Eradication of Established Tumors by Vaccination With Recombinant *Bordetella pertussis* Adenylate Cyclase Carrying the Human Papillomavirus 16 E7 Oncoprotein

Xavier Préville,[1,3] Daniel Ladant,[2] Benedikt Timmerman,[1] and Claude Leclerc[3]

[1]BT PHARMA, Biotop, [2]Unité Postulante de Biochimie des Interactions Macromoléculaires, Centre National de la Recherche Scientifique, and [3]Unité de Biologie des Régulations Immunitaires, Institut National de la Sante et de la Recherche Medicale E352, Institut Pasteur, Paris Cedex 15, France

Abstract

High-risk human papillomaviruses (HPV) such as HPV16 are associated with the development of cervical cancer. The HPV16-E6 and HPV16-E7 oncoproteins are expressed throughout the replicative cycle of the virus and are necessary for the onset and maintenance of malignant transformation. Both these tumor-specific antigens are considered as potential targets for specific CTL-mediated immunotherapy. The adenylate cyclase (CyaA) of *Bordetella pertussis* is able to target dendritic cells through specific interaction with the $\alpha_M\beta_2$ integrin. It has been previously shown that this bacterial protein could be used to deliver $CD4^+$ and $CD8^+$ T cell epitopes to the MHC class II and class I presentation pathways to trigger specific Th and CTL responses *in vivo*, providing protection against subsequent viral or tumoral challenge. Here, we constructed recombinant CyaA containing either the full sequence or various subfragments from the HPV16-E7 protein. We show that, when injected to C57BL/6 mice in absence of any adjuvant, these HPV16-recombinant CyaAs are able to induce specific Th1 and CTL responses. Furthermore, when injected into mice grafted with HPV16-E7-expressing tumor cells (TC-1), one of these recombinant proteins was able to trigger complete tumor regression in 100% of the animals tested. This therapeutic efficacy compared favorably to that of strongly adjuvanted peptide and was marginally affected by prior immunity to CyaA protein. This study represents the first *in vivo* demonstration of the antitumoral therapeutic activity of recombinant CyaA proteins carrying human tumor–associated antigens and paves the way for the testing of this vector in clinical trials. (Cancer Res 2005; 65(2): 641-9)

Introduction

Carcinomas of the anogenital tract account for nearly 12% of all cancers in women, making cervical carcinoma (CxCa) the second most frequent gynecologic cancer in the world (1). The critical observation that infection with human papillomavirus (HPV) might be the causative agent for CxCa (2) was subsequently confirmed by epidemiologic studies (3). HPVs are double-stranded DNA viruses, which replicate exclusively in stratified squamous epithelia, using the differentiation of the epithelium to regulate their replication. Approximately 40 distinct HPV types are known to infect the anogenital tract but roughly a third of these, referred to as high-risk types, are significantly associated with progression to CxCa (4). In this subgroup, the most prevalent HPV types associated with CxCa are HPV16 and HPV18 (55% and 12% prevalence, respectively; ref. 5).

The oncogenic potential of high-risk HPVs is attributed to the products of early genes *E6* and *E7*, whose expression is detected throughout the replication cycle of the virus and is necessary for the onset and the maintenance of malignant transformation. This occurs through the interaction of the E6 and E7 proteins, respectively, with p53 and RB, which blocks the activity of these tumor suppressors, and consequently causes alterations in cyclin-dependent kinases complexes predisposing infected keratinocytes to neoplastic transformation (6).

One striking feature of high-risk HPV infection is that its incidence far exceeds the number of individuals who develop HPV-associated malignancies as approximately 95% of HPV infections of the anogenital tract resolve spontaneously (7). The higher prevalence of high-risk HPV-associated malignancies among immunocompromised patients suggests that immune responses may control HPV infection. Indeed, cellular immunity to HPV16-E7 was found to be associated with clinical and cytologic resolution of HPV-induced lesions (8). Furthermore, $CD4^+$ and $CD8^+$ T cell responses against HPV16-E6 and/or E7 epitopes are detected in blood and tissues of patients diagnosed with HPV16-associated malignancies (9–11), as well as in the blood of healthy individuals (12, 13). Altogether, these observations constitute a strong rationale for the development of immunotherapeutic strategies to prime or boost endogenous immune responses to the tumor-specific antigens that constitute the E6 and E7 proteins of HPV16.

Many vaccine approaches have been successfully developed to prevent tumor growth of HPV16-E6 and HPV16-E7-positive tumorigenic cell lines in C57BL/6 mice by generating immune responses to the HPV16-E7 H-$2D^b$-restricted epitope. These vaccination strategies have included plasmid DNA, viral or bacterial vectors, chimeric virus-like particles, synthetic peptides, and recombinant proteins (14). Some of these approaches that were safe enough for testing in humans were brought to the clinic where their safety profiles were confirmed along with their potency to induce specific cellular immunity in phase I/II studies, albeit with limited clinical support. Optimized clinical trials in terms of inclusion/exclusion criteria and vaccine schedule/dosage should improve therapeutic results. Yet, it remains of interest to evaluate novel tools to target HPV16

Requests for reprints: Xavier Préville, Unité de Biologie des Régulations Immunitaires, Institut National de la Sante et de la Recherche Medicale E352, Institut Pasteur, 25-28 rue de Dr Roux, 75724 Paris Cedex 15, France. Phone: 33-1-4061-3910; Fax: 33-1-4568-8540; E-mail: xprevil@pasteur.fr.

epitopes to the immune system for induction of cellular-mediated responses.

The adenylate cyclase (CyaA) of *Bordetella pertussis* has the capacity to deliver its catalytic domain into the cytosol of eukaryotic cells (15). This ability has been exploited to show that $CD4^+$ and $CD8^+$ T cell epitopes inserted into the catalytic site of CyaA are processed and presented by MHC class II and I molecules, respectively, at the surface of antigen-presenting cells (APC; ref. 16). Furthermore, CyaA was recently shown to bind specifically to the $\alpha_M\beta_2$ integrin (CD11b/CD18; ref. 17), and therefore to target these T cell epitopes to the $CD11b^+$ dendritic cell subpopulation (18). Immunization of mice with recombinant CyaAs bearing appropriate T cell epitopes led to the induction of strong CTL responses, full protection against a lethal viral challenge, and efficient prophylactic and therapeutic antitumor immunity in an artificial model composed of murine tumorigenic cell lines expressing the OVA CTL epitope (19, 20).

To test the ability of CyaA to deliver large antigens, and also its suitability as a vaccine vector to deliver human tumor-associated antigens, we constructed recombinant CyaAs containing either the full-length sequence of the E7 protein from HPV16 or different subfragments of this polypeptide (including in particular, a peptide encompassing residues 49-57 of E7 that corresponds to a H-$2D^b$-restricted epitope). We show that, when injected to C57BL/6 mice in the absence of any adjuvant, these HPV16-E7-recombinant CyaAs are able to induce strong specific CTL and Th1 responses characterized by the secretion of IFN-γ. Furthermore, a single injection of these recombinant proteins was able to provide up to 100% therapeutic efficacy against the s.c. graft of TC-1 cells. This therapeutic efficacy compared favorably to that of a strongly adjuvanted HPV16-E7-derived peptide and was marginally affected by prior immunity to CyaA protein. This study shows the *in vivo* antitumoral therapeutic activity of CyaA-based vaccines in a model validated for testing the efficacy of immunotherapeutics against HPV-associated neoplasia.

