Safety and Immunogenicity of TA-HPV, a Recombinant Vaccinia Virus Expressing Modified Human Papillomavirus (HPV)-16 and HPV-18 *E6* and *E7* Genes, in Women with Progressive Cervical Cancer[1]

Andreas M. Kaufmann,[2] Peter L. Stern, Elaine M. Rankin, Harald Sommer, Volkmar Nuessler, Achim Schneider, Malcom Adams, Toli S. Onon, Thomas Bauknecht, Uwe Wagner, Karlijn Kroon, Julian Hickling, Christopher M. Boswell, Simon N. Stacey, Henry C. Kitchener, Jennifer Gillard, Jantien Wanders, John St. C. Roberts, and Heinz Zwierzina

Frauenklinik, Friedrich-Schiller-University of Jena, 07743 Jena, Germany [A. M. K., A. S.]; Paterson Institute for Cancer Research, Christie Hospital, Manchester, England, United Kingdom [P. L. S., T. S. O., S. N. S.]; Ninewells Hospital and Medical School, Department of Cancer Medicine, University of Dundee, Scotland, United Kingdom [E. M. R.]; Frauenklinik, Klinikum Innenstadt, Ludwig-Maximilians-University, Munich, Germany [H. S., V. N.]; Velindre Hospital, Cardiff, Wales, United Kingdom [M. A.]; Department of Obstetrics and Gynaecology, St Mary's Hospital, Manchester, England, United Kingdom [T. S. O., H. C. K.]; Frauenklinik, University of Bonn, Bonn, Germany [T. B.]; Frauenklinik, University of Tübingen, Tübingen, Germany [U. W.]; EORTC New Drug Development Office Oncology, Amsterdam, the Netherlands [K. K., J. W.]; Xenova Research Limited, Cambridge, England, United Kingdom [J. H., C. M. B., J. G., J. S. C. R.]; and University Clinic, Innsbruck, Austria [H. Z.]

ABSTRACT

***Purpose*: Cervical cancer, the second most common malignancy in women worldwide, is almost invariably associated with infection by human papillomavirus (HPV). HPV-16 or -18 is commonly present in 70% of cervical cancers. HPV-positive tumor cells present antigens of the viral protein in the context of human leukocyte antigen (HLA) class I that can be recognized by CTLs. We have conducted a study in patients with early-stage cervical cancer to assess the safety and immunological effects of vaccination with TA-HPV, a live recombinant vaccinia virus expressing modified forms of the HPV-16 and -18 E6 and E7 proteins.**

***Experimental Design:* Twenty-nine patients with clinical International Federation of Gynecologists and Obstetricians (FIGO) stage Ib or IIa cervical cancer were given two vaccinations with TA-HPV at least 4 weeks apart, starting 2 weeks before radical hysterectomy. Patients were monitored closely for side effects of the vaccination. Serial blood samples were examined for HPV-specific CTLs or changes in levels of antibodies to HPV-16 or -18 E6 and E7 proteins and to vaccinia virus.**

***Results*: Vaccination with recombinant vaccinia was well tolerated in all patients with only mild to moderate local toxicity, and no serious adverse events were attributable to the vaccine. After a single vaccination, HPV-specific CTLs were found in four patients (HLA A1, A3, three patients; HLA A1, A24, one patient). Eight patients developed HPV-specific serological responses.**

***Conclusions*: This study confirmed the safety and immunogenicity of the vaccine in a proportion of those patients vaccinated. Additional clinical studies using TA-HPV in combination with an additional experimental vaccine for HPV-16 are currently under way.**

INTRODUCTION

Cervical cancer is the second most common malignancy in women and is responsible for 400,000 deaths/year worldwide (1). Infection with the HPV[3] (the most prevalent types are HPV-16 and -18) could be demonstrated in 99.7% of patients (2, 3). The oncoproteins E6 and E7 are always expressed in tumor cells because they are mechanistically involved in the process of carcinogenesis (4–6). Epitopes of these proteins can be presented in the context of HLA class I molecules (7–9). Thus, "tagged" with these unique viral tumor antigens, cervical cancer cells may be recognized specifically by CTLs. Such CTLs can be activated by HPV-positive tumor cells *in vitro* (10). In CIN and cervical cancer patients, HPV oncogene-specific memory T

Received 5/20/02; revised 7/24/02; accepted 7/24/02.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Presented at the American Association for Cancer Research Annual Meeting, April 2000 (abstract 2992) and the 6th International Symposium on Biological Therapy of Cancer, EORTC, September 2001. P. L. S. and S. N. S. were supported by the Cancer Research Campaign. T. S. O. was supported by a Joseph Starkey Clinical Research fellowship from the Wigan Cancer Research Fund.
[2] To whom requests for reprints should be addressed, at Friedrich-Schiller Universität Jena, Frauenklinik, Gynäkologische Molekularbiologie, Bachstrasse 18, 07743 Jena, Germany. Phone: 49-3641-934273; Fax: 49-3641-934216; E-mail: a.kaufmann@med.uni-jena.de.

[3] The abbreviations used are: HPV, human papillomavirus; HLA, human leukocyte antigen; CIN, cervical intraepithelial neoplasia; PBMC, peripheral blood mononuclear cell; FIGO, International Federation of Gynecologists and Obstetricians; EORTC, European Organization for Research and Treatment of Cancer; pfu, plaque-forming unit(s); B-LCL, B-lymphoblastoid cell line; MBP, maltose-binding protein; IHC, immunohistochemistry; mAb, monoclonal antibody.

cells have been demonstrated in peripheral blood and in tumor-infiltrating lymphocytes (11–14). In about 50% of patients with cervical cancer, an antibody response to HPV oncoproteins, which correlates with disease progression, can be detected (15–17). The E6 and E7 proteins may provide a tumor-specific target for immunotherapeutic interventions in patients with cervical cancer.

Immunotherapy using HPV proteins as targets has been shown to control tumor growth in experimental models (7, 18, 19), and virus-like particles have shown prophylactic efficacy in animal papillomavirus infection (20, 21). Several vaccination strategies are being pursued to circumvent the low immunogenicity of the virus including HLA-restricted peptides or antigen-loaded dendritic cells (22, 23). Some promising results have been obtained with recombinant viral vaccines based on vaccinia (24–26). Vaccinia virus has been widely used as immunogen and was well tolerated. It has been accepted as safe when it was used during the smallpox eradication program of the WHO (27). In addition, vaccinia induces a strong immune response by itself that may deliver "danger signals" for the immune system that represent an adjuvant effect. Therefore, based on the Wyeth vaccinia strain, a recombinant virus was constructed carrying functionally modified *E6* and *E7* genes of HPV-16 and -18 (28). During the lytic life cycle of the virus, the inserted HPV genes are expressed, and their proteins are processed and presented by antigen-presenting cells to induce a MHC class I-restricted T-cell response (29).

In women with advanced cervical cancer, a single treatment with this modified vaccinia virus produced HPV-specific CTLs in one of three patients and a HPV-specific antibody response in three of eight patients (30). We have used the same construct, called TA-HPV, in patients with early-stage cervical cancer (FIGO stage I or II). The main objective of the study was an assessment of the effects on the cellular immune response as reflected by changes in circulating HPV-specific CTLs. Antibodies to E7 from HPV-16 or -18 and to the vaccinia virus were also measured. We have been able to verify that the vaccination is feasible in an outpatient setting and is safe and well tolerated. A serological response to vaccinia was observed in 18 of 29 patients. A seroconversion was induced to at least one of the HPV antigens in 8 of 15 patients. Most importantly, a HPV-specific *de novo* CTL response could be demonstrated in 4 of 29 evaluable vaccinees.

