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Regression of Human Papillomavirus Intraepithelial Lesions Is Induced by MVA E2 Therapeutic Vaccine

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Abstract

Human papilloma viruses can induce warts, condylomas, and other intraepithelial cervical lesions that can progress to cancer. Cervical cancer is a serious problem in developing countries because early detection is difficult, and thus proper early treatment is many times missing. In this phase III clinical trial, we evaluated the potential use of MVA E2 recombinant vaccinia virus to treat intraepithelial lesions associated with papillomavirus infection. A total of 1176 female and 180 male patients with intraepithelial lesions were studied. They were injected with 10^7 MVA E2 virus particles directly into their uterus, urethra, vulva, or anus. Patients were monitored by colposcopy and cytology. Immune response was determined by measuring the antibody titer against MVA E2 virus and by analyzing the cytotoxic activity against cancer cells bearing papillomavirus DNA. Papillomavirus was determined by the Hybrid Capture method or by polymerase chain reaction analysis. By histology, 1051 (89.3%) female patients showed complete elimination of lesions after treatment with MVA E2. In 28 (2.4%) female patients, the lesion was reduced to CIN 1. Another 97 (8.3%) female patients presented isolated koilocytes after treatment. In men, all lesions were completely eliminated. All MVA E2–treated patients developed antibodies against the MVA E2 vaccine and generated a specific cytotoxic response against papilloma-transformed cells. Papillomavirus DNA was not detected after treatment in 83% of total patients treated. MVA E2 did not generate any apparent side effects. These data suggest that therapeutic vaccination with MVA E2 vaccine is an excellent candidate to stimulate the immune system and generate regression in intraepithelial lesions when applied locally.

Introduction

Cervical carcinoma is the seventh most common cancer in the world. It is known that approximately 291 million women worldwide present human papillomavirus (HPV) in their body (de Sanjose *et al.*, 2007), accounting for 15% of all cancers that represent 274,000 deaths every year (Hakim and Dinh, 2009). Benign lesions, named papillomas, are small wartlike neoplasias that usually regress on their own. In some cases, however, lesions undergo malignant transformation and develop into large tumors (McLaughlin-Drubin *et al.*, 2012). Research shows that 95% of all cervical carcinomas contain DNA of some HPV (Stanley, 2002; Hossein *et al.*, 2013; Powell *et al.*, 2013; Rahman *et al.*, 2013; Ghosh *et al.*, 2014), with types 16 and 18 accounting for about 50% and 14% of all cases, respectively (Nuovo *et al.*, 1990; Muñoz *et al.*, 1996).

The preferred method to diagnose an HPV infection is to confirm the presence of HPV DNA in the lesion by hybridization or by polymerase chain reaction (PCR) (Nuovo

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and Richart, 1990; Cuzick *et al.*, 2012). Unfortunately, the expense of this type of testing prevents its widespread use in parts of the world with limited resources (Poljak *et al.*, 2012). Thus, regular screening of abnormal cervical cytology (Pap smear) remains the mostly used preventive strategy for cervical cancer around the world (Walsh, 1998). However, despite the implementation of screening programs, many deaths are still recorded each year. The main problem lies with the interpretation of abnormalities in the Pap smear. Because the test requires trained personnel, a highly variable false-negative rate is still associated with it (Cuzick *et al.*, 1998). Therefore, therapy options are usually carried out late in the infection process. This is most probably the main factor responsible for mortality from invasive cancer in developed countries (Stanley, 2002).

Once HPV lesions are detected, the main therapeutic approach involves physical elimination of the lesion (Martin-Hirsch *et al.*, 2010). In precancerous lesions, however, surgical procedures alone are not very effective, since recurrences occur at rates of 20–30% or more with lesions both at previously treated sites because of failure of the procedure to eliminate the HPV, and at new sites because of new infections (Lacey *et al.*, 2013). Persistence of high-risk HPV can lead to the development of cancer lesions. When this occurs, radiotherapy and chemotherapy are then used with relative success, since about 50% of the HPV cancer patients still die. Clearly, new therapeutic strategies are in urgent need to control the burden of HPV-related cancer (Jemal *et al.*, 2011).

Recently, the development of anticancer vaccines and intralesional immunotherapy are becoming a promising alternative therapy for this type of cancer and the most effective way to treat and eradicate virus-induced tumors (Rosales and Rosales, 2014). The recombinant vaccinia virus MVA E2 is a vaccinia virus Ankara (MVA) containing the bovine papilloma virus E2 protein (Rosales *et al.*, 2000; Valadez *et al.*, 2000). MVA E2 has been shown to stop human tumor growth in mice, and to induce tumor regression in tumor-bearing rabbits (Rosales *et al.*, 2000; Valadez *et al.*, 2000). In a series of studies, MVA E2 was evaluated in patients who had established HPV-induced CIN lesions (Corona-Gutierrez *et al.*, 2004; Garcia-Hernandez *et al.*, 2006; Albarran *et al.*, 2007). In a phase I/II clinical trial, for CIN 1 to CIN 3 lesions, 36 women received 10^7 MVA E2 virus particles injected directly into the uterus once every week over a 6-week period. Thirty-four (94%) patients showed complete elimination of precancerous lesions after treatment (Corona-Gutierrez *et al.*, 2004). In the other two patients, precancerous lesions were reduced from grade CIN 3 to CIN 1. In addition, 50% of patients eliminated completely the HPV, and in the remaining 50% of patients, HPV DNA was only 10% of the original viral load (Corona-Gutierrez *et al.*, 2004).

Later, in a phase II clinical trial for high-grade lesions (CIN 2 and CIN 3), 19 out of 34 (56%) patients had a complete regression, while in 11 (32%) more patients the lesions were reduced by 60–90% (Garcia-Hernandez *et al.*, 2006). In addition, specific cytotoxic activity against cancer cells correlated with clinical outcome (Garcia-Hernandez *et al.*, 2006). In another phase I/II clinical trial to evaluate MVA E2 for the treatment of flat condyloma lesions associated with oncogenic HPV in men, 50 patients were treated with either MVA E2 or 5-fluorouracil (Albarran *et al.*, 2007). Thirty men received 10^6 MVA E2 virus particles per dose, administered directly into the urethra once a week over a 4-week period. Twenty control patients were treated with 1 ml of 5% 5-fluorouracil directly into the urethra twice a week over a 4-week period. Twenty-eight (93%) of MVA E2–treated patients did not have lesions or presence of HPV after 4 weeks of treatment, and generated a specific cytotoxic response against papilloma-transformed cells. These patients did not show any recurrence of lesions after 1 year of treatment. In 2 (7%) other patients, the flat condyloma did not diminish. In the control group, 13 (65%) patients were free of lesions, and 3 of these patients had recurrence of lesions after 3 months of treatment (Albarran *et al.*, 2007).

In the present report, we evaluated the therapeutic potential of MVA E2 in the treatment of HPV-induced anogenital intraepithelial lesions in a phase III study. A total of 1176 female and 180 male patients were injected with MVA E2 directly into the uterus, urethra, vulva, or anus; 1051 (89%) of female treated patients showed complete elimination of lesions, and other 28 (2.4%) female patients showed reduction of lesions to CIN 1. In male patients, all lesions were completely eliminated. All patients developed antibodies against the MVA E2 vaccine and generated a specific cytotoxic response against papilloma-transformed cells. Papillomavirus DNA was not detected after treatment in 83% of patients. Our data suggest that intralesion treatment with MVA E2 generated specific cytotoxic responses that were able to completely eliminate lesions, and also induced eradication of HPV virus from infected patients.

Materials and Methods

Study design and subjects

A phase III clinical protocol was conducted in which 1356 patients (1176 female and 180 male) with HPV intraepithelial lesions were treated with MVA E2 recombinant virus. Patients were recruited from the following medical institutions in Mexico: Hospital de Cuautitlan, in Estado de Mexico; Sanatorio San Francisco, in Veracruz; Hospital General de Veracruz, in Veracruz; Hospital Angeles Xalapa, in Veracruz; Hospital Militar, in Veracruz; Hospital de Nutricion, Instituto Nacional de Cancerología, in Veracruz; Hospital de la Mujer, in Michoacan; and Hospital Español, in Mexico City; and in Venezuela: Inversiones Milfred Medical.