Materials and Methods

Mice and Cell Lines. Specific pathogen-free 6- to 10-week-old female C57BL/6 mice were obtained from CER Janvier (Le Gesnet St-Isle, France) or Charles River (L'Arbresle, France). TAP1 $^{-/-}$ (21), MHC class II $^{-/-}$ (22), and CD40 $^{-/-}$ (23) bred onto a C57BL/6 background were also used in this study. Animals were kept in the Pasteur Institute animal facilities under pathogen-free conditions with water and food *ad libitum*. Experiments involving animals were conducted according to the institutional guidelines for animal care.

TC-1 cells expressing HPV16-E6 and HPV16-E7 proteins (24) and mouse thymoma EL4 cells (20) were obtained from the American Type Culture Collection (LGC Promochem, Molsheim, France). Cells were maintained in RPMI 1640 with GlutaMAX supplemented with 10% heat-inactivated FCS, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.4 mg/mL geneticin, and 0.2 mg/mL hygromycin B (the latter two for TC-1 cells only) and 5×10^{-5} mol/L 2-mercaptoethanol (Life Technologies, Cergy-Pontoise, France).

Peptides and Oligonucleotides. The synthetic peptides $E7_{49-57}$ (RAHYNIVTF, one-letter code for amino acid) corresponding to the HPV16-E7 H2-D^b-restricted epitope (25) and $E7_{43-77}$ (GQAEP**DRAHYNIVTF**CCKCDSTLRLCVQSTHVDIR) corresponding to the $E7_{49-57}$ CTL epitope with its natural flanking sequence and a Th epitope (in boldface; ref. 26) were purchased from Neosystem (Strasbourg, France). CpG ODN 1826 was purchased from Proligo (Paris, France).

Construction and Purification of Recombinant *B. pertussis* CyaA Carrying HPV16-E7 Epitopes. Recombinant adenylate cyclase used in this article were expressed in *Escherichia coli* by using derivatives of plasmid pTRACE5 which codes for an enzymatically inactive CyaA (27). The adenylate cyclase activity of CyaA was inactivated by inserting the dipeptide leucine-glutamine between codons 188 and 189 within the catalytic site (28). The *E. coli* strain XL1-Blue (Stratagene, La Jolla, CA) was used for all DNA manipulations that were done according to standard protocols.

CyaA-$E7_{49-57}$ contains a nine-amino acid–long polypeptide sequence (RAHYNIVTF) inserted between codons 224 and 235 of CyaA. To do so, two synthetic oligonucleotides (MWG, Courtaboeuf, France), BTP1 (5′-CTA GCC GTG CCC ATT ACA ATA TTG TAA CCT TTG GTA C-3′, coding strand) and BTP2 (5′-CAA AGG TTA CAA TAT TGT AAT GGG CAC GG-3′, noncoding strand) were annealed and ligated into the pTRACE5 plasmid digested with *Nhe*I and *Kpn*I. CyaA-$E7_{Full}$ contains the entire sequence (98 amino acids) of the HPV16-E7 protein inserted at the same 224 position of CyaA. The DNA sequence encoding the E7 protein was amplified from HPV16 DNA (kindly given by F. Thierry, Unité d'Expression génétique et Maladies, Institut Pasteur, Paris, France) using specific primers BTP3 (5′-GGG CGC TAG CAT GCA TGG AGA TAC ACC TAC-3′) and BTP4 (5′-GGG CGG TAC CTG GTT TCT GAG AAC AGA TGG G-3′). The resulting PCR product was digested by *Nhe*I and *Kpn*I and ligated into pTRACE5 cleaved by *Nhe*I and *Kpn*I as described above. CyaA-$E7_{\Delta30-42}$ contains the first 29 amino acid residues of HPV16-E7 inserted between codons 319 and 320 of CyaA as well as residues 43-98 of HPV16-E7 inserted between codons 224 and 235 of CyaA. The expression plasmid for CyaA-$E7_{\Delta30-42}$ was constructed in two steps. A first DNA fragment encoding amino acid residues 1-29 of HPV16-E7 was PCR-amplified using DNA as the target, a synthetic HPV16-E7 gene (optimized for production in *E. coli*; designed by GTP Technology, Labège, France) and primers BTP5 (5′-GGG CAC CGG TAA ACG TAT GCA CGG CGA TAC TCC G-3′) and BTP6 (5′-CGT GAG CAT CTG GCT TTC ACT AGT ACG TTT GTT CAG CTG CTC GTA GCA-3′). A second DNA fragment encoding codons 320-372 of CyaA was PCR-amplified using pTRACE5 as target DNA and primers BTP7 (5′-GGG CAC TAG TGA AAG CCA GAT GCT CAC GCG CGG G-3′) and BTP8 (5′-AGT ACA TCC GGC GAG AAC-3′). These two DNA fragments (which partly overlap) were purified and combined with primers BTP5 and BTP8 in a third PCR to amplify a 294-bp-long DNA fragment. This fragment was digested by *Age*I and *Bst*BI and inserted between the corresponding sites of pTRACE5 to yield plasmid pTRACE5-$E7_{1-29}$. Then, a DNA fragment encoding the amino acid residues 43 to 98 of HPV16-E7 was PCR-amplified using the synthetic HPV16-E7 gene as target DNA and primers BTP9 (5′-GGG CGC TAG CGG TCA AGC AGA ACC GGA C-3′) and BTP10 (5′-GGG CGG TAC CAG GTT TTT GAG AGC AAA TCG GAC AAA CAA TCC CCA GAG TAC CCA TC-3′). The purified PCR fragment was digested by *Nhe*I and *Kpn*I, and ligated into plasmid pTRACE5-$E7_{1-29}$ digested by the same restriction enzymes. All constructions were verified by DNA sequencing (Genome Express, Meylan, France).

All recombinant adenylate cyclase were produced in the *E. coli* strain BLR (Novagen, Madison, WI) as previously described (29). The recombinant proteins were purified to homogeneity from inclusion bodies by a two-step procedure that includes DEAE-Sepharose and phenyl-Sepharose chromatography (29). An additional washing step with 60% isopropanol was done (30) in order to eliminate most of the contaminating lipopolysaccharides. Lipopolysaccharide contents were determined using the kit QCL-1000 (BioWhittaker, Walkersville, MD). Purified recombinant proteins were analyzed by Coomassie blue–stained SDS-PAGE. Protein concentrations were determined spectrophotometrically from the absorption at 280 nm using a molecular extinction coefficient of 142,000 mol/L^{-1} cm^{-1}.

