when it is coadministered with protein Ags (61). In addition, in a previous study performed with replicating VV recombinants expressing the measles virus hemagglutinin, the coadministration of the VVV with CT by nasal route enhanced systemic cellular immune responses and mucosal-specific Abs (62).

Heterologous prime-boost approaches using poxvirus vectors during booster is an efficient and widely accepted protocol that has been used in different animal models to induce specific cellular immune responses and also to trigger protection to pathogens (9, 13, 63–66). Currently, this strategy has been investigated in situations where both immunizations were administered systemically, and few studies have applied them by mucosal routes (17, 28, 30, 67). Therefore, because immunization by peripheral routes typically does not induce immune responses in mucosal compartments, the immunogenicity afforded by prime-boost regimens with rMVA to direct immune responses to mucosal sites must be improved. In this sense, we have recently described that by the i.n. route MVA generated an effective specific immune response against HIV-env Ag in mice that have been previously primed i.n. with recombinant influenza virus (17). In this report, we show that mucosal administration of rMVA expressing the HIV-1 Env Ag by the i.n. route, coadministered with a potent mucosal adjuvant as CT, markedly improved the efficiency of rMVA to induce both systemic and mucosal immune responses against HIV.

Lymphocytes derived from one mucosal tissue can recirculate throughout and localize selectively within other mucosal surfaces, including the respiratory tract and uterus (68). Importantly, here we found that both homologous (MVA/MVA) and heterologous (DNA/MVA) prime-boost intranasal immunizations schemes induced specific cellular immune responses locally (in CLNs) and at distal mucosal sites such as the genital tract. Therefore, we demonstrate that by i.n. immunization it is feasible to induce HIV-specific immune responses in the respiratory and genital tracts, corroborating the concept that HIV i.n. immunization is an effective immunization route to induce HIV-specific genital immune responses (17, 34, 69). Although the mucosa-associated lymphoid tissue is organized as a common mucosal immune system, it is nearly impossible to cover all mucosal sites by a single mucosal immunization. In this sense, the prime-boost mucosal strategy combining different mucosal routes in the prime and boost doses may be useful in inducing mucosal immune responses at multiple mucosal sites (vaginal, rectal, etc.), an approach similar to that followed by Lehner et al. (70).

The number of specific IFN-γ-secreting cells found in the LNs (CLNs and ILNs) was low compared with that found in a nonlymphoid tissue such as the genital tissue, which can be explained by the propensity of effector T cells to migrate out of LN and preferentially reside in nonlymphoid organs (47, 48). In this sense, a recent report has also showed that lower percentages of Ag-specific CD4⁺ and CD8⁺ lymphocytes were found in LN, compared with those found in a nonlymphoid organ such as the lung (71). The finding of IgA- and IgG-specific Abs in vaginal washings of mice inoculated with the DNA/MVA or MVA/MVA protocol is also relevant in protection against HIV, due to the importance of local Abs in genital mucosa. The crucial role that local immune responses have in HIV infections is illustrated by the observation that HIV-exposed uninfected sex workers have HIV-1-specific CD8⁺ T cells in the cervix (72) and HIV-1-specific neutralizing IgA in genital tract secretions (45, 46, 73). In experimental studies, the possibility that local genital tract CTLs might play a key role in protection against sexually acquired HIV-1 infection was suggested by Belyakov et al. (41), who demonstrated that intrarectal immunization with HIV-1 peptide vaccine induced mucosal and systemic CTL and protective immunity against intrarectal recombinant HIV-vaccinia challenge, whereas systemic immunization induced specific CTL only in the spleen. Another study corroborated this issue, demonstrating that local mucosal specific CD8⁺ CTL conferred long-lasting immune resistance to mucosal viral transmission in mice, whereas systemic (splenic) CTL alone were unable to protect against mucosal transmission (74). Moreover, the same authors in a more recent study in macaques (75) showed the relevance of mucosal CTLs in protection, demonstrating that intrarectal immunization induced a higher elimination of viral titers in both blood and intestine after mucosal challenge with the pathogenic SHIV-Ku2 than in s.c. immunized macaques. Other authors in different studies performed in macaques demonstrated a direct correlation between protection from colonic SIV challenge and jejunal lamina propria CTL directed against viral Env (76). Moreover, those animals either vaginally or colonically exposed to subinfectious SIV doses showed a CTL detectable response in jejunal LP, revealing association of mucosal CTLs with protection or delayed disease upon challenge (77). Therefore, several experimental results have suggested that a successful vaccination conferring protection against SIV/HIV may require CTL responses in the mucosa.

Considering previous studies conducted with rMVA, our findings are important for the optimal use of MVA as a recombinant vaccine by the i.n. route. To our knowledge, this is the first study reporting that i.n. immunization with a rMVA vector induced specific HIV immunity in the genital tract. A previous study in mice have demonstrated the effectiveness of rMVA to generate a mucosal immune response against HIV; one intrarectal immunization with rMVA expressing gp160 of the HIV-1 isolate 89.6 induced a systemic and gut-associated lymphoid tissue-specific CTL response in mice (8). The i.n. route of inoculation with MVA have been previously used to evaluate its immunogenicity against different pathogens, such as influenza (14), parainfluenza (15), or respiratory syncytial virus (16). Moreover, a recent report emphasizes the importance of Ag i.n. mucosal delivery from rMVA using a prime-boost approach to induce a complete protective immunity against the aerosol challenge with Mycobacterium tuberculosis (78). With the exception of a study of our laboratory (17), there are no other reports indicating immunogenicity against HIV Ags after i.n. delivery of rMVA.

There is firm evidence that CC chemokines such as MIP-1α, MIP-1β, and RANTES can block the CCR5 coreceptor usage and prevent HIV infection in vitro (44) and SIV infection in vivo (79). Here, we have also demonstrated the ability of DNA/MVA and MVA/MVA immunizations by the i.n. route to stimulate MHC class I-restricted production of these three chemokines. Thus, the specific production of these β-chemokines by CD8⁺ T cells may contribute to amplify their antiviral protective activity.

In summary, we have demonstrated that i.n. immunization by MVA/MVA and DNA/MVA schemes is an efficient mucosal route
and vaccinia virus vectors potentiate cellular immune responses against human immunodeficiency virus Env protein systematically and in the genitoracral draining lymph nodes. J. Virol. 77:7048.