Patients were admitted to the protocol once the eligibility criteria had been met. Patients needed to be positive for any type of oncogenic or nononcogenic HPV infection, and to be between 29 and 49 years of age, because it is considered that at this age their immune system is completely mature and functional. Female patients needed also to present cervical intraepithelial neoplasia (CIN 1, CIN 2, CIN 3) or condyloma lesions, and not to be pregnant. Male patients needed also to present condyloma lesions in urethra or to have anal lesions. All patients had a complete physical examination and clinical history made. Laboratory examinations, including hematology, blood chemistry, and urinalysis, were performed for each patient. Before treatment with MVA E2, the physician revised all data and confirmed that the patient was eligible for the protocol. Each patient signed an informed consent form after the physician explained all the

procedures and the need of compliance to the clinical protocol and treatment plan. The attending physician maintained an accurate and complete record of each visit, and the principal investigator maintained confidentiality of the information. Patients could be contacted at any time for their safety.

Clinical and demographic characteristics of patients with intraepithelial lesions diagnosed between 2007 and 2012 were taken into consideration. Approximately 3000 female patients were screened following the inclusion and exclusion criteria (Corona-Gutierrez *et al.*, 2002) in order to select 1176 women and 180 men with intraepithelial lesions and a confirmed HPV infection. Out of the 1356 total selected patients, 1349 were from Hispanic, and 7 were from white ethnical groups. Patients had a mean age of 36.7 years, an average height of 157.3 cm for women and 166.4 cm for men, and an average weight of 68.7 kg for women and 76.4 kg for men. Within the female patients, 876 presented low-grade lesions and 300 presented high-grade lesions. The male patients presented condyloma lesions.

Protocol

The protocol has been approved by the Ethics and Scientific Committee of all the hospitals and by Mexican Health Authorities from Estado de Mexico, Mexico.

MVA E2 recombinant virus was injected directly into the uterus of female patients in a radial clockwise fashion at 3, 6, 9, and 12 o'clock once a week for 6 weeks, or instilled into the urethra of male patients with a catheter for feeding babies (3 mm wide; Becton Dickinson) once a week for 5 weeks. Each dose consisted of 10^7 virus particles. In cases where lesions were visible, for example, in the vulva and anus, MVA E2 was applied locally at the base of each lesion with an insulin syringe. There was 1 week between the selection interview (visit 1, week 0, day -7) and the beginning of treatment (visit 2, week 1, day 0). The treatment plan and procedures are shown in the Appendix. Lesions were monitored by colposcopy weekly, and by histology at the end of the protocol. The type of immune response generated after the treatment was determined by measuring antibodies against the MVA E2 virus, and cytotoxic activity against papillomavirus-bearing cancer cells. Presence of HPV DNA was determined by a hybrid Capture assay or by PCR (see below).

Control group

A group of 141 female and 26 male patients, all having intraepithelial lesions and treated with different therapeutic methods (such as cryosurgery, laser, conization, electrosurgery, 0.5% podophillin, trichoroacetic acid, or 5-fluorouracil), was used as a control group for this study. These patients were followed up from the time when they were treated to the time when they returned to the physician (gynecologist or urologist) with a new lesion.

Adverse events

Any possible adverse events were registered following the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) (National Cancer Institute, 2010). The occurrence of adverse events related to blood, gastrointestinal, pulmonary, liver, kidney, bladder, heart, or neuronal disorders was monitored weekly by the physician. In addition, the appearance of skin allergy, fever, pain, weight gain, weight loss, alopecia, metabolic changes, blood cell changes, and alterations on vital signs were also monitored during each visit at the hospital. All data were recorded into the Final Clinical Report and were supervised by the principal investigator.

Virus and cells

Chicken embryo fibroblasts (CEF) were obtained from 11-day fertile eggs. Briefly, chicken body was minced with scissors and put with 0.25% trypsin. Cells were harvested by centrifugation and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FBS; Gibco BRL), 20 μM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin in a humidified air–5% CO_2 atmosphere at 37°C (Earl *et al.*, 2001). MVA E2 recombinant virus containing the *E2* gene from bovine papillomavirus was constructed and characterized as previously described (Valadez *et al.*, 2000). Virus was titrated on CEF by endpoint dilution to obtain the 50% tissue culture infectious dose (TCID50). One single batch of MVA E2 vaccinia virus was prepared at Lemery S.A. de C.V. (Huixquilucan) under aseptic conditions following current good manufacturing practices (U.S. FDA, 2003).

Lemery is a pharmaceutical company, part of TEVA Mexico, certified to elaborate various pharmaceutical products by the Comisión Federal para la Proteccion contra Riesgos Sanitarios (COFEPRIS), the Federal Health Regulatory Agency in Mexico. COFEPRIS is the equivalent of the FDA in the United States. The MVA E2 virus was prepared as follows: CEFs were attached to microcarriers (Cytodex) and grown in a 15-liter Bioreactor Celligen-Plus. Cells were infected with MVA E2 virus and incubated for 24–48 hr. To recover the virus, fibroblasts were harvested by centrifugation and immediately freeze-thawed three times. Viruses were purified from this cell lysate by two successive sucrose (40–10%) zonal centrifugation steps. The purified virus was titrated on CEF and stored frozen at -70°C. Subsequently, aliquots of MVA E2 at 10^7 plaque-forming units (pfu)/ml were lyophilized. Vials were finally sealed with rubber stoppers and aluminum seals.

Collection of biopsies and HPV DNA detection

Tissue biopsies were collected from infected tissues and were divided in two portions. One portion (0.2 cm) was processed for histology, and the other portion was placed in 1 ml of Digene Specimen Transport Medium (Digene, Inc.) and stored at -20°C for DNA detection. Samples for viral DNA analysis were processed as described previously (Rosales *et al.*, 2000), using the Hybrid Capture Kit (Digene, Inc.). This method detected the presence of oncogenic and nononcogenic HPV DNA in tissue samples without typifying the exact HPV genotype. This diagnosis kit does not include separate antibodies for each HPV genotype. Biopsies from patients in Venezuela were frozen at -20°C until processed. Briefly, presence of DNA was analyzed by PCR using first MY09/11 standard primers followed by specific primers for each different HPV. The amplified DNA fragments were identified by electroforesis in 1.5% agarose

gel with ethidium bromide. All specimens were tested by one-step PCR assay using specific primers for HPV types. The PCR was performed in 2.5 m*M* $MgCl_2$, 250 μmol of each deoxynucleotide, 0/5 μmol of sense and antisense primer, 5 μl template, and 1 unit Taq DNA polymerase (Applied Biosystems) by 40 cycles: denaturation, 94°C, 1 min; annealing, 56°C, 1 min; extension, 72°C, 1 min. PCR samples were purified and sequence by using BidDye terminator v3.2 Cycle Sequencing Kit (PE Applied Biosystems). Sequences were compared with the reported sequences in GenBank server (NCBI/BLAST).

Histology

Histology was performed on samples taken on visits 1 and 8. Briefly, biopsies were isolated and fixed in 10% formaldehyde. Semi-thin sections were cut and stained with hematoxylin/eosin as described next. Sections of 4–5 μm were fixed for 10 min in 2% paraformaldehyde and washed immediately with water. Hematoxylin (0.5%) was added for 1 min and the section was then rinsed for 3 min with tap water and then with distilled water. The sections were placed in 0.1% Li_2CO_3 for a few seconds and rinsed successively, 3 min each time, with alcohol (70%) containing 1% HCl, tap water, 50% alcohol, and 70% alcohol. Eosin (1%) was then added for 2 min and the sections were rinsed with distilled water. Then, several washes (5 min each) were performed with increasing concentrations of alcohol (70%, 80%, 90%, 95%, and 100%) to dehydrate the sample. Xylol was finally added for 5 min. Sections were mounted on Accuo Mount 280 (Baxter Healthcare Corporation). On each occasion, the same pathologist interpreted the results. The cervix lesions CIN 1, CIN 2, and CIN 3 were found to correspond to low, moderate, and severe dysplasia, respectively.