Construction and Purification of Recombinant HPV16-E7 Protein. The *E. coli*-optimized cDNA coding for HPV16-E7 protein (GTP Technology) was subcloned into pIVEX2.4b vector (Roche Molecular Biochemicals, Meylan, France) between the *Nco*I and *Xho*I restrictions sites. The resulting plasmid was then transformed into the *E. coli* strain BL21λDE3 (Novagen). The His-Tag-HPV16-E7 protein was expressed upon induction with 0.5 mmol isopropyl-β-D-thiogalactopyranoside (Euromedex, Souffelweyersheim,

France) and purified on Ni-NTA agarose (Qiagen, Hilden, Germany). Isopropanol washes were used in order to remove lipopolysaccharide contamination.

Immunoblotting. Proteins were separated by SDS-PAGE and electrotransferred to a nitrocellulose membrane (0.45 μm; Bio-Rad, Marnes la Coquette, France) that was probed either with a mouse monoclonal anti-HPV16-E7 antibody (Zymed, San Francisco, CA) or with a polyclonal anti-*E. coli* BLR serum prepared in C57BL/6 mice. Immune complexes were detected with goat anti-mouse immunoglobulins conjugated to phosphatase alkaline (Chemicon, Temecula, CA) and revealed with 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (Sigma, St. Louis, MO).

Mice Immunization and Tumor Rejection Experiments. Animals were immunized either with one i.v. injection (50 μg), or with two i.d. injections (10 μg each) of control or HPV16-E7 recombinant CyaAs diluted in PBS (Life Technologies). i.d. injections were done in the ear dermis (31). For tumor rejection experiments, mice received 5×10^4 TC-1 cells s.c. and were treated by HPV16-E7 recombinant CyaAs 1, 5, or 10 days after tumor inoculation. TC-1 tumor growth was monitored using a caliper and expressed in cubic millimeters using the formula $V = (L \times w^2)/2$, where L, length; w, width (32).

***In vitro* Cytotoxic Assay.** Splenocytes from immunized mice were stimulated *in vitro* with 1 μg/mL of either $E7_{49-57}$ or $E7_{43-77}$ peptides in the presence of syngeneic irradiated naive spleen cells in complete medium for 5 days. The cytolytic activity of these effector cells was tested in a 5-hour ^{51}Cr-release assay on TC-1 cells. Radiolabeling was done as follows: exponentially growing TC-1 cells cultured in a 7.5% carbon dioxide atmosphere at 37°C were quickly trypsinized (trypsin-EDTA, Life Technologies) and incubated with 100 μCi of ^{51}Cr for 1 hour at 37°C. Various E/T ratios were used and all assays were done in duplicate. The radioactivity released in the supernatant of each well was measured. The percentage of specific lysis was calculated as 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). Maximum release was obtained by adding 10% Triton X-405 to target cells and spontaneous release was obtained with target cells incubated in complete medium alone.

Single IFN-γ Producing Cell Enzyme-Linked Immunospot Assay for Secreting Cells. Multiscreen filtration plates (96 wells; Millipore, Molshein, France) were coated with 4 μg/mL of rat anti-mouse IFN-γ antibody (clone R4-6A2; PharMingen, San Diego, CA), overnight at room temperature. Then the plates were washed and blocked with complete medium. Serial 2-fold dilutions of spleen cells from immunized mice were added to the wells along with 5×10^5 γ-irradiated (2,500 rad) syngeneic feeder cells. The cells were incubated for 36 hours with or without $E7_{49-57}$ peptide at 1 μg/mL. After extensive washes, the plates were revealed by incubation with 5 μg/mL of biotinylated rat anti-mouse IFN-γ antibody (clone XMG 1.2; PharMingen) followed by incubation with streptavidin-alkaline phosphatase (PharMingen). Finally, spots were revealed using 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium as substrate. The number of IFN-γ-producing cells were determined by counting the number of spot-forming cells in each well (Bioreader, Karben, Germany), and the results were expressed as the total number of spot-forming cells per spleen (20).

Enzyme-Linked Immunosorbent Assay. Mice immunized i.d. with empty vector CyaAE5 were bled 30 or 90 days later and individual mouse sera were tested for antibody responses by ELISA. Microplates (Nunc, Roskilde, Denmark) were coated overnight with empty vector CyaAE5 (3 μg/mL) in PBS. After washes in PBS-Tween 20 (0.1%), diluted sera were added to the wells and incubated for 1 hour at 37°C. Following washes in PBS-Tween 20, plates were incubated with goat anti-mouse IgG peroxidase conjugate (Sigma) for 1 hour at 37°C. Plates were developed using *o*-phenylenediamine and hydrogen peroxide (Sigma). The reaction was stopped with sulfuric acid and the plates analyzed at 492 nm in an ELISA reader (Dynatech, Marnes la Coquette, France). Results are expressed as antibody titers calculated by linear regression analysis plotting dilution versus A_{492}. The titers were calculated to be the $\log_{10}$ highest dilution that gives twice the absorbance of pooled control sera diluted 1:100.

To assess the cytokine production by splenocytes from immunized mice, spleen cells were stimulated *in vitro* with 10 μg/mL of His-Tag-HPV16-E7 protein or 1 μg/mL $E7_{43-77}$ peptide in complete medium for 72 hours. IFN-γ and IL-5 production were determined in culture supernatants by sandwich ELISA (33). All assays were standardized with corresponding recombinant murine cytokines (PharMingen).

Statistical Analysis. Nonparametric statistical tests (34) were used (StatXact 4 software, Cytel Corporation, Cambridge, MA). Survival curves were plotted using Prism software (GraphPad Software, Inc., CA) and compared with the software's built-in logrank test. Data were considered significantly different at $P < 0.05$.

Results

Construction and Characterization of Recombinant Adenylate Cyclases Bearing HPV16-E7 Epitopes. To study the ability of the CyaA vector to induce HPV16-E7-specific T cell responses, we constructed three different recombinant molecules. CyaA-$E7_{49-57}$ contains a nine-amino acid–long peptide sequence (RAHYNIVTF) corresponding to the previously described H-2D^b restricted CTL epitope (Fig. 1*A*; ref. 25), which was inserted between codons 224 and 235 of an enzymatically inactive (hence nontoxic) CyaA. CyaA-$E7_{Full}$ contains the entire sequence (98 amino acids) of the HPV16-E7 protein inserted at the same 224 position of the enzymatically inactive CyaA (Fig. 1*A*). CyaA-$E7_{\Delta 30-42}$ contains the first 29 amino acid residues of HPV16-E7 inserted between codons 319 and 320 of the enzymatically inactive CyaA as well as residues 43-98 of HPV16-E7 inserted between codons 224 and 235 (Fig. 1*A*; ref. 25). This construction was designed to remove the amino acid sequence extending from residues 30-42 of HPV16-E7, as this sequence is highly acid and might potentially inhibit the translocation of the catalytic domain of CyaA into APCs (35). Besides, the NH_2-terminal and COOH-terminal sequences of HPV16-E7 were inserted into two distinct permissive sites within CyaA so as to prevent potential refolding of the HPV16-E7 protein, that could also interfere with the translocation into the cytosol of APCs (36). To allow *in vitro* and *in vivo* assays, the CyaA constructs were produced in *E. coli* and purified to homogeneity (Fig. 1*B*). A lipopolysaccharide elimination procedure was introduced in the purification protocol (29) to obtain recombinant proteins containing < 100 units of endotoxin per dose of 50 μg (24, 93, and 52 for CyaA-$E7_{49-57}$, -$E7_{Full}$, and -$E7_{\Delta 30-42}$, respectively). The presence of the E7 protein in CyaA-$E7_{Full}$ and CyaA-$E7_{\Delta 30-42}$ was confirmed by Western blotting using a specific monoclonal antibody (Fig. 1*C*). The contamination level by unrelated *E. coli* proteins, of the different purified recombinant CyaAs was assessed by Western blot analysis. Ten micrograms of the different HPV recombinant CyaAs as well as different amounts of *E. coli* BLR proteins (1, 0.5, 0.25, 0.12, and 0.06 μg) were probed with a murine anti-*E. coli* BLR serum. As shown in Fig. 1*D*, the highest level of contamination of the HPV recombinant CyaAs is below 2.5%, a value commonly accepted by regular authorities in vaccine development.