PATIENTS AND METHODS

Patients. The study was approved by the EORTC Protocol Review Committee, the United Kingdom Medicines Control Agency, the United Kingdom Gene Therapy Advisory Committee, the Dutch Gene Therapy Commission, the local ethics committees of each participating institution, and the relevant safety and environmental monitoring agencies according to local laws. All investigations involving humans have been performed in accordance with the principles embodied in the Declaration of Helsinki. The study was performed in compliance with the guidelines of Good Clinical Practice as described in the International Conference on Harmonization of Good Clinical Practices guidelines step 4 (1996).

Eligible patients had histologically confirmed untreated clinical stage Ib or IIa squamous cell or adenocarcinoma of the uterine cervix. They had to be age 18 years or older with an Eastern Cooperative Oncology Group performance status of ≤ 2 and a life expectancy of >3 months. White cell and platelet counts, serum creatinine, serum transaminases and bilirubin (≤ 1.5 times the upper normal limit), prothrombin and partial prothrombin (time $\leq$ twice upper normal limit) had to be within the normal range. Immune parameters evaluated were a CD4 count exceeding 400/mm^3 and a reaction to at least two antigens in the Multitest CMI for cell-mediated immunity (Pasteur Merieux, Lyon, France). Patients were excluded if they had eczema, were known to be immunodeficient, had a history or acute hepatitis B or C infection, had HIV infection, had regular contact with children ≤ 5 years, or had a second primary tumor requiring treatment in the preceding 5 years. Written informed consent was obtained from all patients before inclusion in the study.

Vaccine and Treatment. The TA-HPV vaccine is a live recombinant vaccinia virus that has been engineered to express the E6 and E7 proteins of HPV-16 and -18 (30). The vaccine was supplied and vaccine investigation was sponsored by Cantab, now Xenova Research Limited (Cambridge, United Kingdom). It was provided in vials of 50 μl containing 10^8 pfu recombinant virus/ml and stored frozen at −80°C. Production, analysis, storage, and application were done under Good Manufacturing Practice standards, especially regarding the guidelines for genetically modified organisms.

The treatment consisted of two applications of the vaccine, 4–12 weeks apart. The first was given 2 weeks before radical hysterectomy, and the second was given 4–8 weeks after surgery, when disease burden was considered minimal or nil. The vaccinations were given with 20 μl of TA-HPV (2×10^6 pfu) using a scarification technique estimated to deliver approximately 2.5×10^5 pfu (27). The site was allowed to dry in air and then covered with an occlusive dressing that was changed regularly until scab dehiscence. Radiotherapy, when required, was given after the second vaccination when the scab had dried, and, as with surgery, local institution guidelines were followed. Tolerability was monitored using a daily patient diary for 1 month after each vaccination. Patients were evaluated pretreatment and on days 28, 56, 84, and 168 after the first vaccination, and before radiotherapy if performed with history, physical examination, toxicity scoring using Common Toxicity Criteria grades, full blood count, urea, electrolytes and liver function tests, and serum immunoglobulins. Before study entry a chest radiograph, magnetic resonance imaging, or computed tomography scan of abdomen and pelvis and a pregnancy test were done. Absolute numbers of circulating CD4 and CD8 cells were checked before study entry, at days 56 and 168, and before radiotherapy. Patients needed to receive at least one vaccination to be evaluable. Eligible patients were entered in the study via the New Drug Development Office of the EORTC, which was also responsible for data collection and study monitoring.

Statistical Methods. An optimal two-stage design by Simon (31) was used. In the initial phase, 28 evaluable patients were to be enrolled. The definition of an induced immune response was prospectively defined as *de novo* CTL induction. If fewer than 2 vaccinees showed a response, then the trial was to be terminated; if more than 5 vaccinees responded, then

further investigation was appropriate. If 2–4 patients showed a response, then recruitment was to be continued until 44 evaluable patients had been enrolled. This gave a type 1 error of 10% and a type 2 error of 5%.

Cytotoxicity Assays. For analysis of cytotoxic T cells, blood samples (100 ml before first vaccination, 50 ml at days 28, 56, 84, and 168 and before radiotherapy) were collected in monovettes containing citrate and shipped overnight at ambient temperature to Cantab Pharmaceuticals (Cambridge, United Kingdom). Briefly, PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation. An aliquot was removed to establish a B-LCL and monocyte cultures, and the remaining PBMCs were frozen and stored in vapor phase nitrogen until use. Autologous monocytes were cultured in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and granulocyte macrophage colony-stimulating factor (1000 units/ml) for 3 days and then infected with recombinant adenovirus RAd 101 (containing modified versions of HPV-16 *E6* and *E7*) or RAd 102 (containing modified versions of HPV-18 *E6* and *E7*) at a multiplicity of infection of 2000:1 and used as stimulator cells for the CTL cultures (30). For an individual patient, samples of PBMCs from each time point were tested simultaneously. Frozen PBMCs were thawed, suspended in interleukin 7 (20 ng/ml)-containing medium, and added to autologous monocytes infected with RAd 101 or RAd 102 (at least 2 wells each for each time point and virus) at a ratio of 1:2 (stimulator:responder cells). After 7 days, fresh culture medium was added containing interleukin 2 (25 IU/ml) plus fresh autologous adenovirus-infected monocytes treated with mitomycin C (50 μg/ml), and culture was continued for 6–7 days. Alloreactive CTL cultures were set up using PBMCs from each time point cultured with mitomycin C (50 μg/ml)-treated, HLA-mismatched B-LCLs as stimulator cells.

Cytotoxicity of the cultured T cells was determined by ^{51}chromium release assay. A panel of target cells, EBV immortalized B-LCLs, labeled with ^{51}Cr (4.6 MBq/10^6cells) was used: autologous and allogeneic B-LCL; TA-HPV-infected autologous and allogeneic B-LCL; and Wyeth strain vaccinia-infected autologous B-LCL. The virus-infected target cells were infected for 1 h at 37°C with TA-HPV or Wyeth at 2–20 pfu/cell and used after 16–18 h.

The effector cells from each time point were suspended in medium to give the highest possible E:T ratio (typically, 60:1), and serial 3-fold dilutions in triplicate were made. Natural killer cell activity was blocked using unlabeled K562 cells at a 20-fold excess of target cells. After 4 h of culture, the supernatant was counted in a liquid scintillation counter and expressed as cpm. Specific lysis was determined for each well using the following equation: % specific lysis = 100 × (experimental release − spontaneous release) × (maximal release − spontaneous release)$^{-1}$.

Maximum release was determined from target cells lysed with Triton X-100 (1%, v/v). A response was defined as positive when the lysis of TA-HPV targets was ≥10% above the lysis of any of the control targets at two or more of the E:T cell ratios tested.

Serology. The determination of antibodies to HPV and vaccinia virus was performed in pre- and postvaccination sera of the patients. Venous blood (5–10 ml) was taken before the first vaccination; on days 28, 56, 84, and 168; and before radiotherapy and was shipped overnight to Cantab Pharmaceuticals. Serum was separated, aliquoted, and stored at −20°C. Serum from patients in Munich was shipped frozen and then aliquoted and stored.

Anti-vaccinia IgG levels were determined by ELISA using Wyeth strain vaccinia-infected CV-1 cell lysates as antigen as described previously (30). Mock-infected cells were used as control antigen, and results were expressed compared with a known high-titer anti-vaccinia IgG included in every assay. Anti-HPV-16 E7 and anti-HPV-18 E7 antibodies were detected by ELISA using, as antigen, E7 expressed as MBP fusions. The assays had been validated previously on a population of women with CIN3 and cervical cancer (data not shown). Briefly, the HPV-16 *E7* and HPV-18 *E7* open reading frames were cloned in vector pMal-c2 (New England Biolabs) and expressed in *Escherichia coli* strain BLR. E7-MBP fusions were purified according to the manufacturer's instructions. As a control antigen, unfused MBP was also purified. For the ELISA, each serum was tested against equal amounts (300 ng) of E7-MBP and unfused MBP coated onto adjacent wells. E7-specific reactivity was determined as the difference in absorbances between E7-MBP and unfused MBP wells. Antibodies to HPV-16 E6 and HPV-18 E6 were not performed because specific assays were not available.