Enzyme-linked immunosorbent assay

Antibody responses to both the vaccinia virus MVA E2 and the HPV-E2 protein were evaluated as previously described (Rosales *et al.*, 2000). Briefly, ELISA plates were coated with 5×10^5 purified virus particles, or with 5 μg of a mixture of peptides from the E2 protein (Albarran *et al.*, 2007). Serum dilutions were added to plates and incubated overnight at 4°C. Plates were then washed three times with phosphate buffer saline (PBS), and incubated with a 1/2000 dilution of horseradish peroxidase-conjugated Protein A (Sigma Aldrich) for 1 hr. Following three more washes, the plates were incubated with the peroxidase substrate o-phenylene diamine (Sigma Aldrich) at room temperature during 30 min. Absorbance was read at 405 nm on an ELISA plate reader (Bio-Tek Instruments).

Cell extract of HPV-transformed cell lines

The HPV-transformed cell lines HeLa (HPV-18), CALO (HPV-18), VIPA (HPV-18), SIHA (HPV-16), and CASKI (HPV-16) were grown and kept in culture with DMEM-10% FBS. The cell extract was prepared as follows: 15×10^6 HPV-transformed cells were collected by centrifugation, resuspended in 5 ml of buffer (20 m*M* KCl, 20 m*M* Tris-HCl, pH 8.0), and sonicated with three pulses of ultrasound lasting 2 min each. The cell extract was then dialyzed against PBS, and its protein concentration was determined. The extract was stored at −80°C until used.

Target and effector cells

Target cells for cytotoxic assays were prepared from HPV-infected tissue of individual patients. A piece of a biopsy was minced in PBS with scissors. Then, 0.25% trypsin (Gibco BRL) was added and the tissue incubated for 10 min at 37°C. Cells in suspension were then washed with PBS by centrifugation and re-suspended in DMEM-10% FBS. Cells were finally frozen in DMEM-10% FBS with 10% DMSO and stored at −20°C until used. Effector cells were prepared as follows: lymphocytes isolated from peripheral blood were stimulated with phytohemagglutinin 1/1,000 dilution from the commercial solution (catalog No. 10567-015; Gibco, Life Technologies) for 7 days. Next, lymphocytes were induced with a HPV-transformed cell extract by using 500 μg of cell extract per ml of lymphocytes culture. The mixture was incubated for 7 days at 37°C. Immediately, lymphocytes were washed twice with PBS buffer and resuspended in DMEM.

Cytotoxicity assay

Lymphocytes from 146 randomly chosen patients were isolated at the beginning and at the end of the study (see Appendix). Effector (lymphocytes) and target (tumor) cells were mixed at 1:1, 10:1, 30:1, 50:1, and 100:1 effector-to-target cell ratios in wells of a 96-well microtiter plate. After an 8 hr incubation at 37°C in a 5% CO_2 atmosphere, the percentage of specific cell lysis (or cell killing) was determined by assessing the lactate dehydrogenase (LDH) release with the Cyto Tox Non-Radioactive Cytotoxicity kit following the manufacturer's instructions (Promega Corporation). The percentage of specific LDH released was calculated as follows: [(experimental release − spontaneous release)/(total release − spontaneous release)] × 100. All assays were performed in triplicate (Corona-Gutierrez *et al.*, 2004).

Adverse events

All the possible adverse events were classified into the following categories: body in general, skeleton muscle, gastrointestinal, urogenital, nervous system, skin, and respiratory system, following the CTCAE of the National Cancer Institute (Institute, 2010). Judged by the criteria, physicians registered certain adverse events as to be related to the application of the MVA E2. Adverse events were considered to be of grade 1 (mild) when an intervention was not indicated; of grade 2 (moderate) when minimal, local, or noninvasive intervention was indicated; of grade 3 (severe) when symptoms were medically significant but not immediately life-threatening; and grade 4 (life-threatening) when life was compromised and urgent intervention was indicated (National Cancer Institute, 2010).

Statistical analysis

The efficacy of the MVA E2 treatment was assessed by comparing the number of patients with recurrences during a 2-year period after treatment in the control group and in the MVA E2-treated group. The mean time of recurrence (X) for appearance of new lesions after treatment was calculated as follows: X = number of patients with recurrence/time (24 months) after treatment. For this comparison, patients from

the control group who returned to the hospital with a new lesion in a period of 24 months were compared with patients from the MVA E2-treated group who also returned to the hospital with a new lesion in a period of 24 months. A paired Student's *t*-test was used for evaluating the difference in recurrence index between the two groups. Differences were considered statistically significant at $p < 0.05$.

Results

MVA E2 recombinant virus stimulated regression of intraepithelial lesions

Patients with intraepithelial lesions and an HPV infection, diagnosed by colposcopy and histology, and confirmed by the presence of HPV DNA, were included in this study and were treated with MVA E2 recombinant virus. At the end of treatment (visit 8, 14 weeks since the beginning of the protocol), 825 (94.82%) out of 870 female patients with low-grade lesions, and 220 (73.33%) out of 300 female patients with high-grade lesions, were free of lesions as diagnosed by histology (Table 1). Eighteen (5.67%) out of 317 female patients with low-grade lesions who had at least one previous treatment (such as cryosurgery, laser, or conization) presented few koilocytes after treatment, and 27 (4.88%) out of 553 female patients with low-grade lesions and no previous treatment showed some koilocytes after treatment (Table 1).

In 13 female patients (11.06%) out of 112 patients with initial high-grade lesions and having had one previous conization, the lesion was reduced to a CIN 1 (low-grade) lesion. Other 34 (30.35%) patients out of the same 112 patients with high-grade lesions and a previous conization presented some koilocytes after treatment (Table 1). Fifteen (7.97%) out of 188 female patients with high-grade lesions and no previous treatment had a reduction to a CIN 1 (low-grade) lesion after treatment (Table 1). Another 18 (9.57%) patients out of the same 188 female patients with high-grade lesions and no previous treatment had few koilocytes after treatment (Table 1). In addition, the six patients with condyloma in vulva were completely free of lesions after MVA E2 treatment. These results represent an overall efficacy of 90% for the MVA E2 in the treatment of HPV-induced CIN lesions (Table 1). All 180 male patients, independently of a previous treatment showed complete elimination of condyloma (anus and urethra) lesions after MVA E2 treatment (Table 1). The efficacy of the MVA E2 treatment does not seem to be affected by a previous treatment for HPV infections. There was no difference in the elimination of lesions after MVA E2 treatment among patients without previous treatment or patients previously treated with conventional procedures (Table 1).

Colposcopy and histology analyses confirmed that lesions in cervix were completely eliminated (Fig. 1A and B). At the beginning of treatment, aceto-white staining in the uterus revealed the presence of spots with possible papillomavirus infection. Histology of biopsies taken from these lesions also revealed an abnormal damaged epithelium (Fig. 1A and B). After treatment, the cervix presented a pinkish, smooth normal appearance free of lesions. Histology of biopsies taken from these tissues showed that a normal epithelium had been regenerated and it was free of koilocytes (Fig. 1B). Similarly, external lesions in anus and vulva were completely eliminated after treatment with MVA E2 (Fig. 2). In addition, condyloma lesions near anus were also eliminated after treatment with MVA E2 (Fig. 3).

MVA E2-treated patients did not have recurrence of lesions

In the control group, 141 female and 26 male patients who were treated with conventional methods also eliminated

Table 1. Clinical Characteristics of Human Papillomavirus–Infected Patients with Intraepithelial Lesions, Before and After Treatment with MVA E2

Patients before MVA E2 treatment			*Patients after MVA E2 treatment*		
Group	*Type of lesion*	*Number*	*Without lesions,* n *(%)*	*Low-grade lesions (CIN-1),* n *(%)*	*Koilocytes,* n *(%)*
Women					
1	Low grade[a]	317	299 (94.32)		18 (5.67)
	Low grade[b]	553	526 (95.11)		27 (4.88)
	Total low grade	870	825 (94.82)		45 (5.18)
2	High grade[c]	112	65 (58.03)	13 (11.60)	34 (30.35)
	High grade[b]	188	155 (82.44)	15 (7.97)	18 (9.57)
	Total high grade	300	220 (73.33)	28 (9.33)	52 (17.33)
3	Condyloma (vulva)[b]	6	6 (100)		
	Total women	1176	1051 (89.37)	28 (2.38)	97 (8.25)
Men					
4	Condyloma (urethra)[d]	8	8 (100)		
	Condyloma (urethra)[b]	160	160 (100)		
5	Condyloma (anus)[b]	12	12 (100)		
Total men		180	180 (100)		
Total patients		1356	1231 (90.78)		

[a]Previous treatment: cryosurgery, conization, or laser.
[b]No previous treatment.
[c]Previous treatment: conization.
[d]Previous treatment: electrofulguration.