Immunization with HPV16-E7 Recombinant CyaAs Induces E7-Specific CTL responses. To test whether CyaA can induce CTL responses against HPV16-E7 epitopes, C57BL/6 mice were immunized i.v. with 50 μg of the different HPV16-E7 recombinant CyaAs. Splenocytes were harvested and stimulated *in vitro* with 1 μg/mL of the $E7_{43-77}$ peptide. Their ability to lyse TC-1 cells was determined 5 days later using a ^{51}Cr release assay. A single i.v. immunization of C57BL/6 mice with HPV16-E7 recombinant CyaAs induced strong and specific CTL responses to TC-1 cells (Fig. 2*A*). Similar results were obtained when the peptide $E7_{49-57}$ was used for *in vitro*

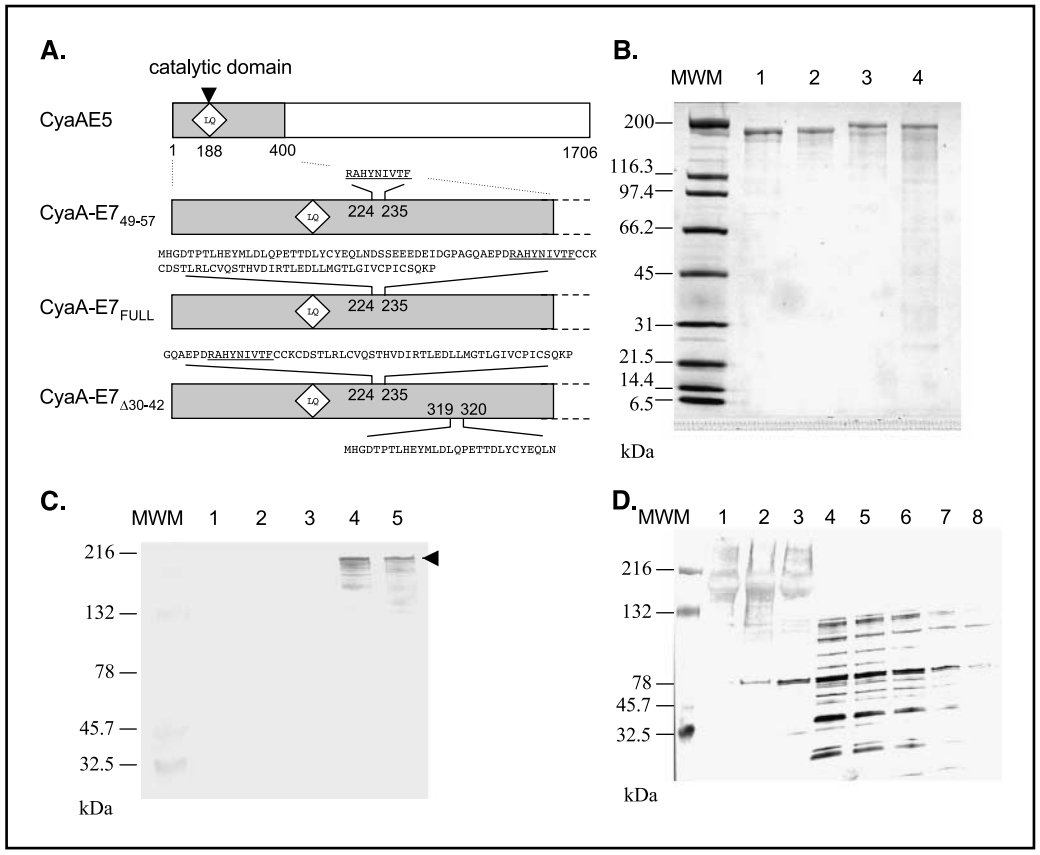

Figure 1. Construction and purification of HPV16-E7 recombinant CyaAs. *A*, schematic representation of CyaA showing the site of insertion of the dipeptide LQ which inactivates the enzymatic activity of CyaA. Positions of the HPV16-E7 protein inserts are also shown. The HPV16-E7 H-2^b-restricted epitope is underlined. *B*, SDS-PAGE analysis of the HPV16-E7 recombinant CyaAs. Five micrograms of the purified proteins were separated on a 4% to 15% SDS polyacrylamide gel and stained by Coomassie blue. *Lane 1,* wild-type CyaA; *lane 2,* CyaA-E7$_{49\text{-}57}$; *lane 3,* CyaA-E7$_{Full}$; *lane 4,* CyaA-E7$_{\Delta 30\text{-}42}$. *C*, immunoblot probed with a mouse monoclonal anti-HPV16-E7 antibody. *Lanes 1* and *2,* wild-type CyaA (2 and 0.4 μg, respectively); *lanes 3, 4* and *5,* CyaA-E7$_{49\text{-}57}$, CyaA-E7$_{Full}$, and CyaA-E7$_{\Delta 30\text{-}42}$, respectively, 0.4 μg of each protein. *D,* immunoblot probed with a mouse anti-*E. coli* BLR serum. *Lanes 1* to *3,* CyaA-E7$_{49\text{-}57}$, CyaA-E7$_{Full}$, and CyaA-E7$_{\Delta 30\text{-}42}$, respectively (10 μg); *lanes 4* to *8, E. coli* BLR total extract (1, 0.5, 0.25, 0.12, and 0.06 μg, respectively).

restimulation (data not shown). Splenocytes from mice vaccinated with a recombinant CyaA carrying a nonrelevant epitope (OVA$_{257\text{-}264}$) and restimulated *in vitro* with 1 μg/mL E7$_{43\text{-}77}$ peptide yielded a weak nonspecific TC-1 cell lysis (Fig. 2*A*). It has been previously shown that the delivery of the OVA CD8$^+$ T cell epitope (SIINFEKL) to the MHC class I molecule by CyaA *in vivo* is dependent on TAP1 function and independent of the presence of CD4$^+$ T cells (18). This is also the case for CyaA-E7$_{\Delta 30\text{-}42}$ as *in vitro*-stimulated splenocytes from i.v.-vaccinated TAP1$^{-/-}$ mice were unable to lyse TC-1 cells (Fig. 2*B*). In agreement with earlier observations (18), we noted that i.v. vaccination of MHC class II $^{-/-}$ mice using CyaA-E7$_{\Delta 30\text{-}42}$ resulted in the induction of high levels of specific CTL responses to TC-1 cells (Fig. 2*B*). However, in this model, we observed some dependency towards CD40 signaling, as only a low level of CTL response to TC-1 cells was detected in CD40$^{-/-}$ mice (Fig. 2*B*).