HLA Genotyping of Tumor Tissue. HLA genotyping for HLA-A, -B, -C, -DR, and -DQ was performed by PCR-single-strand conformational polymorphism method at the Oxford Transplant Centre on DNA extracted from patient PBMCs.

Cervical Biopsies. Tumor biopsies were taken after definitive surgery. Pieces of 3 mm^3 were coated with Tissue-Tek OCT (Miles Inc.) and snap-frozen in liquid nitrogen for storage in cryovials at −80°C or in liquid nitrogen. Trial biopsies from Germany were dispatched to the Paterson Institute on dry ice. No frozen biopsies were obtained from the Cardiff trial subjects. Where necessary and available, paraffin-embedded tumor material was used. Cervical biopsies were sectioned for three purposes: (*a*) histological examination; (*b*) HPV testing by PCR; and (*c*) HLA phenotyping by IHC.

HPV Typing. DNA was extracted from the tumor biopsy sections, using mouse liver sections between specimens to monitor possible contamination, as described previously (32). All biopsies were shown to have amplifiable DNA as assessed using primers for the "housekeeping" gene, glyceraldehyde-3-phosphate dehydrogenase. HPV DNA was detected using the general primer GP5+/GP6+ PCR system. As determined on purified DNA, the sensitivity of this assay was at the femtogram level for those HPV genotypes that match strongly with the primers (*e.g.*, HPV-16) and at the picogram level for HPV types (*e.g.*, HPV-39) having four or more mismatches with one or both primers (33). Specific HPV genotyping used type-specific primers for HPV-6/11, -16, -18, -31, and -33 (34–36). Amplification products were resolved on 2% agarose and visualized by ethidium bromide staining. Sequencing of the PCR product identified HPV types 58 and 39.

IHC. The expression of HLA class I was assessed by IHC of cryostat sections of 14 clinical trial biopsies. The mAbs used detected HLA monomorphic (W6/32), β_2-microglobulin (BM63; DAKO), HLA-A loci (A131; from J. Kornbluth, Uni-

Table 1 Patient and tumor characteristics

Patient	Age (yrs)	Site	Postsurgery stage	Postsurgery histology	Day radiotherapy started	Section type pathology HPV/HLA[a]		HPV typing[b]
1	66	Munich	IIb	Squamous	80	F	Squamous	HPV-16
2	40	Munich	Ib	Squamous		F	Not interpretable	−
3	67	Freiburg	Ib	Squamous		NR		
4	58	Munich	IIb	Squamous	79	NR		
5	36	Munich	IIa	Squamous		W	Squamous	−
6	33	Munich	Ib	Squamous		W	No lesion	−
7	44	Munich	IIb	Mucinous-AC	72	F	AC	+
8	49	Munich	IIb	Adenocarcinoma	−14, 82	W	Squamous	−
9	55	Freiburg	Ib	Adenocarcinoma		NR		
10	48	Cardiff	Ib	Squamous		W	Squamous	HPV-18
11	47	Munich	IIb	Squamous	69	W	Squamous	HPV-16
12	39	Bonn	Ib	Squamous	88	F	CIN	−
13	37	Munich	Ib	Squamous		W	Squamous	−
14[c]	47	Jena	IIa	Squamous	31	F	Squamous	HPV-16
15	49	Jena	Ib	Squamous		F	CIN	HPV-58
16	34	Cardiff	Ib	Squamous		W	Squamous	−
17[d]	47	Cardiff	Ib	Squamous		W	Squamous	−
18	34	Manchester	Ib	Squamous		F	Squamous	HPV-16/39
19	65	Jena	Ib	Squamous		F	CIN	−
20	43	Jena	Ib	Squamous		F	Squamous	HPV-18
21	47	Jena	Ib	Squamous		NR		
22	47	Jena	Ia	Adenocarcinoma		F	Warty	−
23[c]	41	Jena	IIb	Squamous		F	Squamous	HPV-16
24	42	Manchester	Ib	Squamous	112	F	Squamous	HPV-16
25	41	Cardiff	Ib	Squamous		W	No lesion	+/−
26	44	Jena	Ib	Adenocarcinoma		F	No lesion	−
27	32	Manchester	Ib	Squamous		F	No lesion	−
28	36	Cardiff	CINIII	CINIII		W	CINIII	HPV-16
29	47	Munich	Uterine Ca	Mucinous Adenocarcinoma	90	NR		

[a] Sections used for analysis of the HPV (and HLA) expression were different from those used to assign pathology after surgery (F, frozen sections; W, wax-embedded; NR, not received).
[b] HPV typing positive (+) but not identified, negative (−), or uncertain (+/−).
[c] Patient received only one vaccination due to radiotherapy (14) or progressive disease (23).
[d] The first vaccination failed to take, but the second vaccination resulted in a strong local response.

versity of Arkansas), and BW4 and BW6 epitopes (116.5.28 and 126.39, respectively, from K. Gelsthorpe, Sheffield, Blood Transfusion Center, Sheffield, United Kingdom). The epithelial cells were identified using mAb LP34 staining and HLA expression determined by comparison to the stroma by the criteria of Keating *et al.* (37). In five wax biopsies, mAb HC10 detecting a HLA-B/C epitope was used to investigate HLA-B/C locus expression as described by Cromme *et al.* (38).

RESULTS

Demographics and Clinical Characteristics of the Patients. Twenty-nine patients with presurgical clinical stage FIGO Ib or IIa disease from six European centers entered the trial (Table 1). Patients had a median age of 44 years (age range, 32–67 years). After surgery, the tumor histology confirmed that 22 patients had squamous cell carcinoma, 5 patients had adenocarcinoma, 1 patient had adenocarcinoma of the endometrium, and 1 patient had CIN III with no evidence of invasive disease. Revision of staging or histology was required after definitive surgery in nine patients: six patients were upstaged to pT IIb; one patient was downstaged to pT Ia; one patient had CIN III; and one patient had endometrial rather than cervical adenocarcinoma. This resulted in a retrospective cohort of 1x pT Ia, 18x pT Ib, 2x pT IIa, and 6x pT IIb. Thus, 20 patients had histopathologically confirmed stage pT Ib or pT IIa cervical cancer.

Two vaccinations were given to 27 patients. Patient 23 developed rapid tumor progression; therefore, no further blood drawing was clinically acceptable after day 56. Patient 14 had radiotherapy after surgery for FIGO stage IIa disease. The first vaccination did not "take" in patient 17. It was not repeated because this would have delayed surgery; however, the second vaccination was successful. Patient 8 had a single brachytherapy (800 cGy) to the cervix 1 week before the first vaccination. All 29 patients were deemed evaluable for immunological response because they had all received at least one vaccination. The protocol provided for replacement of ineligible patients and for recruitment of up to 44 available patients, depending on response rates. However, this was not done because of an unexpected shortage of vaccine.

Table 1 also summarizes the HPV analysis of patient biopsies. The histology of the sections preceding and following those used for the PCR was examined to check for evidence of tumor. Fourteen cases were analyzed from frozen biopsies, of which eight were HPV positive and six were HPV negative, but the latter had no obvious tumor in the flanking sections. In 10 cases, HPV analysis was from wax sections that yielded 3 positives and 7 negatives, of which 2 had no apparent tumor.