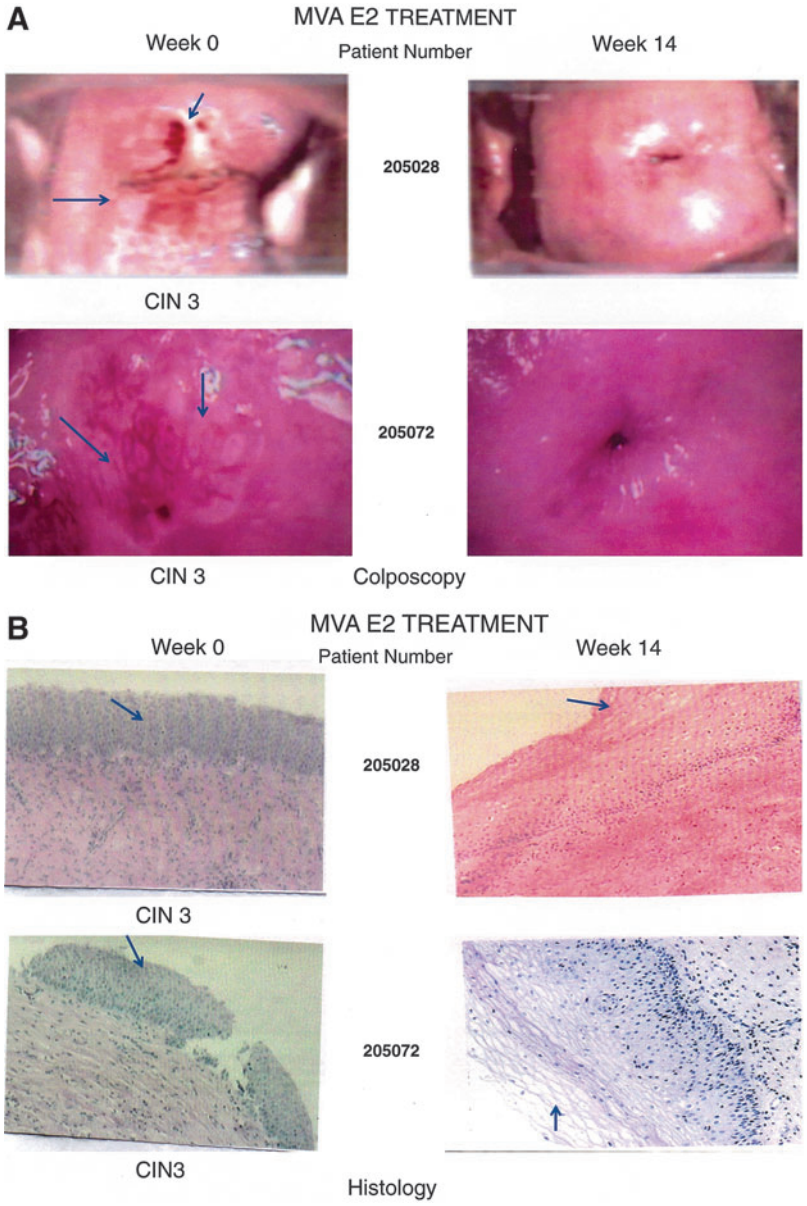

FIG. 1. **(A)** Colposcopy and histology of intraepithelial lesions from patients treated with MVA E2 therapeutic vaccine. Photographs of cervix (left panels) and of histology samples (right panels) from representative patients. Patients 205028 and 205072 had initial CIN 3 lesions at week 0. The acetowhite staining in the uterus reveals the presence of possible spots with papillomavirus infection (arrows). **(B)** In the histology pictures, the epithelium is indicated by arrows. Patients were free of lesions at week 14 after MVA E2 treatment, and the epithelium was free of koilocytes at week 14.

their lesions by 14 weeks after treatment. However, most of these patients, 126 out of 141 female (89.36%) and 26 out of 26 male (100%) patients, showed recurrence of new lesions during the next 24 months after treatment. Most recurrences appeared between 4 and 10 months after treatment (Fig. 4). Other 19 (13.4%) female patients with a good initial response from the control group presented recurrences within 36 months after treatment. In the MVA E2-treated group, only 5 female patients out of 141 (3.54%) with high-grade lesions (from group 2 in Table 1) showed the appearance of the same lesion during a period of 2 years after treatment (Fig. 4). None of the MVA E2-treated male patients showed recurrences a period of 2 years after treatment. Both groups were kept under observation for a total of 5 years after treatment. There were not any more patients with recurrences in the MVA E2-treated group during this period (Fig. 4).

To look more carefully at the efficacy of the MVA E2 treatment, the recurrence index (X) was calculated for both treatment groups. The number of patients who returned with a new lesion in a period of 2 years was compared in both groups. In the control group, 126 female patients/24 months and 26 male patients/24 months (Fig. 4) give a recurrence index of $X = 5.25$ patients/month for women and $X = 1.083$ patients/month for men. In contrast, in the MVA E2-treated group, 5 female patients/24 months and 0 male patients/24 months give a recurrence index of $X = 0.208$ patients/month for women and $X = 0$ patients/month for men. These data strongly suggested that therapeutic treatment with MVA E2 stimulated regression of HPV-induced intraepithelial lesions in the ano-genital region, and was capable of maintaining patients free of lesions for up to 2 years after treatment. In addition, a follow-up of most of MVA E2-treated patients who enrolled at the beginning of protocol remained after 5 years free of lesions (Fig. 4). Student's t-test between women or men treated with MVA E2 and the control group showed a significant difference between the time of recurrence of new lesions ($p = 0.00850$ for women and $p = 0.03066$ for men). These results showed that therapeutic

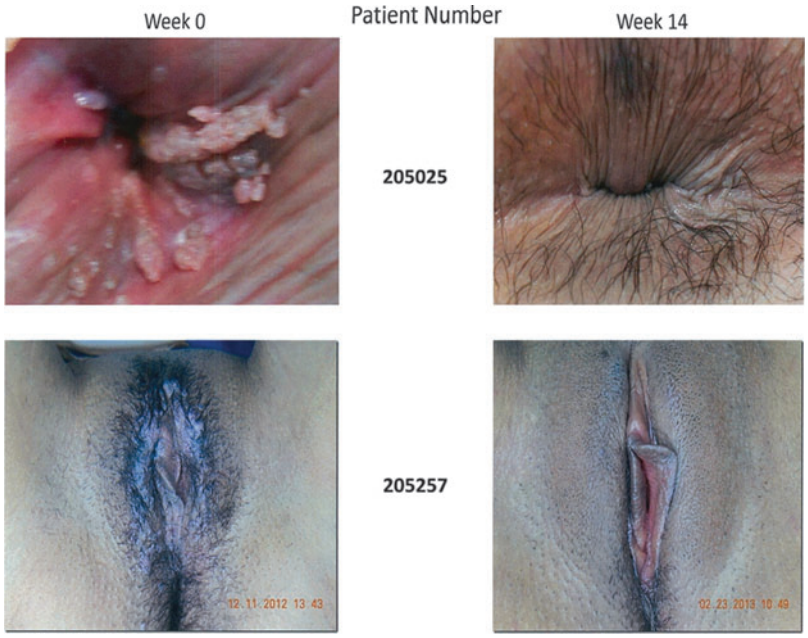

FIG. 2. Photographs of anus (top) and vulva (bottom) with papillomavirus-induced lesions in representative patients 205025 and 205257. Tissues are shown before (week 0) and after (week 14) MVA E2 treatment.

treatment with MVA E2 efficiently stimulates regression of intraepithelial lesions in the anogenital region and is capable of maintaining patients free of lesions for long periods of time.