To estimate the frequencies of HPV16-E7-specific splenocytes in mice immunized with recombinant CyaAs, the number of cells producing IFN-γ in response to *in vitro* stimulation with HPV16-E7$_{49\text{-}57}$ peptide was quantified by enzyme-linked immunospots (ELISPOT). Figure 2*C* shows that there was only a slight difference in the number of IFN-γ-producing splenocytes obtained from mice immunized with CyaE5-CysOVA as compared with those immunized with CyaA-E7$_{49\text{-}57}$. In contrast, the number of IFN-γ-producing splenocytes obtained was much higher ($P < 0.05$) in mice vaccinated with HPV16-E7 recombinant CyaAs containing either the full HPV16-E7 protein or its deleted form (Δ30-42). The observed responses were epitope-specific as very few splenocytes from these mice produced IFN-γ in the absence of stimulation by the HPV16-E7$_{49\text{-}57}$ peptide (Fig. 2*C*).

These results show that CyaA is able to deliver *in vivo* the immunodominant CD8$^+$ H-2D^b-restricted T cell epitope of the HPV16-E7 protein into the cytosol of immunocompetent cells and elicit strong CTL responses. We confirm that CyaA is tolerant to the insertion of large polypeptidic fragments (36, 37) as CyaAs carrying the full HPV16-E7 protein or its deleted form, were also able to induce strong CTL responses.

Th1 responses play an important role in protection against intracellular pathogens and tumor development (38, 39). We therefore characterized the type of T cell responses induced by i.v. immunization with HPV16-E7 recombinant CyaAs. Cytokine synthesis was determined after *in vitro* stimulation of splenocytes with 10 μg/mL of the purified His-Tag HPV16-E7 protein. As shown in Fig. 2*D*, immunization with CyaAs carrying the full HPV16-E7 protein or its deleted form resulted in a Th1-like profile characterized by the production of high levels of IFN-γ and the lack of detectable levels of IL-5. This response was specific because the IFN-γ levels obtained after immunization with CyaA-E7$_{Full}$ or CyaA-E7$_{\Delta 30\text{-}42}$ were significantly higher than those obtained in mice mock-immunized with CyaAE5-CysOVA ($P < 0.05$). Similar results were achieved when the restimulation was carried out with 1 μg/mL of E7$_{43\text{-}77}$ peptide (data not shown). These results indicate that cognate help provided by CD4$^+$ T cells play an important role in the secretion of IFN-γ as levels obtained with CyaAs carrying the HPV16-E7 protein which contains class II H-2^b-restricted T cell epitopes, are much higher than those obtained with CyaA-E7$_{49\text{-}57}$ which contains only the class I H-2D^b-restricted epitope.

Immunization With HPV16-E7 Recombinant CyaAs Induces Regression of Established HPV16-Expressing Tumors. Considering the robust cellular immune responses obtained, we then

evaluated *in vivo* the therapeutic activity of HPV16-E7 CyaAs in a preclinical model consisting of C57BL/6 mice injected s.c. with a H-2^b tumorigenic cell line expressing HPV16-E6 and E7 proteins (TC-1 cells). In this model, tumor rejection is mediated by $E7_{49\text{-}57}$-specific $CD8^+$ T cells (24, 25, 40, 41). Thus, 5×10^4 TC-1 cells were injected s.c. in the right flank of C57BL/6 mice and 50 μg of CyaA-$E7_{49\text{-}57}$, -$E7_{Full}$, or -$E7_{\Delta 30\text{-}42}$ were injected i.v. to mice 1, 5, or 10 days later. Figure 3*A* represents the tumor growth in mice treated therapeutically 10 days after tumor grafting. Noticeably, in these conditions, 100% of the animals developed palpable tumors by the time therapeutic vaccination was given (Fig. 3*A, c, d, e, insets*). Control animals developed tumors of a size > 1,000 mm^3 within a maximum of 49 days (Fig. 3*A, a, b*). In sharp contrast, the majority of animals treated with HPV16-E7 recombinant CyaAs had their growing tumors eradicated and remained tumor-free throughout the experiment (Fig. 3*A, c, d, e*). The median survival times of untreated and mock-treated animals were 39 and 33 days, respectively. In contrast, the survival of mice vaccinated with CyaAs carrying HPV16-E7 antigens was significantly superior to that of control animals ($P < 0.05$) (Fig. 3*A, f*). CyaA-$E7_{\Delta 30\text{-}42}$ was clearly superior in terms of tumor regression and growth inhibition because the survival rate was 100%. Similar results were obtained with CyaA-E7 therapy applied 1 or 5 days after TC-1 grafting (data not shown).