Overall, the HPV typing of the patient material yielded 11 of 24 as HPV positive (1 not assigned; 7 with HPV-16 and 1 of these was also positive with HPV-39; 2 HPV-18; and 1 HPV-58). Lack of HPV detection certainly coincided with absence of any tumor in the section area being analyzed in some cases (Table 1). The HPV negatives derive from specimens with no lesion (3), uninterpretable pathology (1), wart or CIN but no carcinoma pathology (3), and, finally, five specimens where there was evidence of squamous carcinoma. The latter were all HPV typed from the wax-embedded specimens, which might cause reduced sensitivity.

Safety. Inoculation with vaccinia typically causes local erythema and swelling followed by ulceration with scab formation. Ulceration started a median of 6 days (range, 3–19 days) after the first TA-HPV vaccination and had resolved by 17 days (range, 6–35 days), reaching grade 2 toxicity in 10 patients. The reaction to the second vaccination was milder (grade 1 in 13 patients). Erythema and swelling were seen after the first vaccination in 28 and 24 patients, respectively (maximum grade 3: 1 patient each) and were again milder and less frequent after the second dose. Itching at the vaccination site was noted by eight patients, especially after the second vaccination. The systemic effects attributable to TA-HPV were minimal; the most common were malaise (grade 2, 6 patients), myalgia (≤grade 2, 2 patients), and headache (≤grade 2, 4 patients). There were no serious adverse events attributable to TA-HPV.

Serological Responses to Vaccination with TA-HPV. Serum samples for serological testing were complete for 20 patients. Postvaccination samples were incomplete from 7 patients (one sample from patients 1 and 12; two samples from patients 14, 15, 19, and 20; and all samples after day 28 from patient 23). No prevaccination day 0 sample was received from two patients (patients 6 and 11).

All samples were assayed for anti-vaccinia antibodies to verify a successful "take" of the immunization. The presence and induction of anti-vaccinia antibodies were determined by ELISA using whole cell lysates of vaccinia-infected CV-1 cells. Nine patients had previously been immunized with vaccinia as part of the smallpox eradication program (as determined by clinical history), 13 had not, and in 7 patients, the status was unknown. Measurable anti-vaccinia IgG was present before vaccination only in patient 8, who was not known to have been previously immunized, which, given the patient's age, indicated an inaccurate history of vaccination. Eighteen patients developed anti-vaccinia antibodies after TA-HPV vaccination, with 13 producing a response by day 28. In 10 patients, the maximum response was at day 28, although only 4 patients had previously been immunized. Only one patient responded to the booster vaccination with an increase in antibody titer after the second vaccination, but she had not had previous exposure to vaccinia. Thus, at least 62% of the patients showed serological evidence of effective vaccination. However, all of the patients gave a clinical "take" to the vaccination as indicated by a classical injection site reaction to vaccinia. The reason for discrepancy between the number serological responses and the clinical take rate is not clear and has not been observed in other studies with this vaccine (Ref. 30; data not shown).

The analysis of specific antibody responses to HPV E7 proteins was performed by ELISA using maltose-binding fusion proteins as antigens. Overall antibodies to HPV-16 E7 were detected in 11 of 29 patients, and overall antibodies to HPV-18 E7 were detected in 15 of 29 patients. Of the 27 patients from whom samples were available before vaccination, 2 and 6 patients had pre-existent antibodies to either HPV-16 E7 or HPV-18 E7, respectively, and 4 patients (patients 4, 15, 20, and 23) had antibodies to both proteins. *De novo* antibodies to HPV-16 E7 or HPV-18 E7 developed in four patients each (Table 2). Antibodies to both HPV-16 and HPV-18 E7 were induced in patient 17 only. Vaccination with TA-HPV was able to induce *de novo* seroconversion in half the patients, excluding those with pre-existing anti-E7 antibodies. In nine patients, no HPV E7-specific antibodies could be demonstrated at any time point.

Analysis of CTLs *versus* TA-HPV. Blood samples for CTL analysis were received from all 29 patients and were complete in 23 patients. Samples were missing from patients 12 and 14 (day 28), patient 15 (days 28 and 56), patients 19 and 20 (day 84), and patient 23 (day 56 onward). All but 10 of the samples reached Cambridge within 24 h, and all were tested except for day 28 from patient 12, which took 5 days in transit.

As a positive control, parallel cultures were set up from each patient stimulated with allogeneic B-cell lines to determine whether the cells were sufficiently viable to mount an allogeneic response (indicated as "allogeneic" in Table 3). Only three patients (patients 6, 17, and 29) did not show an allogeneic response at any individual time point. Before vaccination, only one patient (patient 11) had detectable HPV-specific CTL activity. However, four patients (patients 11, 12, 14, and 18) developed a specific CTL response as a result of vaccination (Table 3). Patient 11 developed a HPV-18-specific CTL response, whereas the prevaccination response was *versus* HPV-16. Representative examples of the responses in patients 14 and 18 are shown in Fig. 1. Although serial samples/patient were tested, the CTLs were found at only one time point, respectively: on day 28 in patient 11 to HPV-18 E6 and E7 proteins and on day 56 in patients 12, 14, and 18 to HPV-16 E6 and E7 proteins. However, because some blood samples were missing in patients 12 and 14, we cannot rule out the presence of HPV-specific CTLs at the earlier time point. The tumors of patients 11, 14, and 18 were HPV-16 positive by PCR. The biopsy of patient 12 used for HPV analysis contained only CIN, and no HPV was detected. Therefore, two of the patients who developed anti-HPV-16 CTLs had HPV-16 associated with the tumor biopsy.

HLA Expression in Tumor Biopsy. In 11 patients, the presence of tumor cells in the biopsies taken after surgery could be confirmed by histology and HPV typing. Because expression of HLA alleles is critical for any therapeutic effect of CTLs induced by vaccination, the presence of HLA-A and -B was investigated by IHC (Table 4). In 5 of 10 evaluable patients, there was evidence of loss of expression of one or more HLA-A and/or -B alleles. Thus, one patient tumor showed total HLA-B locus loss, two patients showed HLA-B allele loss, one patient showed HLA-A locus loss and HLA-B allele loss, and one patient showed heterogeneous HLA-B locus loss. These results suggest that at least some patient tumors may be nonsusceptible, through immune evasion, to vaccine-generated CTLs. At least

Table 2 IgG responses to vaccinia, HPV-16 E7 and HPV-18 E7

Patient	Sample	Mean ELISA value [a]		
		Vaccinia	HPV16 E7	HPV18 E7
1	Day 0	0.09	0.00	0.00
	Post vaccination	0.37	0.04	0.21
2	Day 0	0.04	0.00	0.45
	Post vaccination	0.24	0.00	0.53
3	Day 0	0.02	0.00	0.00
	Post vaccination	0.20	0.05	0.00
4	Day 0	0.03	0.23	0.27
	Post vaccination	0.05	0.21	0.29
5	Day 0	0.09	0.00	0.73
	Post vaccination	0.07	0.00	0.65
6	Day 0	ND	ND	ND
	Post vaccination	0.07	0.00	0.33
7	Day 0	0.02	0.00	0.18
	Post vaccination	0.05	0.27	0.21
8	Day 0	0.26	0.00	0.00
	Post vaccination	0.68	0.00	0.00
9	Day 0	0.02	0.00	0.16
	Post vaccination	0.14	0.00	0.16
10	Day 0	0.02	0.06	0.00
	Post vaccination	0.04	0.10	0.00
11	Day 0	ND	ND	ND
	Post vaccination	0.05	0.00	0.00
12	Day 0	0.01	0.00	0.00
	Post vaccination	0.05	0.00	0.00
13	Day 0	0.05	0.00	0.06
	Post vaccination	0.12	0.00	0.08
14	Day 0	0.05	1.01	0.05
	Post vaccination	0.10	0.90	0.08
15	Day 0	0.02	0.11	0.10
	Post vaccination	0.58	0.16	0.08
16	Day 0	0.00	0.00	0.00
	Post vaccination	0.04	0.00	0.10[b]
17	Day 0	0.01	0.00	0.00
	Post vaccination	2.09	0.36	0.48
18	Day 0	0.02	0.00	0.00
	Post vaccination	0.26	0.00	0.00
19	Day 0	0.04	0.00	0.00
	Post vaccination	0.19	0.00	0.00
20	Day 0	0.02	0.16	0.22
	Post vaccination	0.05	0.08	0.15
21	Day 0	0.01	0.08	0.52
	Post vaccination	1.22	0.09	0.30
22	Day 0	0.01	0.00	0.00
	Post vaccination	0.16	0.06	0.00
23	Day 0	0.04	0.13	0.16
	Post vaccination	0.89	0.04	0.08
24	Day 0	0.03	0.00	0.00
	Post vaccination	0.01	0.00	0.16
25	Day 0	0.01	0.00	0.00
	Post vaccination	0.04	0.15	0.08
26	Day 0	0.08	0.00	0.00
	Post vaccination	0.14	0.00	0.00
27	Day 0	0.02	0.00	0.11
	Post vaccination	0.10	0.00	0.10
28	Day 0	0.02	0.00	0.05
	Post vaccination	0.26	0.14	0.00
29	Day 0	0.04	0.10	0.08
	Post vaccination	0.17	0.20	0.29