Humoral immune response generated against the HPV-E2 protein and the MVA E2 therapeutic vaccine

The data presented above suggested that a long-term response against HPV-induced lesions was obtained after treatment with MVA E2. The most likely mechanism for this type of response is an activation of the adaptive immune response, as suggested previously (Rosales *et al.*, 2000; Valadez *et al.*, 2000). Thus, indicators of both humoral and cellular immune responses were explored in the MVA E2-treated patients. Serum from all patients were collected at the beginning (week 0) and at the end of the treatment (week 14) in order to determine the humoral immune response generated against the MVA E2 virus itself or against the HPV-E2 protein. Antibodies against MVA are indicative that the immune system of patients is responding properly, while antibodies against the E2 protein are indicative that an immune response against proteins from infected cells is being generated. Presence of antibodies was assessed by ELISA. Specific antibodies against the MVA E2 virus were

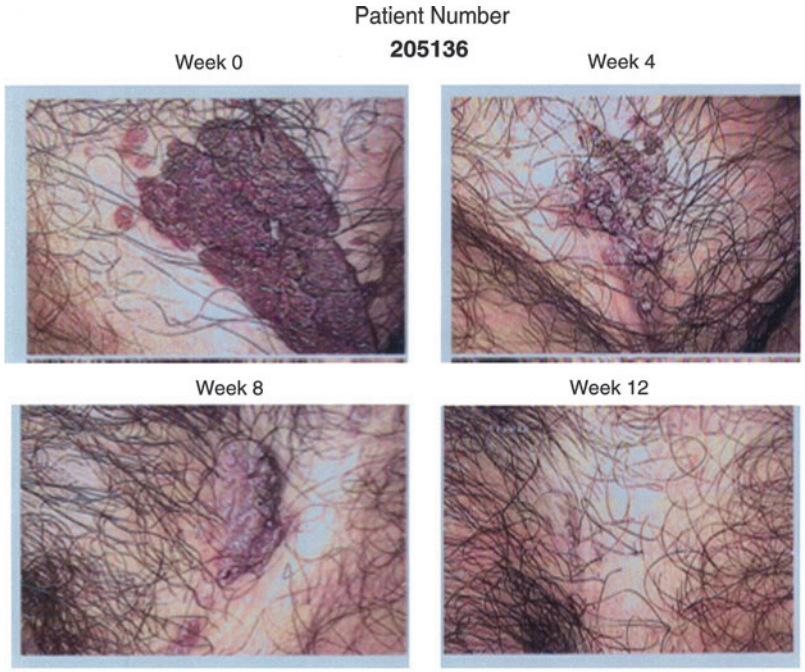

FIG. 3. Photographs of the anus with papillomavirus-induced condyloma lesions from representative patient 205136. Tissue is shown at week 0, 4, 8, and 12 weeks of MVA E2 treatment. Patient was completely free of lesions after treatment.

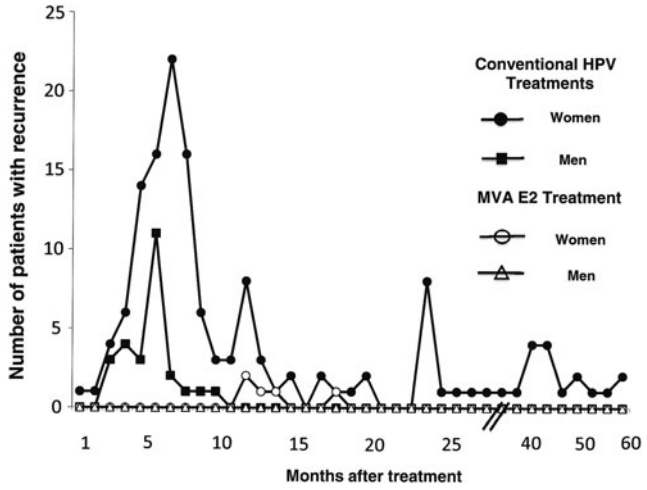

FIG. 4. MVA E2-treated patients did not have recurrence of lesions. **(A)** Recurrence of HPV-induced lesions in control patients (black symbols) treated by conventional methods (including cryosurgery, laser, conization, electrosurgery, 0.5% podophillin, trichoroacetic acid, or 5-fluorouracil) or MVA E2-treated patients (open symbols) was recorded during a 2-year period after the corresponding treatment. **(B)** Recurrence index (X = number of patients with recurrence/mean time for recurrence) was calculated for control patients (black bars) and for MVA E2-treated patients (white bars). *Differences from control were statistically significant at $p \leq 0.008$ (Student's t-test).

detected in all patients treated. Sera titers increased during treatment with MVA E2, and titers were between 1/500 and 1/1000 dilutions. Antibodies against the HPV-E2 protein were also detected in all treated patients. Serum titers ranged from 1/128 to 1/256 (data not shown). In contrast, no antibodies were detected in untreated patients. These data suggested that all treated patients mounted a humoral immune response against both against MVA E2 vaccinia proteins, and the HPV-E2 protein. Similar results were obtained in our previous phase I and phase II studies (Corona-Gutierrez *et al.*, 2004; Garcia-Hernandez *et al.*, 2006; Albarran *et al.*, 2007).

Cellular immune response generated against the HPV-infected cells with the MVA E2 therapeutic vaccine

In addition to antibodies against the HPV E2 protein, effector cells against HPV-infected cells were generated in the MVA E2-treated patients. First, we observed that all intraepithelial lesions were eliminated by the therapeutic application of MVA E2. Thus, it was possible that cytotoxic cells were being generated in these patients. In order to confirm this idea, lymphocytes were isolated from patients and tested for their cytotoxic activity against HPV- transformed cells. More than 10% of all patients were randomly selected for lymphocyte isolation and testing of cytotoxic activity of these cells. Lymphocytes from all tested patients were able to kill the target papilloma-transformed cells at different effector/target ratios (Fig. 5). In contrast, lymphocytes isolated from patients before treatment (Fig. 5) or

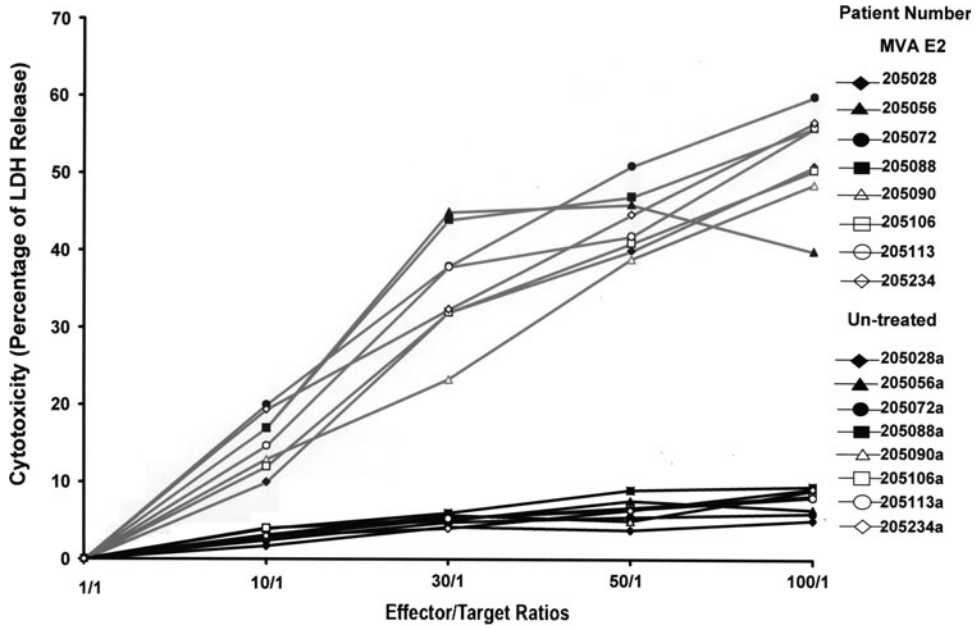

FIG. 5. Cytotoxic activity of lymphocytes from MVA E2–treated patients against HPV-transformed cells. Lymphocytes (effector cells) from HPV-infected patients before (black lines) or after MVA E2 treatment (blue lines) were stimulated with HPV antigens as described in Materials and Methods, and then mixed with HPV-infected cells (target cells) isolated from biopsy preparations, at several effector-to-target ratios. After an 8 hr incubation, supernatants were collected and the percentage of specific cell lysis was determined by measuring the lactate dehydrogenase (LDH) release. Cytotoxicity of lymphocytes from representative patients (out of 146 patients) before treatment (205028a, 205056a, 205072a, 205088a, 205090a, 205106a, 205113a, 205234a) and from the same patients (205028, 205056, 205072, 205088, 205090, 205106, 205113, 205234) after MVA E2 treatment is shown.