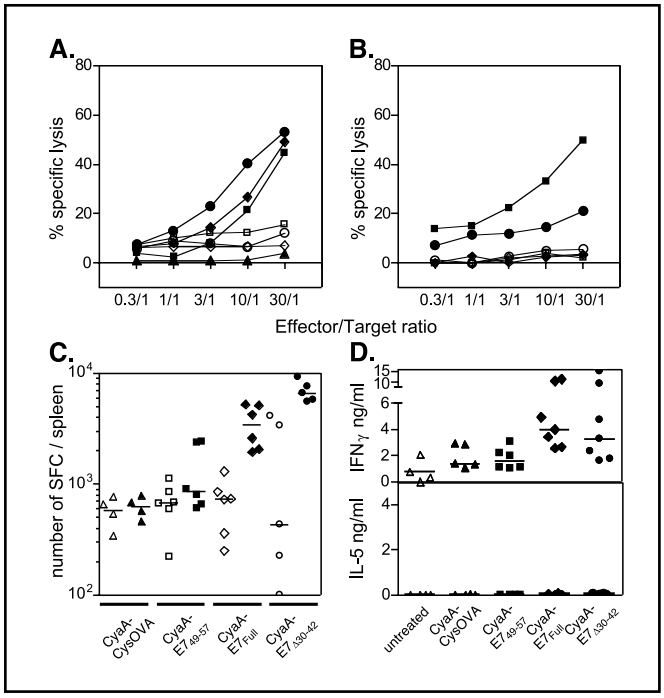

Figure 2. Characterization of T cell responses induced by recombinant HPV 16-E7 CyaAs. *A,* C57BL/6 mice were immunized i.v. on day 0 with 50 μg of CyaA-$E7_{49\text{-}57}$ (□, ■), CyaA-$E7_{Full}$ (◇, ◆), or CyaA-$E7_{\Delta 30\text{-}42}$ (○, ●). Animals were sacrificed 7 days later and their splenocytes were stimulated *in vitro* for 5 days with 1 μg/mL of the HPV16-$E7_{43\text{-}77}$ peptide in the presence of irradiated syngeneic splenocytes, and used as effectors against TC-1 target cells (*closed symbols*) or EL4 (*open symbols*). The responses of splenocytes from mice treated with mock CyaAE5-CysOVA carrying the nonrelevant $CD8^+$ T cell epitope $OVA_{257\text{-}264}$ and restimulated *in vitro* for 5 days with 1 μg/mL of the HPV16-$E7_{43\text{-}77}$ peptide in the presence of irradiated syngeneic splenocytes are also represented (▲). Target lysis was evaluated by ^{51}Cr release. The data represent the median percentage of the specific lysis values ($n = 8$). *B,* $TAP^{-/-}$ mice (◇, ◆), MHC class $II^{-/-}$ mice (□, ■) and $CD40^{-/-}$ mice (○, ●) were immunized i.v. with 50 μg of CyaA-$E7_{\Delta 30\text{-}42}$. *In vitro* stimulated splenocytes were used as effectors against TC-1 target cells (*closed symbols*) or EL4 (*open symbols*; $n = 6$). *C,* detection of HPV16-E7-specific IFN-γ-producing cells after immunization with the recombinant HPV16-E7 CyaAs. C57BL/6 mice were immunized as in (*A*) with CyaAE5-CysOVA (△, ▲), CyaA-$E7_{49\text{-}57}$ (□, ■), CyaA-$E7_{Full}$ (◇, ◆), or CyaA-$E7_{\Delta 30\text{-}42}$ (○, ●). Seven days later, spleen cells isolated from immunized mice were cultured *in vitro* for 36 hours without stimulation (*open symbols*) or with 1 μg/mL of the $E7_{49\text{-}57}$ peptide (*closed symbols*) in the presence of syngeneic irradiated splenocytes. The data are expressed as the number of spot-forming cells per spleen and the results of individual mice from three independent experiments are represented for each group. Horizontal bars represent the median response of each group. *D,* cytokine profile induced by recombinant HPV16-E7 CyaAs. *Top,* C57BL/6 mice were either left untreated (△) or were immunized as in (*A*) with CyaAE5-CysOVA (▲), CyaA-$E7_{49\text{-}57}$ (■), CyaA-$E7_{Full}$ (◆), or CyaA-$E7_{\Delta 30\text{-}42}$ (●). Seven days later, spleen cells were stimulated *in vitro* with 10 μg/mL of the His-Tag-HPV16-E7 protein, and the supernatants were tested for IFN-γ content 72 hours later. Results of individual mice from four independent experiments are represented and expressed as the concentration of IFN-γ released in the supernatant from duplicate wells. Backgrounds obtained with nonrestimulated splenocytes are subtracted. Horizontal bars represent the median response of each group. *Bottom,* same as in *top* except that supernatants were tested for IL-5 content. Results of individual mice from two independent experiments are represented and expressed as the concentration of IL-5 released in the supernatant from duplicate wells.

We tested another injection route of clinical interest: hence, 10 μg of CyaA-$E7_{\Delta 30\text{-}42}$ were injected i.d. twice at a 7-day intervals starting 10 days after TC-1 graft. Interestingly, as all untreated and mock-treated animals developed tumors, we observed tumor regression in all of the animals treated with CyaA-$E7_{\Delta 30\text{-}42}$ (Fig. 3*B, a, b*). This therapeutic immunization resulted in a 100% survival at 90 days of the CyaA-$E7_{\Delta 30\text{-}42}$-treated mice, whereas the survival medians of untreated and mock-treated animals were 30 and 32 days, respectively (Fig. 3*B, c*).

Taken together, these results show the efficacy of the adenylate cyclase vector as a suitable therapeutic vaccine for inducing the regression of HPV16-expressing tumors in a preclinical model.

Therapeutic Efficacy of CyaA-$E7_{\Delta 30\text{-}42}$ Compares Favorably to That of Peptide Administered with CpG ODN 1826. To better evaluate the potency of CyaA as an antigen delivery system, we compared the therapeutic efficacy of CyaA-$E7_{\Delta 30\text{-}42}$ to that of HPV16-$E7_{43\text{-}77}$ peptide supplemented with CpG ODN 1826 (42). Mice were therefore injected s.c. with 5×10^4 TC-1 cells and treated therapeutically 10 and 17 days later via the i.d. route with 10 μg of CyaA-$E7_{\Delta 30\text{-}42}$ or 10 μg of HPV16-$E7_{43\text{-}77}$ peptide given with 1 μg of CpG ODN 1826. The survival rates were similar in these two groups (Fig. 4), although results obtained with CyaA-$E7_{\Delta 30\text{-}42}$ were slightly better but not statistically different from those obtained with HPV16-$E7_{43\text{-}77}$ peptide mixed with CpG ODN 1826. Of note, this result was obtained using 50 times more HPV16-$E7_{43\text{-}77}$ peptide than CyaA-$E7_{\Delta 30\text{-}42}$ on a molar basis. When used alone, the peptide HPV16-$E7_{43\text{-}77}$ had no effect on TC-1 tumor growth.

Prior Immunity to CyaA Vector Marginally Affects the Therapeutic Efficacy of CyaA-$E7_{\Delta 30\text{-}42}$. In a clinical setting, multiple boosts will probably have to be given to patients with lesions in order to obtain efficient cellular immune responses. It is therefore essential to show that preimmunity to the CyaA vector does not impair its ability to trigger tumor rejection. To do so, we immunized mice i.d. twice at a 7-day interval with 10 μg of empty vector CyaAE5, 90 or 30 days prior to s.c. injection with 5×10^4 TC-1 cells. Therapeutic treatment with two i.d. injections at a 7-day interval of 10 μg CyaA-$E7_{\Delta 30\text{-}42}$ was set on day 10. Analysis of antibody responses showed that empty vector immunized–mice were immune to CyaA at the time of TC-1 injection (Fig. 5*A*). We then compared the ability of CyaA-$E7_{\Delta 30\text{-}42}$ treatment to induce tumor rejection in age-matched naive

animals and in CyaA immune animals. Whatever their immune status towards CyaA, the majority of mice treated with CyaA-$E7_{\Delta 30\text{-}42}$ remained tumor-free throughout the experiment (Fig. 5*B*). Only one animal in the day 30 immune mice group, and two in the day 90 immune mice group developed tumors (Fig. 5*B b, d, f*). In contrast, 100% of mock-treated animals developed tumors and were sacrificed (Fig. 5*B, a, c, e*). We did not observe any correlation between the level of anti-CyaA antibody titers and the development of TC-1 tumors (data not shown). Furthermore, survival curves of the CyaA-$E7_{\Delta 30\text{-}42}$-treated mice (Fig. 5*B, b, d, f*) were not statistically different ($P = 0.324$).

These data therefore indicate that immunity towards CyaA has only very limited effect on the ability of this vector to subsequently induce efficient responses against a foreign given antigen.