[a] Highest pre- and postvaccination responses to vaccinia and HPV-16 or HPV-18 E7 antigens expressed as mean ELISA value, compared with a positive control serum. A positive signal is defined as a mean ELISA value greater or equal 0.1. A positive response (indicated by shaded boxes) is a signal 2× the prevaccination response.

[b] Patient 16 is not defined as positive because actual ELISA value = 0.099. (ND, not done).

Table 3 Maximum specific lysis of TA-HPV-infected autologous target cells

Patient	Stimulation[a]	Day 0[b]	Day 28	Day 56	Day 84
11	RAd 101	**13.5**[c]	5.8	4	4.9
	RAd 102	6.6	**10.4**	7.1	4.7
	Allogeneic	15.5	10.5	46.2	19.6
12	RAd 101	−2.7	ND	**27.1**	0.8
	RAd 102	−3.1	ND	−10.8	−4.1
	Allogeneic	43.9	ND	45.2	31.7
14	RAd 101	7.3	ND	**22.1**	10.9
	RAd 102	2.8	ND	1.1	0.3
	Allogeneic	10.8	ND	11.5	8.1
18	RAd 101	0.9	4.6	**16.3**	4.4
	RAd 102	3.1	2.6	1.2	2.3
	Allogeneic	4.9	64.7	52.3	34.9

[a] Restimulation with autologous monocytes infected with recombinant adenovirus containing HPV-16 *E6* and *E7* (RAd 101) or HPV-18 *E6* and *E7* (RAd 102) or with allogeneic B-cell lines.

[b] Day after first vaccination when blood sample was taken.

[c] Positive result (shown in bold) with specific lysis ≥ 10% above lysis of control targets at ≥2 E:T ratios. (ND, not done).

one patient with vaccine-generated CTLs showed HLA loss in the tumor.

DISCUSSION

The spectrum of HPV-associated clinical findings in women ranges from genital warts to squamous intraepithelial neoplasia and invasive carcinoma (39). HPV-16 and -18 are associated with intraepithelial and invasive lesions. HPV-16 is the most common type found in squamous carcinoma (40), and HPV-18 is most common in adenocarcinomas and small cell neuroendocrine cervical carcinomas (41, 42). The association of virus infection with risk of development of cervical neoplasia has provided strong justification for devising immunization strategies against high-risk HPV types for therapy and prevention of progression and recurrence of invasive cervical cancer. We have performed one of the first multinational, multicenter studies of a genetically modified vaccinia-based vaccine. The study confirms that outpatient vaccination with TA-HPV is feasible, safe, and associated with little toxicity. Our results show that vaccination with TA-HPV is able to induce a measurable immune response in patients with active cervical cancer. A single vaccination was sufficient to produce an immunological effect in 12 of 29 patients (4 patients generated *de novo* CTLs, and 8 patients generated *de novo* anti-HPV antibody responses).

The patient group was chosen for good performance criteria in particular in relation to the immune response and prognosis. All patients recruited to the study were tested for their immunocompetence. They passed a delayed type of hypersensitivity test (Pasteur Merieux) with ≥2 recall antigens positive and had absolute CD4 counts above 400/mm^3. As a positive control in the chromium release assay we investigated allogen-specific T-cell activity as a surrogate marker for lytic CTL function. The presence of allogen-specific CTLs indicates the ability of autologous lymphocytes to recognize and kill cells bearing foreign antigen. The absence of allogen-specific CTLs and the cause of such anergy in patient 6, 17, and 29 are unclear because all patients reacted in the delayed type of hypersensitivity test and had sufficient numbers of CD4 and CD8 cells but may be due to T-cell immunosuppression via down-modulation of the CD3 ζ chain of the T-cell receptor complex that has been described in cervical cancer patients (43).

The good clinical prognosis in these patients requires follow-up for 5 years to assess any clinical efficacy. Ultimately, only randomized studies looking at the effects on progression-free and overall survival can determine whether the immunological responses have clinical significance.

The postvaccination CTL response was restricted to a single HPV type in each patient. The CTLs were only transiently present in the circulation and were not detectable 4 weeks earlier or later, although in some cases, not all samples necessary to confirm this were available for analysis. Such transience, providing only a narrow window of detection, means that a CTL response could easily be missed. The difficulties are further compounded by the individual variation in the timing of CTL appearance in the blood after TA-HPV vaccination. In a previous trial vaccinating patients with recurrent cervical cancer with TA-HPV (30), a HPV-specific CTL response was induced in one of three evaluable patients. In this case, CTLs were detected to both HPV-16 and -18, but again the response induced was transient. Additional studies in larger groups of patients will be needed to determine whether this is a consistent pattern of responsiveness to the vaccine.

Immunization with TA-HPV was successful in all patients independently of previous vaccinia immunizations as documented by the local reactions induced. Only 1 of 55 vaccinations did not lead to overt local vaccinia infection. However, none of the four patients mounting a CTL response to the HPV proteins had previously been immunized with vaccinia. It is postulated that the induction of an active immune response to vaccinia is beneficial also for the immunization to accompanying antigens (44–46). Therefore, vaccinia is currently being used as a vector in combination with other vaccination strategies to enhance immune responses (44, 47). Our vaccination strategy relied solely on TA-HPV; therefore, it is possible that a strong and early memory response to vaccinia may override a specific response induction to HPV antigens.

We used B-cell lines as antigen-presenting targets in the cytotoxicity assay. Therefore, CD8+ or cytolytic CD4+ CTLs may have been measured, the latter being less active against MHC class II-negative cervical cancer cells. Moreover, effective T-cell activity against tumor cells is dependent on the expression of suitable HLA molecules by the target cells. In cervical cancer, a selective loss of HLA expression can occur in up to 90% of tumors, and multiple mechanisms have been identified (48, 49). Some of the tumors of the cohort of patients recruited in our study were available for studying HLA-A and -B expression. The immunohistochemical approach can detect HLA-A or -B locus and some B-allele loss of expression, and the results should be considered a minimum loss estimate (37). Overall, tumor specimens of 10 evaluable patients were investigated, and 5 showed some evidence of down-regulated HLA class I expression, with alterations in six different alleles, which might compromise any CTL activity.