Table 2. Presence of Total Human Papillomavirus DNA in 1262 Patients Before and After Treatment with MVA E2 Diagnosed by Hybrid Capture Method

	Patients before treatment			*Patients after MVA E2 treatment*	
Category	*Total*	*Women*	*Men*	*Women,* n *(%)*	*Men,* n *(%)*
Oncogenic HPV	1190	1097	93	203 (18.50)	14 (15.05)
Nononcogenic HPV	72	16	56	2 (12.5)	3 (5.35)
Total	1262	1113	149		

HPV, human papillomavirus.

from patients in the control group (data not shown) did not show any cytotoxicity. These results strongly suggested that indeed cytotoxic lymphocytes were induced in MVA E2-treated patients.

MVA E2 eliminated the papillomavirus from patients presenting intraepithelial low- and high-grade lesions

Presence of papillomavirus in patients with low- and high-grade lesions before and after treatment with MVA E2 was assessed with the Hybrid Capture method or by PCR. Samples from 1262 patients were assayed by Hybrid Capture. Among these, 203 out of 1097 female and 14 out of 93 male patients still had oncogenic HPV after treatment with MVA E2 (Table 2). Also, 2 out of 16 female and 3 out of 56 male patients still had nononcogenic HPV after treatment with MVA E2 (Table 2). Another 94 patients (from Venezuela) were assessed for the presence of HPV by PCR. After treatment, 3 out of 18 female patients still had oncogenic HPV DNA (Table 3). None of the nine male patients had oncogenic HPV after treatment (Table 3). Five out of 45 female patients and 2 out of 22 male patients still had nononcogenic HPV 6 or HPV11 after treatment (Table 3). These data showed that 82.9% of patients treated with MVA E2 could efficiently eliminate the papillomavirus DNA. No correlation was observed between elimination of different types of HPV DNA and the type of lesion presented in patients.

Table 3. Presence of Human Papillomavirus DNA in 94 Patients Before and After Treatment with MVA E2 Diagnosed by Polymerase Chain Reaction

	Patients before treatment			*Patients after MVA E2 treatment*	
Category	*Total*	*Women*	*Men*	*Women,* n *(%)*	*Men,* n *(%)*
Oncogenic	27				
HPV 16		1	0	0	0
HPV 31		7	4	1 (14.2)	0
HPV 33		1	2	0	0
HPV 35		1	1	0	0
HPV 45		4	0	1 (25)	0
HPV 53		4	2	1 (25)	0
Total		18	9		
Nononcogenic	67				
HPV 6		26	8	3 (11.53)	1 (12.5)
HPV 11		19	13	2 (10.52)	1 (7.69)
HPV 43		0	1	0	0
Total		45	22		

Therapy with MVA E2 recombinant virus did not produce uncomfortable adverse events

Possible adverse events were classified into the following categories: body in general, skeleton muscle, gastrointestinal, urogenital, nervous system, skin, and respiratory system, following the CTCAE of the National Cancer Institute (Institute, 2010). Judged by the physicians, certain adverse events were considered to be related to the application of MVA E2. Among the total patients, the only adverse events observed during the MVA E2 application were as follows: 543 presented headaches, 934 presented flu symptoms, 746 had a temperature above 39 degrees for 1 day, 856 presented chills, 678 had abdominal ache, and 367 had joint pain lasting for 1 day. These adverse events are all considered to be of grade 1 (very mild) uncomfortable side effects, because these symptoms are similar to those presented on a normal flu infection (Institute, 2010). Up to now, no other adverse events have been registered for any patient included in the clinical protocols, using MVA E2 therapeutic vaccine, performed during the past 15 years. These results showed that the MVA E2 therapeutic vaccine is a very mild and effective agent to be used in the treatment of intraepithelial lesions (Table 4).

Discussion

Persistence of high-risk HPV can lead to the development of cancer lesions. Cervical cancer remains a serious problem in developing countries because early detection is difficult, and thus proper early treatment is many times missing.

Table 4. Adverse Events Associated with the Administration of MEL-1 in 1356: Patients with Intraepithelial Lesions

Symptoms	*Number of patients (%)*
Headache	543 (44.04)
Cold	934 (68.87)
Fever	746 (55.01)
Chill	856 (63.12)
Abdominal ache	678 (50.0)
Articulation pain	367 (27.06)

When HPV-mediated cancer develops, radiotherapy and chemotherapy are used with relative success, since about 50% of the HPV cancer patients still die (Powell *et al.*, 2013). Clearly, new therapeutic strategies are in urgent need to control the burden of HPV-related cancer (Jemal *et al.*, 2011). Recently, the development of anticancer vaccines and intralesional immunotherapy are becoming a promising alternative therapy for this type of cancer and the most effective way to treat and eradicate virus-induced tumors (Rosales and Rosales, 2014).

The recombinant vaccinia virus MVA E2 is a vaccinia virus Ankara (MVA) containing the bovine papilloma virus E2 protein (Rosales *et al.*, 2000; Valadez *et al.*, 2000). This recombinant virus is based on the highly attenuated poxvirus strain-modified vaccinia virus Ankara (MVA). MVA is a nonreplicating derivative of the uniquely successful smallpox vaccine. Thus, its use in humans is completely safe, as proven by many clinical trials, which have been conducted with it (Kaufmann *et al.*, 2002; Cosma *et al.*, 2003; Cebere *et al.*, 2006; Dorrell *et al.*, 2007; Jaoko *et al.*, 2008; Currier *et al.*, 2010; Wilck *et al.*, 2010; Cavenaugh *et al.*, 2011; Garcia *et al.*, 2011; Goepfert *et al.*, 2011; Sheehy *et al.*, 2012; Verheust *et al.*, 2012; Gilbert, 2013; Ogwang *et al.*, 2013). In addition, MVA is genetically stable, easy to manufacture, and very immunogenic (Cottingham and Carroll, 2013; Gilbert, 2013) because of cross-presentation of dying vaccinia virus-infected cells by dendritic cells to T cells (Iborra *et al.*, 2012). For these reasons, MVA has become the vector of choice for novel HPV therapeutic vaccines (Su *et al.*, 2010; Rosales and Rosales, 2014).

MVA E2 has been shown to stop human tumor growth in mice, and to induce tumor regression in tumor-bearing rabbits (Rosales *et al.*, 2000; Valadez *et al.*, 2000). In a series of studies, MVA E2 was evaluated in patients who had established HPV-induced CIN lesions (Corona-Gutierrez *et al.*, 2004; Garcia-Hernandez *et al.*, 2006; Albarran *et al.*, 2007). In a phase I/II clinical trial for CIN 1 to CIN 3 lesions (Corona-Gutierrez *et al.*, 2004), in a phase II clinical trial for high-grade lesions (CIN 2 and CIN 3) (Garcia-Hernandez *et al.*, 2006), and in another phase I/II clinical trial to evaluate MVA E2 for the treatment of flat condyloma lesions associated with oncogenic HPV in men (Albarran *et al.*, 2007), it was established that MVA E2 is a promising treatment for HPV-induced lesions. MVA E2 treatment eliminated most CIN 1 lesions, many CIN 3 lesions were reduced to CIN 1, and most patients did not show any recurrence of lesions after 1 year of treatment (Corona-Gutierrez *et al.*, 2004; Garcia-Hernandez *et al.*, 2006; Albarran *et al.*, 2007). In addition, no severe adverse events were registered for the use of this therapeutic recombinant vaccinia virus MVA E2.