CyaA Immunization Induces Long-Term Persistent HPV16-$E7_{49\text{-}57}$-Specific $CD8^+$ T Cells. To assess the persistence of immune response induced by HPV16-E7 recombinant CyaAs, mice surviving from therapeutic experiments after 3 months were sacrificed and their splenocytes subjected to *in vitro* stimulation for 5 days with 1 μg/mL $E7_{43\text{-}77}$ peptide. Their ability to lyse TC-1 cells was then determined by a ^{51}Cr release assay. As shown in Fig. 6*A*, specific CTL responses to HPV16-$E7_{43\text{-}77}$ peptide were still shown from splenocytes of animals immunized 3 months earlier. To assess the physiologic relevance of such a long-lasting immunogenicity, remaining animals were re-challenged s.c. with 5×10^4 TC-1 cells at day 100. Under such conditions, all naive age-matched control animals developed tumors and displayed a survival median time of 37.5 days (Fig. 6*B*). In contrast, mice immunized 3 months earlier with HPV16-E7 recombinant CyaAs were efficiently protected from tumor development for an additional period of 3 months. Animals vaccinated with CyaA-$E7_{Full}$ and CyaA-$E7_{\Delta 30\text{-}42}$ displayed a higher level of protection than those vaccinated with CyaA-$E7_{49\text{-}57}$. This observation suggests that cognate T cell help provided by CyaA carrying the full or partly deleted HPV16-E7 protein is of importance for efficient long-lasting responses against TC-1 cells.

Discussion

Previous studies have shown that the adenylate cyclase from *B. pertussis* is a powerful tool to deliver $CD4^+$ and $CD8^+$ T cell epitopes to the MHC class II and I presentation pathways of dendritic cells. In experimental and artificial tumor murine models, this system has been used to trigger efficient Th1 and CTL responses providing antiviral and antitumoral protection (16). As an evaluation of the potential application of CyaA in humans for the treatment of HPV16-associated cervical malignancies, we showed *in vivo* that this vector efficiently delivers epitopes from the HPV16-E7 protein. We constructed various HPV16 recombinant CyaAs containing the full E7 protein from HPV16 or subfragments of this polypeptide, including the H-$2D^b$-restricted CTL epitope. We showed that these different recombinant proteins were able to prime specific and strong CTL responses when injected to C57BL/6 mice in the absence of any adjuvant. Our data indicated that the

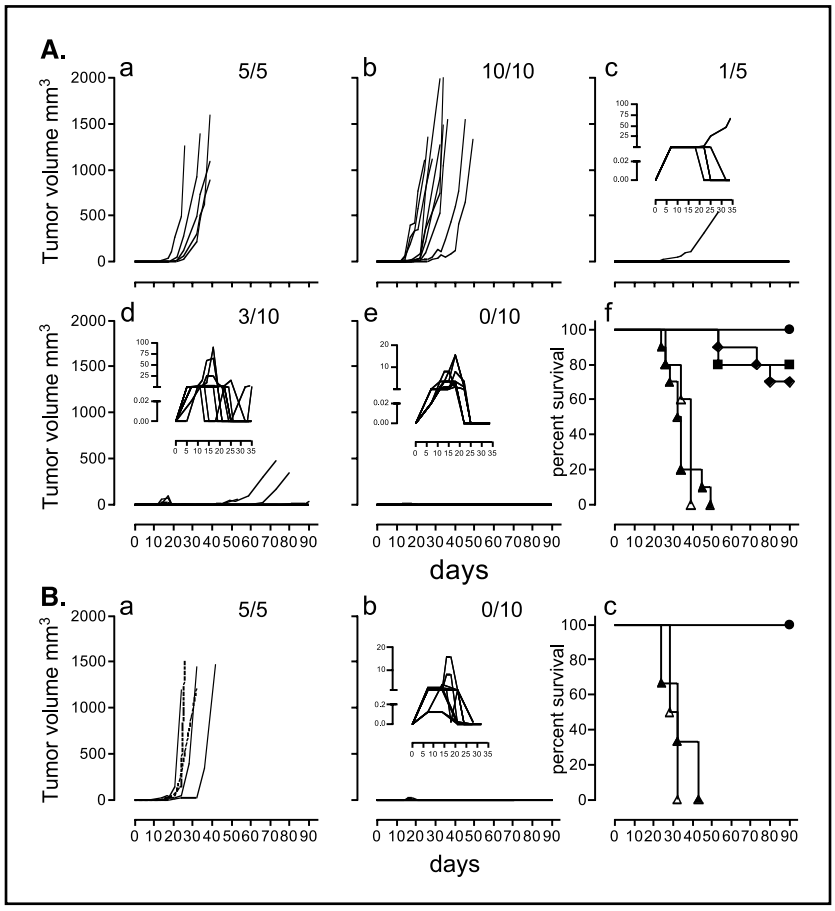

Figure 3. Eradication of established tumors and prolongation of mice survival following therapeutic vaccination with recombinant HPV16-E7 CyaAs. *A,* C57BL/6 mice were grafted on day 0 with 5×10^4 TC-1 tumor cells. Mice were either left untreated (*a*) or treated on day +10, with one i.v. injection of 50 μg CyaAE5-CysOVA (*b*), CyaA-$E7_{49\text{-}57}$ (*c*), CyaA-$E7_{Full}$ (*d*), or CyaA-$E7_{\Delta 30\text{-}42}$ (*e*). *Insets* (*c*, *d*, *e*) are close-ups of the 0- to 35-day period to show that all animals had palpable tumors at the time of vaccination. Each curve represents the tumor growth in a single animal. Mice were killed when the tumor sizes were > 1,000 mm^3 or whenever the sanitary status of the animals commanded (necrosed tumor, rapid weight loss > 20%) so as to avoid unnecessary suffering. *Top right,* number of sacrificed animals versus the total number of animals included. Survival curves of these mice are shown (*f*). Untreated (△), mock-treated with CyaAE5-CysOVA (▲), treated with CyaA-$E7_{49\text{-}57}$ (■), CyaA-$E7_{Full}$ (♦), CyaA-$E7_{\Delta 30\text{-}42}$ (●). *B,* same as in (*A*) for the experimental setting. Therapeutic vaccination was done in the ear dermis on days +10 and +17 with 10 μg of CyaAE5-CysOVA (*a, solid lines*) or 10 μg of CyaA-$E7_{\Delta 30\text{-}42}$ (*b*). Each curve represents the tumor growth in a single animal. Two untreated animals were included (*a, dashed lines*). *Top right* (*a* and *b*), number of sacrificed animals versus the total number of animals included. Survival curves of these mice are shown (*c*). Untreated (△), mock-treated with CyaAE5-CysOVA (▲), treated with CyaA-$E7_{\Delta 30\text{-}42}$ (●).