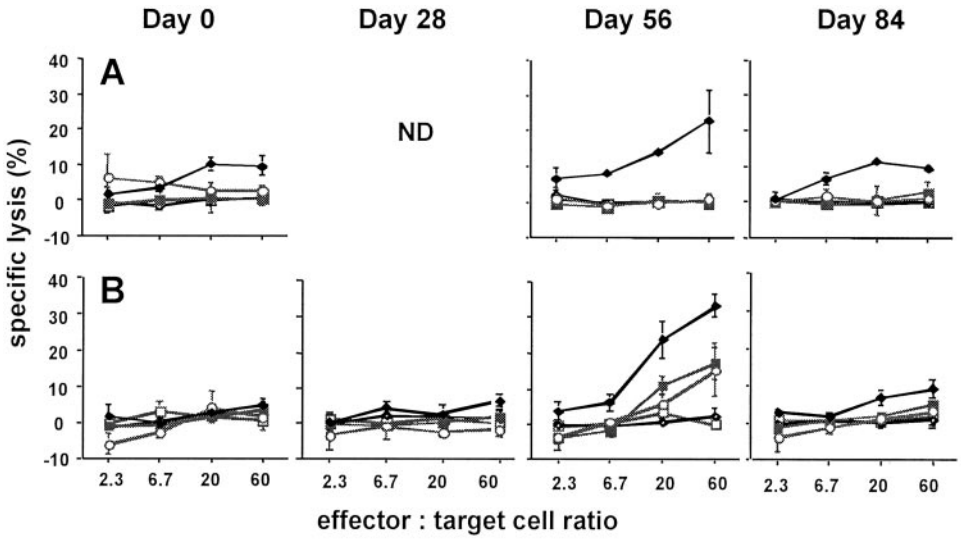

Fig. 1 Representative CTL analysis of peripheral blood lymphocytes (*A*, patient 14, *B*, patient 18) drawn at the indicated time points and restimulated twice *in vitro*. ^{51}Chromium release assay was performed with labeled target cells (◆, autologous B-LCL infected with TA-HPV; ○, autologous B-LCL infected with Wyeth; ■, autologous B-LCL, uninfected; ◇, allogeneic B-LCL, TA-HPV infected; □, allogeneic B-LCL, uninfected). *ND*, not done.

Table 4 HLA phenotyping on confirmed HPV-positive tumor tissue

Patient no.	HPV typing[a]	HLA genotyping of tumor cells[b]		CTL(+) (Virus)[c]
		HLA-A	HLA-B	
1	16	++	++	
7	+	++	−	
10	18	ND	−/+	
11	16	ND	ND	CTL (HPV-18)
14	16	++	Bw6-ve	CTL (HPV-16)
15	58	−	Bw4-ve	
18	16/39	++	++	CTL (HPV-16)
20	18	++	++	
23	16	++	Bw4-ve	
24	16	++	++	
28	16	ND	++	

[a] HPV typing from tumour sections by PCR (+, positive for HPV but no type specified).

[b] HLA expression was investigated by immunohistochemistry on frozen and wax-embedded material (−, complete loss of HLA-A or HLA-B locus expression if no alleles indicated).

[c] *De novo* induction of HPV-specific CTL (ND, not done).

The HLA genotyping of the patients showed evidence of increased HLA-B7 (52%) and HLA-DR15 (55%) antigen frequency with other common alleles not obviously over- or underrepresented (data not shown). Both HLA-B7 and DR15 have previously been found to be overrepresented in patients with HPV-associated disease in some studies (50). However, the patients have been recruited from several different centers, and there is no suitable control population for strict comparison, and the group is small. All four patients who developed a CTL response carried HLA-A1, and three also carried HLA-A3 (patients 11, 12, and 18), whereas patient 14 carried A1, A24. Patient 23 also carried the A1, A3 alleles but received only one vaccination, and no specific CTL activity was detected on days 0 and 28. In the study by Borysiewicz *et al.* (30), the patients with long-term disease-free survival after a single TA-HPV vaccination had HLA types A1 and A24. Thus far, 37 patients with cervical cancer have been given TA-HPV, and CTL responses have been seen only in those with these HLA types. More patients need to be treated to see whether these early observations are significant. If so, then further exploration of TA-HPV in patients with HLA A1, A3, or A1, A24 and cervical cancer or high risk premalignant disease may be fruitful.

Patients developed either a CTL response or a serological response to TA-HPV. There was no patient that developed both specific CTLs and antibodies. One interpretation may be that the vaccination induced an immune response biased toward a T helper 1 or a T helper 2 type. Which response will be more pronounced might depend on genetic (HLA) and epigenetic factors (lymphokine pattern) of the individual patient. Because a humoral response to HPV E6 and E7 proteins is not likely to affect a therapeutic outcome, strategies to enhance the T helper 1 arm of the response may support the generation of the more favorable CTLs (51).

However, specific antibodies to HPV E6 and/or E7 proteins have been shown to correlate with progression to invasive cancer and represent useful specific tumor markers for cervical cancer (16, 52). The peak serological response to HPV-16 and -18 E7 protein occurred after the first vaccination in five of the nine patients (data not shown). The HPV-specific antibody response was obviously not hindered by a primary or secondary antibody response to vaccinia. Patients 6 and 29 had not previously been vaccinated with vaccinia. Patient 6 did not develop anti-vaccinia antibodies, although antibodies to HPV were found. Patient 17 had previously been vaccinated, and she, like patient 29, showed an increase in antibodies to vaccinia and HPV.

This is the first multicenter study run by the EORTC Biological Therapeutics Development Group using a genetically modified organism. We encountered unexpected difficulties in obtaining approval at national levels, so the study was done in two rather than the planned six countries. There is no agreement among the different national regulatory agencies in Europe about what constitutes gene therapy, the level of monitoring of patients, the environment required, or the documentation re-

quired when submitting a clinical protocol for approval. Despite these obstacles, the trial was successfully finished.

In conclusion, this study shows that a single vaccination with TA-HPV can induce immunity to HPV proteins in patients with active malignancy. Both a humoral and cell-mediated response could be demonstrated. However, the down-regulation of MHC class I molecules in the tumor in many cases of cervical cancer (48, 49) could mitigate against an antitumor effect of the CTLs *in vivo*. To date, 26 patients are on study for 5 years of follow-up. This number is much too small for any prospective evaluation on tumor recurrence or metastasis. In the patients in whom HPV-specific CTLs were detected after vaccination with TA-HPV, the responses appeared to be transient. This could be due to homing of lymphocytes out of the peripheral blood or may indicate that the immunogenicity of the vaccine needs to be enhanced. Preclinical studies have indicated that a priming vaccination with a fusion protein consisting of E7E6L2 of HPV-16 (designated TA-CIN) followed by a booster vaccination with TA-HPV results in a greatly enhanced CD8+ T-cell response against HPV-16 E7 (53). Studies to evaluate TA-HPV immunization regimen in patients with high-grade vulval intraepithelial neoplasia are currently in progress.

ACKNOWLEDGMENTS

We acknowledge the support and expertise of the Cardiff HPV Group, particularly Prof. L. K. Borysiewicz, Dr. S. Man, Dr. A. Fiander, and A. Evans. We thank R. Fallon for assistance with the chromium release assays.

REFERENCES

1. Pisani, P., Parkin, D. M., and Ferlay, J. Estimates of the world wide mortality from major cancers in 1985. Implications for prevention and projection of future burden. Int. J. Cancer, *55:* 891–903, 1995.

2. Walboomers, J. M., Jacobs, M. V., Manos, M. M., Bosch, F. X., Kummer, J. A., Shah, K. V., Snijders, P. J., Peto, J., Meijer, C. J., and Munoz, N. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J. Pathol., *189:* 12–19, 1999.

3. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Human Papillomaviruses, Vol. 64. Lyon, France: IARC, 1995.