In the present report, we evaluated the therapeutic potential of MVA E2 in the treatment of HPV-induced anogenital intraepithelial lesions in a phase III study and found that 97% of treated patients showed complete elimination of precancerous CIN lesions by 14 weeks after treatment, and other 2.4% female patients showed reduction of lesions to CIN 1. In male patients, all lesions were completely eliminated. In addition, papillomavirus DNA was not detected after treatment in 83% of all patients. Also, patients developed a specific cytotoxic response against papilloma-transformed cells. Our data suggest that the MVA E2 therapeutic vaccine can induce a long-lasting immunity that prevents recurrence of lesions in most patients, and thus MVA E2 promises to be an excellent tool for the treatment of most papillomavirus-related lesions in humans.

When HPV lesions are detected, the main therapeutic approach currently used involves physical elimination of lesions (Martin-Hirsch *et al.*, 2010). Ablative therapies include cryotherapy, excision procedures (conization), laser therapy, and electrosurgery (Sonnex and Lacey, 2001; Rosales and Rosales, 2014). Although these ablative therapies are effective in initial treatments where elimination of lesions can be as high as 80% (Lacey *et al.*, 2013), removal of damaged tissue does not always guarantee elimination of viral DNA. Particularly, in precancerous lesions, surgical procedures alone are not very effective, since recurrences occur at rates of 20–30% or more with lesions both at previously treated sites because of failure of the procedure to eliminate the HPV, and at new sites because of new infections (Lacey *et al.*, 2013), even after several treatments (Maw, 2004). Persistence of high-risk HPV can lead to the development of cancer lesions. When this occurs, radiotherapy and chemotherapy are then used with relative success, since about 50% of the HPV cancer patients still die (Powell *et al.*, 2013). Clearly, new therapeutic strategies are in urgent need to control the burden of HPV-related cancer (Jemal *et al.*, 2011).

In the fight against papillomavirus infections, other options have also been tried for the prevention of these infections and the consequent appearance of primary lesions. Preventive vaccines, such as Gardasil (Merck) (Villa *et al.*, 2006) and Cervarix (GSK) (Harper *et al.*, 2006), have been approved and promise, in the long-term (30–50 years), to reduce the incidence of disease associated with the vaccine HPV types if a large vaccination coverage (larger than 50%) of uninfected people is achieved (Dillner *et al.*, 2011). Unfortunately, full vaccination coverage of large populations will not be easy in many parts of the world, and a high prevalence and mortality of cervical cancer will continue around the world, especially in developing countries. In addition, these vaccines target only HPV 16 and 18 and in the case of Gardasil HPV 6 and 11. Despite some cross-reactivity (Paavonen *et al.*, 2009), these vaccines show a small prophylactic effect on many HPV subtypes not included in the vaccine (Tomljenovic and Shaw, 2013). Also, the HPV subtype distribution in cervical cancer varies throughout the world (Clifford *et al.*, 2005; Smith *et al.*, 2007). Thus, unvaccinated people remain at high risk of HPV-related disease and in need of treatment (Pandhi and Sonthalia, 2011; Markowitz *et al.*, 2012; Draper *et al.*, 2013; Nelson *et al.*, 2013). A more promising therapeutic strategy seems to be the stimulation of an immune response, which can recognize and eliminate virus-infected cells (Best *et al.*, 2012).

MVA E2 is indeed a therapeutic vaccine capable of activating the immune system of the patient and inducing elimination of virus-infected cells. MVA E2 therapeutic vaccine induced antibodies against the E2 protein and also against HPV-induced cancer cells (Rosales *et al.*, 2000; Corona-Gutierrez *et al.*, 2004; Garcia-Hernandez *et al.*, 2006; Albarran *et al.*, 2007). Contrary to the humoral response of preventive vaccines, which mostly induce the production of neutralizing antibodies against papillomavirus

particles to prevent virus infection, the MVA E2-induced humoral response included antibodies against tumor cells. These antibodies bind tumor cells and mark them for destruction by effector cells of the immune system. Indeed, we have previously shown that MVA E2 treatment generated specific antitumor antibodies that could induce antibody-dependent cell-mediated cytotoxicity (Rosales *et al.*, 2000; Valadez *et al.*, 2000). Despite the presence of antitumor antibodies, the efficacy of MVA E2 therapy also involves cytotoxicity mediated by T cells. HPV-specific $CD4^+$ (helper) and $CD8^+$ (cytotoxic) T cells are generated in patients who successfully eliminated previous HPV 16 infections (Welters *et al.*, 2003; Bourgault Villada *et al.*, 2004). In contrast, patients with cervical intraepithelial neoplasia or cervical cancer presented deficient T cell responses (de Jong *et al.*, 2004). Thus, an efficient cytotoxic cell-mediated immune response seems critical for elimination of HPV-related lesions.

In agreement with this hypothesis, previous studies using DNA vaccines carrying the HPV *E6* or *E7* genes alone or combined with different antigens from various viruses could induce specific T cell lymphocytes against papillomavirus *in vitro* and in mouse models (Lescaille *et al.*, 2013; Santana *et al.*, 2013). But in early clinical trials, no correlation between cytotoxic activity and the poor clinical outcome was detected for DNA vaccines (Garcia *et al.*, 2004; Trimble *et al.*, 2009). Also, a recombinant vaccinia-based vaccine (vac-Sig/E7/LAMP-1) was effective for controlling E7-expressing tumors grown in the liver mice *and* E7-specific CD8+ T cell precursors correlated with the antitumor effect (Chen et al., 2000). Other systems using a recombinant vaccinia virus-encoded *L2*, *E6*, and *E7* genes in a prime-boost regimen were also capable of inducing a CTL immunity by preventing tumor growth in a mice model (van der Burg *et al.*, 2001). Another vaccinia virus encoding the *E6* and *E7* genes from HPV 16 and 18 (TA-HPV) was used to intramuscularly immunize patients with HPV 16-positive high-grade vulval intraepithelial neoplasia (VIN). At 24 weeks after treatment, 42% patients showed partial reduction in total lesion diameter (Baldwin *et al.*, 2003). The relatively low efficacy of TA-HPV correlated with no increase in cytotoxic activity against selected individual HLA class I-restricted HPV 16 E6/7 peptides (Baldwin *et al.*, 2003).

In another study, 8 out of 13 patients with high-grade VIN presented a partial reduction in lesion diameter, but no increase in cytotoxic activity was detected (Davidson *et al.*, 2003). For MVA E2, previous studies have shown that therapeutic vaccination with MVA E2 in tumor-bearing animals (Rosales *et al.*, 2000) as well as in HPV-infected humans (Corona-Gutierrez *et al.*, 2004; Garcia-Hernandez *et al.*, 2006; Albarran *et al.*, 2007) can induce the formation of specific cytotoxic cells against tumor cells. In the present study, we also found that all MVA E2-treated patients generated a T-cell immune response. Cytotoxic T cells against tumor cells from each patient were detected (Fig. 5). In contrast, untreated patients or control-group patients did not show cytotoxic activity. Thus, a strong correlation between cytotoxicity against HPV-infected cells and elimination of lesions was always found. Together, all these reports support the idea that the presence of cytotoxic lymphocytes against tumor cells is the main mechanism induced by recombinant vaccinia viruses to eliminate HPV-induced lesions. Experiments are in progress in order to determine whether other immune cells such as NK and dendritic cells are also involved in the MVA E2-induced mechanism for regression of lesions.

Other MVA vectors against HPV have also been evaluated in phase I/II clinical trials. A vaccinia virus carrying the HPV *E6* and *E7* genes together with the interleukin (*IL*)-2 gene (TG4001) was used to treat 21 patients with HPV 16-related high-grade CIN 2/3. Patients received three weekly subcutaneous injections of TG4001. Ten patients (48%) showed promising clinical responses at 6 months after treatment (Brun *et al.*, 2011). Also, as mentioned above, TA-HPV (a vaccinia virus encoding the *E6* and *E7* genes from HPV 16 and 18) was assessed in a phase II trial of HPV 16-positive high-grade VIN. Patients were immunized intramuscularly with TA-HPV, and at 24 weeks, 42% patients showed partial reduction in total lesion diameter (Baldwin *et al.*, 2003). An important difference between those studies and our present report is the way the recombinant virus was administered. In these studies, the virus was injected either subcutaneously or intramuscularly. In contrast, MVA E2 was administered directly into the lesion. Local administration of MVA E2 works in different ways to stop HPV infection. First, the E2 protein delivered into cells by the MVA infection stops production of E6 and E7 oncogenes, and promotes apoptosis in cervical cancer cells (Desaintes *et al.*, 1997). Second, the lytic effect of MVA infection, together with the well-known excellent antigen presenting capacity of vaccinia virus, stimulates the immune system against infected cells. Thus, it seems that the local application of the MVA E2 vaccine is an efficient method for stimulating the immune system and creating regression of HPV-induced intraepithelial lesions.

In support of this idea, a recent report, using the murine model of cervical cancer with HPV 16 E6- and E7-expressing TC-1 tumor cells, indicated that the same TA-HPV vaccine increased its efficacy when it was administered directly into the tumor (Lee *et al.*, 2013). In this case, an increase in E7-specific $CD8^+$ T cells was found in the blood together with a significant decrease in tumor size (Lee *et al.*, 2013). Also, it was recently reported that intravaginal immunization with HPV vectors induces $CD8^+$ T cell responses (Cuburu *et al.*, 2012). In addition, intralesional immunotherapy by injecting *Corinebacterium parvum* induced regression of bovine papillomavirus lesions associated with the increased number of CD8 and $\gamma\delta+$ cells in the dermis, as well as infiltration of neutrophils (Hall *et al.*, 1994). Thus, intralesion injection of recombinant vaccines seems to be the preferred method for stimulating the immune system and creating regression of HPV-induced intraepithelial lesions (Marabelle *et al.*, 2014).

In the present study, a 2-year follow-up showed that HPV DNA could not be detected in 83% of patients after MVA E2 treatment. The eradication of papillomavirus was the result of the efficient elimination of lesions in about 97% of MVA E2-treated patients. This indicates that some patients who eliminated their lesions still had some detectable virus DNA. Presence of papillomavirus in these patients could be detected most likely because DNA analysis is a very sensitive assay. Hence, even small amounts of papillomavirus in few infected cells can be noticed. However, the relative

viral load in DNA-positive patients was less than 5% of the original viral load at the beginning of the study. In a previous study, we showed that 50% of patients presented some papillomavirus DNA after 6 weeks of treatment (Corona-Gutierrez *et al.*, 2004). In contrast, in this phase III study, we show that most patients were free of papillomavirus by 12 weeks after treatment. Therefore, it is recommended to wait for at least 12 weeks after treatment for confirmation of complete disappearance of papillomavirus DNA.

Data discussed above suggested that MVA E2 treatment was very efficient at eliminating not only CIN lesions but also the papillomavirus itself. In the control group, patients who were treated with conventional methods also eliminated their lesions by 14 weeks after treatment. Thus, it seemed that both therapeutic strategies were similar. However, during a 2-year period after treatment, important differences were observed. In the control group, although elimination of lesions was accomplished successfully, 89% of female and 100% of male patients showed recurrence of new lesions during the next 24 months after treatment. Most recurrences appeared between 4 and 10 months after treatment, indicating that most of the conventional procedures used for eliminating HPV-induced lesions are not sufficient to prevent new lesions.

Some patients even required more than one intervention to control their disease. These results are in agreement with those reported previously for therapies such as loop electrosurgical excision procedure (LEEP). In this case, the procedure could eliminate initial lesions and remove most of the HPV in patients (Aerssens *et al.*, 2008). However, many recurrences and persistence of high-risk HPV DNA were still found (Aerssens *et al.*, 2008). Therefore, a follow-up including HPV detection and Pap-smears of all patients after LEEP is strongly recommended (Baloglu *et al.*, 2010). In contrast, in the MVA E2-treated group, only 5 (3.5%) out of 141 female patients with high-grade lesions showed the appearance of lesions in a period of 2 years after treatment. None of the MVA E2-treated male patients showed recurrences in the 2-year period after treatment. Moreover, for a total of 5 years after treatment, there were not any more patients with recurrences in the MVA E2-treated group. These data show that MVA E2 treatment is capable of eliminating lesions and keeping patients free from new lesions for long periods of time. This is most likely because of activation of a cell immune response that eliminates HPV-infected cells, since there is a direct correlation between the cytotoxic immune response and elimination of lesions. This cytotoxic response seems also to have a strong immune memory that explains the long-lasting effect of the MVA E2 therapy.

It is interesting that some groups suggest that HPV infection could be managed conservatively (Ho *et al.*, 1998) because many HPV infections (around 30%) are cleared spontaneously within 2 years from infection and without any clinical manifestation by immune-competent individuals (Woodman *et al.*, 2007; Doorbar *et al.*, 2012). However, it is worth mentioning that this type of regression happens only when the patient is very healthy and young and if it is the first time she/he has a lesion in her/his life. In developing countries the rate of regression is unknown, but it seems to be much lower than the one reported for developed countries (Lacey *et al.*, 2013). In addition, persistence of the virus (Doorbar, 2013) and a high rate of recurrence—30% or more (Lacey *et al.*, 2013)—together with the destructive potential of conventional therapeutic procedures for the cervix, make us think that conservative management of HPV-infected lesions is not an adequate policy. Also, conventional procedures have the disadvantage of recurrences. Thus, novel therapeutic approaches, such as MVA E2, would become very important in the near future.

In addition to its therapeutic potential, the MVA E2 vaccine proved to be a very mild treatment. Administration of the MVA E2 did not interfere with patients' normal activities, and none of the treated patients presented serious adverse events along the length of the study. Symptoms of flu or light pelvic pain during or after the second or third dose were the only side effects associated with MVA E2 treatment. These adverse events were all considered to be of grade 1 (mild) according to the CTCAE of the National Cancer Institute (Institute, 2010).

In summary, we have found in a phase III clinical trial that MVA E2 therapeutic vaccine could eliminate precancerous CIN 1, CIN 2, and CIN 3 lesions, as well as most papillomavirus-induced lesions located in anus, vulva, urethra, and uterus. MVA E2 treatment also eliminated the papillomavirus from most patients and induced a long-lasting immune cytotoxic response that correlated with no recurrence of lesions. Thus, MVA E2 therapeutic vaccine is an excellent new tool for the treatment of most papillomavirus-related lesions in humans and becomes a promising therapy that could reduce the mortality caused by cervical cancer worldwide.

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Author Disclosure Statement

The authors declare that no competing financial interests exist and that there is no conflict of interest among the authors.

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Appendix

SCHEDULE FOR PROGRAMMED VISITS AND PROCEDURES

Study phase	*Selection*	*Beginning of treatment*	*Treatment*					*Final*	*Follow-up*
Study visit	1	2	3	4	5	6	7	8	9
Week of study	0	1	2	3	4	5	6	14	18
Day of the study	−7	0	7	14	21	28	35	91	126
Informed consent	✓								
Exclusion/inclusion criteria	✓	✓							
Demographic data	✓	✓							
Gyneco-obstetric and urologic antecedents	✓								
Medical history	✓								
Physical examination	✓	✓	✓	✓	✓	✓	✓	✓	✓
Concomitant medication	✓	✓	✓	✓	✓	✓	✓	✓	✓
HPV typing	✓							✓	✓
HIV serology test	✓	✓		✓					
Colposcopy or peniscopy examination	✓	✓	✓	✓	✓	✓	✓	✓	✓
Biopsy		✓						✓	
Concomitant diseases	✓	✓	✓	✓	✓	✓	✓	✓	✓
Clinical laboratory security tests	✓							✓	✓
Urinalysis	✓	✓						✓	✓
Electrocardiogram	✓							✓	
Adverse events	✓	✓	✓	✓	✓	✓	✓	✓	✓
Study drug administration		✓	✓	✓	✓	✓	✓		
Immunological response (citotoxity essays)		✓						✓	
Global evaluation		✓	✓	✓	✓	✓	✓	✓	✓