4. Munger, K., Phelps, W. C., Bubb, V., Howley, P. M., and Schlegel, R. The *E6* and *E7* genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J. Virol., *63:* 4417–4424, 1989.

5. Hudson, J. B., Bedell, M. A., McCance, D. J., and Laiminis, L. A. Immortalization and altered differentiation of human keratinocytes *in vitro* by the E6 and E7 open reading frames of human papillomavirus type 18. J. Virol., *64:* 519–526, 1990.

6. Crook, T., Morganstern, J. P., and Crawford, L. Continued expression of HPV 16 E7 protein is required for maintenance of the transformed phenotype of cells co-transformed by HPV 16 plus EJ-ras. EMBO J., *8 :* 513–520, 1989.

7. Chen, L. P., Thomas, E. K., Hu, S. L., Hellstrom, I., and Hellstrom, K. E. Human papillomavirus type 16 nucleoprotein E7 is a tumor rejection antigen. Proc. Natl. Acad. Sci. USA, *88:* 110–114, 1991.

8. Chen, L. P., Ashe, S., Brady, W. A., Hellstrom, I., and Hellstrom, K. E. Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA4. Cell, *71:* 1093–1102, 1992.

9. Kast, W. M., Barndt, R. M. P., Sidney, J., Drifijout, J. W., Kubo, R. T., Grey, H. M., Melief, C. J. M., and Sette, A. Role of HLA-A motifs in identification of potential epitopes in human papilloma virus type 16 E6 and E7 proteins. J. Immunol., *152:* 3904–3912, 1994.

10. Kaufmann, A. M., Gissmann, L., Schreckenberger, C., and Qiao, L. Cervical cancer cells transfected with the CD80 gene elicit a primary cytotoxic T lymphocyte response specific for HPV 16 E7 antigens. Cancer Gene Ther., *4:* 377–382, 1997.

11. Ressing, M. E., van Driel, W. J., Celis, E., Sette, A., Brandt, M. P., Hartman, M., Anholts, J. D., Schreuder, G. M., ter Harmsel, W. B., Fleuren, G. J., Trimbos, B. J., Kast, W. M., and Melief, C. J. Occasional memory cytotoxic T-cell responses of patients with human papillomavirus type-16 cervical lesions against a human leucocyte antigen A*0201-restricted E7 encoded epitope. Cancer Res., *56:* 582–588, 1996.

12. Evans, E. M., Man, S., Evans, A. S., and Borysiewicz, L. K. Infiltration of cervical cancer tissue with human papillomavirus-specific cytotoxic T-lymphocytes. Cancer Res., *57:* 2943–2950, 1997.

13. Shepherd, P., and Luxton, J. T-cell responses to human papillomavirus in cervical dysplasia. Papillomavirus Report, *10:* 53–59, 1999.

14. Nimako, M., Fiander, A., Wilkinson, G. W. G., Borysiewicz, L. K., and Man, S. Human papillomavirus-specific cytotoxic T lymphocytes in patients with cervical intraepithelial neoplasia grade III. Cancer Res., *57:* 4855–4861, 1997.

15. Ghosh, A., Smith, N. K., and Stacey, S. N. Serological responses to HPV16 in cervical dysplasia and neoplasia. Correlation of antibodies to E6 with cervical cancer. Int. J. Cancer, *53:* 591–596, 1993.

16. Meschede, W., Zumbach, K., Braspenning, J., Scheffner, M., Benitez-Bribiesca, L., Luande, J., Gissmann, L., and Pawlita, M. Antibodies against early proteins of human papillomaviruses as diagnostic markers for invasive cervical cancer. J. Clin. Microbiol., *36:* 475–480, 1998.

17. Chee, Y. H., Namkoong, S. E., Kim, D. H., Kim, S. J., and Park, J. S. Immunologic diagnosis and monitoring of cervical cancers using *in vitro* translated HPV proteins. Gynecol. Oncol., *57:* 226–231, 1995.

18. Feltkamp, M. C., Smits, H. L., Vierboom, M. P., Minnaar, R. P., de Jongh, B. M., Drijfhout, J. W., ter Schegget, J., Melief, C. J., and Kast, W. M. Vaccination with cytolytic T lymphocyte epitope containing peptide protects against a tumor induced by human papillomavirus type 16 transformed cells. Eur. J. Immunol., *23:* 2242–2249, 1993.

19. Schäfer, K., Müller, M., Faath, S., Henn, A., Osen, W., Zentgraf, H., Benner, A., Gissmann, L., and Jochmus, I. Immune response to human papillomavirus 16 L1E7 chimeric virus-like particles: induction of cytotoxic T cells and specific tumor protection. Int. J. Cancer, *81:* 881–888, 1999.

20. Breitburd, F., Kirnbauer, R., Hubbert, N. L., Nonnenmacher, B., Trin-Dinh-Desmarquet, C., Orth, G., Schiller, J. T., and Lowy, D. R. Immunization with viruslike particles from cottontail rabbit papillomavirus (CRPV) can protect against experimental CRPV infection. J. Virol., *69:* 3959–3963, 1995.

21. Kirnbauer, R., Chandrachud, L. M., O'Neil, B. W., Wagner, E. R., Grindlay, G. J., Armstrong, A., McGarvie, G. M., Schiller, J. T., Lowy, D. R., and Campo, M. S. Virus-like particles of bovine papillomavirus type 4 in prophylactic and therapeutic immunization. Virology, *219:* 37–44, 1996.

22. Frazer, I. H. Immunology of papillomavirus infection. Curr. Opin. Immunol., *8:* 484–491, 1996.

23. Adams, M., Borysiewicz, L. K., Fiander, A., Man, S., Jasani, B., Navabi, H., Lipetz, C., Evans, A. S., and Mason, M. Clinical studies of human papilloma vaccines in pre-invasive and invasive cancer. Vaccine, *19:* 2549–2556, 2001.

24. Meneguzzi, G., Cerni, C., Kieny, M. P., and Lathe, R. Immunisation against papilloma virus type 16 tumor cells with recombinant vaccinia viruses expressing the E6 and E7. Virology, *181:* 62–69, 1991.

25. Campo, M. S., Grindley, G. J., O'Nell, B. W., Chandrachud, L. M., McGarvie, G. M., and Jarret, W. F. Prophylactic and therapeutic vaccination against mucosal papilloma virus. J. Gen. Virol., *74:* 945–953, 1993.

26. Lin, L. Y., Guarnieri, F. G., Staveley, O., Carroll, K. F., Levitsky, H. I., August, J. T., Pardoll, D. M., and Wu, T. C. Treatment of established tumors with a novel vaccine enhances major histocompatibility class 2 presentation antigen. Cancer Res., *56:* 21–26, 1996.

27. Fenner, F., Henderson, D. A., Arita, L., Jezek, Z., and Ladnyi, L. D. Smallpox and its eradication. Geneva: WHO, 1988.

28. Boursnell, M. E. G., Rutherford, E., Hickling, J. K., Rollinson, E. A. M., Rolley, N., Mclean, C. S., Borysiewicz, L. K., Vousden, K., and Inglis, S. C. Construction and characterisation of recombinant vaccinia virus expressing human papillomavirus proteins for immunotherapy of cervical cancer. Vaccine, *14:* 1485–1494, 1996.

29. Yewdell, J., Bennink, J., Smith, G., and Moss, B. Use of recombinant vaccinia viruses to examine cytotoxic T lymphocyte recognition of individual viral proteins. Adv. Exp. Med. Biol., *239:* 151–161, 1988.

30. Borysiewicz, L. K., Fiander, A., Nimako, M., Man, S., Westmoreland, D., Evans, A. S., Adams, M., Stacey, S. N., Boursnell, M. E. G., Rutherford, E., Hickling, J. K., and Inglis, S. C. A recombinant vaccinia virus encoding human papilloma virus types 16 and 18 E6 and E7 proteins as immunotherapy for cervical cancer. Lancet, *347:* 1523–1527, 1996.

31. Simon, R. Optimal two-stage design for Phase II clinical trials. Control. Clin. Trials, *10:* 1–10, 1989.

32. Abdel-Hady, E. S., Martin-Hirsch, P., Duggan-Keen, M., Stern, P. L., Moore, J. V., Corbitt, G., Kitchener, H. C., and Hampson, I. N. Immunological and viral factors associated with the response of vulval intraepithelial neoplasia to photodynamic therapy. Cancer Res., *61:* 192–196, 2001.

33. De Roda Husman, A. M., Walboomers, J. M. M., van den Brule, A. J. C., Meijer, C. J. L. M., and Snijders, P. J. F. The use of general primers Gp5 and Gp6 elongated at their 3′ ends with adjacent highly conserved sequences improves papillomavirus detection by PCR. J. Gen. Virol., *76:* 1057–1062, 1995.

34. Soler, C., Allibert, P., Chardonnet, Y., Cros, P., Mandrand, B., and Thivolet, J. Detection of human papillomavirus types 6, 11, 16 and 18 in mucosal and cutaneous lesions by the multiplex polymerase chain reaction. J. Virol. Methods, *35:* 143–157, 1991.

35. Van den Brule, A. J. C., Meijer, C. J. L. M., Bakels, V., Kenemans, P., and Walboomers, J. M. M. Rapid human papillomavirus detection in cervical scrapes by combined general primers mediated and type specific polymerase chain reaction. J. Clin. Microbiol., *28:* 2739–2743, 1990.

36. Vandenvelde, C., Scheen, R., van Pachterbeke, C., Loriaux, C., Decelle, J., Hubert, T., Delhaye, C., Cattoor, J. P., and van Beers, D. Prevalence of high risk genital papillomaviruses in the Belgian female population determined by fast multiplex polymerase chain reaction. J. Med. Virol., *36:* 279–282, 1992.

37. Keating, P. J., Cromme, F. V., Duggan-Keen, M., Snijders, P. J., Walboomers, J. M., Hunter, R. D., Dyer, P. A., and Stern, P. L. Frequency of down-regulation of individual HLA-A and -B alleles in cervical carcinomas in relation to TAP-1 expression. Br. J. Cancer, *72:* 405–411, 1995.

38. Cromme, F. V., van Bommel, P. F., Walboomers, J. M., Gallee, M. P., Stern, P. L., Kenemans, P., Helmerhorst, T. J., Stukart, M. J., and Meijer, C. J. Differences in MHC and TAP-1 expression in cervical cancer lymph node metastases as compared with the primary tumours. Br. J. Cancer, *69:* 1176–1187, 1994.

39. Fu, Y. S., Huang, I., Beaudenon, S., Ionesco, M., Barrasso, R., de Brux, J., and Orth, G. Correlative study of HPV, DNA, histopathology and morphometry in cervical condyloma and intraepithelial neoplasia. Int. J. Gynecol. Pathol., *7:* 297–303, 1988.

40. Wilczynski, S. P., Bergen, S., Walker, J., Liao, S. Y., and Pearlman, L. F. Human papillomaviruses and cervical carcinomas: histologic features associated with different viral types. Hum. Pathol., *19:* 697–704, 1998.

41. Wilczynski, S. P., Walker, J., Liao, S. Y., Bergen, S., and Berman, M. Adenocarcinoma of the cervix associated with human papillomaviruses. Cancer (Phila.), *62:* 1332–1336, 1988.

42. Stoler, M. H., Mills, S. E., Gersell, D. J., and Walker, A. J. Small cell neuroendocrine carcinoma of the cervix. A human papillomavirus type 18 associated cancer. Am. J. Surg. Pathol., *15:* 28–32, 1991.

43. Kono, K., Ressing, M. E., Brandt, R. M., Melief, C. J., Potkul, R. K., Andersson, B., Petersson, M., Kast, W. M., Kiessling, R. Decreased expression of signal-transducing ζ chain in peripheral T cells and natural killer cells in patients with cervical cancer. Clin. Cancer Res., *2:* 1825–1828, 1996.

44. Hanke, T., Neumann, V. C., Blanchard, T. J., Sweeney, P., Hill, A. V., Smith, G. L., and McMichael, A. Effective induction of HIV-specific CTL by multi-epitope using gene gun in a combined vaccination regime. Vaccine, *17:* 589–596, 1999.

45. Schneider, J., Gilbert, S. C., Blanchard, T. J., Hanke, T., Robson, K. J., Hannan, C. M., Becker, M., Sinden, R., Smith, G. L., and Hill, A. V. Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara. Nat. Med., *4:* 397–402, 1998.

46. Sedegah, M., Jones, T. R., Kaur, M., Hedstrom, R., Hobart, P., Tine, J. A., and Hoffman, S. L. Boosting with recombinant vaccinia increases immunogenicity and protective efficacy of malaria DNA vaccine. Proc. Natl. Acad. Sci. USA, *95:* 7648–7653, 1998.

47. Chen, C. H., Wang, T. L., Hung, C. F., Pardoll, D. M., and Wu, T. C. Boosting with recombinant vaccinia virus increases HPV-16 E7-specific T cell precursor frequencies of HPV-16 E7-expressing DNA vaccines. Vaccine, *18:* 2015–2022, 2000.

48. Honma, S., Tsukada, S., Honda, S., Nakamura, M., Takakuwa, K., Maruhashi, T., Kodama, S., Kanazawa, T., Takahashi, T., and Tanaka, K. Biological-clinical significance of selective loss of HLA-class-I allelic product expression in squamous-cell carcinoma of the uterine cervix. Int. J. Cancer, *57:* 650–655, 1994.

49. Brady, C. S., Bartholomew, J. S., Burt, D. J., Duggan-Keen, M. F., Glenville, S., Telford, N., Little, A. M., Davidson, J. A., Jimenez, P., Ruiz-Cabello, F., Garrido, F., and Stern, P. L. Multiple mechanisms underlie HLA dysregulation in cervical cancer. Tissue Antigens, *55:* 401–411, 2000.

50. Konya, J., and Dillner, J. Immunity to oncogenic human papillomaviruses. Adv. Cancer Res., *82:* 205–238, 2001.

51. Gherardi, M. M., Ramirez, J. C., and Esteban, M. Interleukin-12 (IL-12) enhancement of the cellular immune response against human immunodeficiency virus type 1 env antigen in a DNA prime/vaccinia virus boost vaccine regimen is time and dose dependent: suppressive effects of IL-12 boost are mediated by nitric oxide. J. Virol., *74:* 6278–6286, 2000.

52. Park, J. S., Park, D. C., Kim, C. J., Ahn, H. K., Um, S. J., Park, S. N., Kim, S. J., and Namkoong, S. E. HPV-16-related proteins as the serologic markers in cervical neoplasia. Gynecol. Oncol., *69:* 47–55, 1998.

53. Van der Burg, S. H., Kwappenberg, K. M., O'Neill T., Brandt, R. M. P., Melief, C. J. M., Hickling, J. K., and Offringa, R. Pre-clinical safety and efficacy of TA-CIN, a recombinant HPV16 L2E6E7 fusion protein vaccine in homologous and heterologous prime-boost regimens. Vaccine, *19:* 3652–3660, 2001.

Clinical Cancer Research

Safety and Immunogenicity of TA-HPV, a Recombinant Vaccinia Virus Expressing Modified Human Papillomavirus (HPV)-16 and HPV-18 *E6* and *E7* Genes, in Women with Progressive Cervical Cancer

Andreas M. Kaufmann, Peter L. Stern, Elaine M. Rankin, et al.

Clin Cancer Res 2002;8:3676-3685.