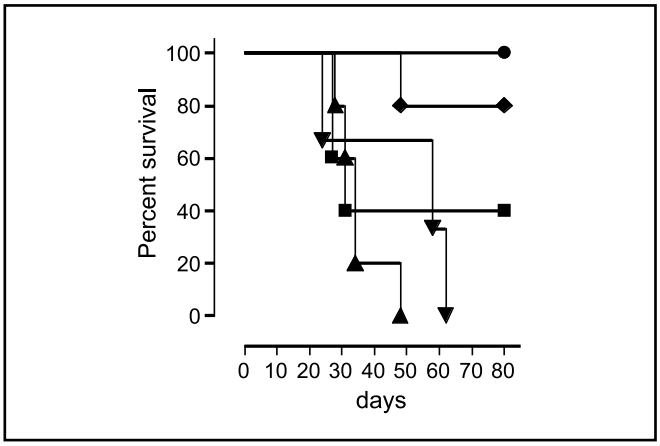

Figure 4. Comparison of CyaA-E7$_{\Delta 30\text{-}42}$ therapeutic activity to that of CpG ODN 1826-adjuvanted HPV16-E7$_{43\text{-}77}$. C57BL/6 mice were grafted s.c. on day 0 with 5 $\times$ 10^4 TC-1 tumor cells. Mice were treated on days +10 and +17, with one i.d. injection of 10 μg HPV16-E7$_{43\text{-}77}$ (n = 5, ▲), 1 μg CpG ODN 1826 (n = 5, ■), 10 μg HPV16-E7$_{43\text{-}77}$ + 1 μg CpG ODN 1826 (n = 5, ♦), 10 μg CyaA-CysOVA (n = 3, ▼), or 10 μg CyaA-E7$_{\Delta 30\text{-}42}$ (n = 7, ●). Mice were killed when the tumor sizes were > 1,000 mm^3 or whenever the sanitary status of the animals commanded.

delivery of the HPV16-E7 H-2D^b CTL epitope by CyaA required a fully functional class I presentation pathway as CyaA-E7$_{\Delta 30\text{-}42}$ was unable to prime CTL responses in TAP1 $^{-/-}$ mice. The CTL response induced by CyaA was independent of the presence of CD4$^+$ T cells as indicated by the efficient CTL responses obtained in MHC class II $^{-/-}$ mice. This characteristic of CyaA as a vaccine vector is of great importance when considering the vaccination of patients presenting a reduced number of CD4$^+$ T cells. Low CTL responses were obtained in CD40 $^{-/-}$ mice indicating that optimal CTL priming was dependent upon CD40 signaling. This suggests that in this model, in contrast to adjuvants such as ODN-CpG (42), CyaA might not be able to bypass the CD40L-CD40 signaling to allow effective stimulation of CTLs (43).

Although single i.v. immunizations with the different HPV16-E7 recombinant CyaAs induced similar levels of E7$_{49\text{-}57}$-specific CD8$^+$ T cells, we observed that the frequencies of HPV16-E7-specific IFN-γ-secreting T cells induced by CyaA-E7$_{Full}$ and CyaA-E7$_{\Delta 30\text{-}42}$ were superior to those induced by CyaA-E7$_{49\text{-}57}$. These observations suggest that the induction of cognate help by CD4$^+$ T cells following the simultaneous delivery of CTL and Th epitopes by CyaA results in a more complete adaptive cellular immune response, including cytolytic activity and secretion of Th1 cytokines. This is in agreement with previously published data in other preclinical (42) and clinical models (44) and was further evidenced in our study, by the analysis of the cytokines produced by HPV16-E7-specific splenocytes upon *in vitro* restimulation with recombinant His-Tag-HPV16-E7 protein or E7$_{43\text{-}77}$ peptide.

In a tumor rejection model based on tumorigenic TC-1 cells (24), we observed that HPV16-E7 recombinant CyaAs were able to eradicate established tumors.

Because of the abundance of CD11b$^+$ Langerhans cells and dermal dendritic cells in murine skin (45, 46), the i.d. route seemed of interest to enhance vaccine immunogenicity. Indeed, therapeutic i.d. immunizations with CyaA-E7$_{\Delta 30\text{-}42}$ yielded outstanding results in the tumor rejection experiments when considering the very low amount of CyaA used for each injection (10 μg, about 53 pmol of recombinant protein).

Using the i.d. route, we compared CyaA-E7$_{\Delta 30\text{-}42}$ immunization to that of peptide HPV16-E7$_{43\text{-}77}$ given with CpG ODN 1826 (42). Results highlighted the potency of CyaA-mediated immunization because it compared favorably to a therapeutic scheme based on

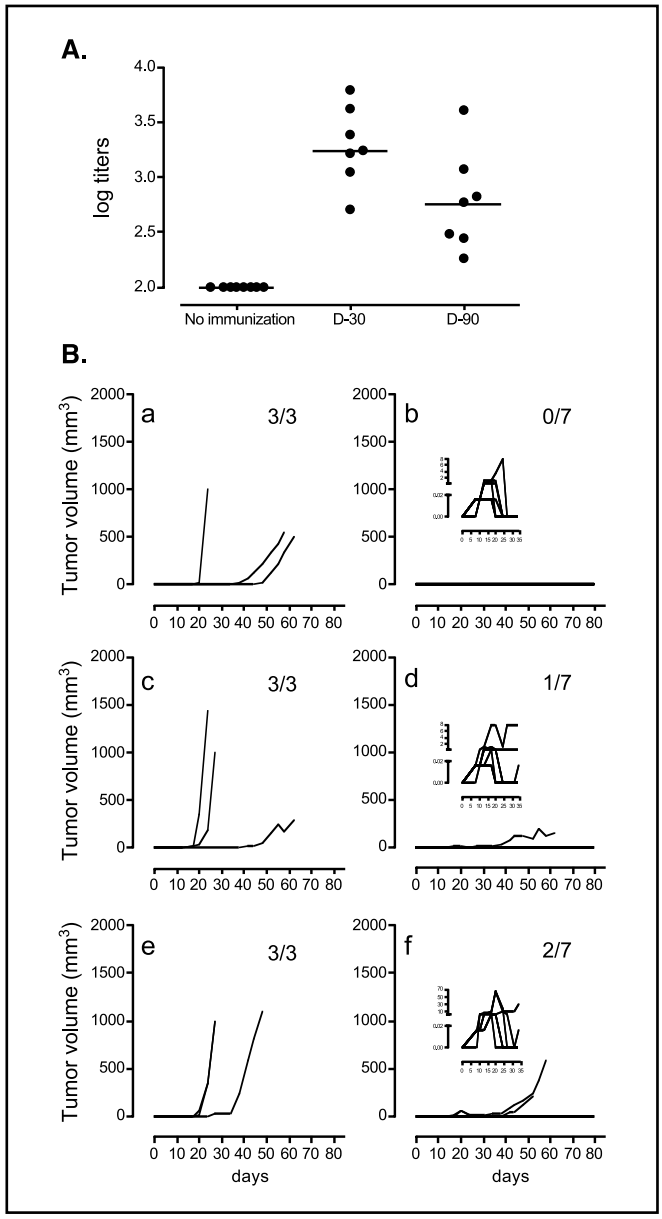

Figure 5. Analysis of the effect of preimmunity to CyaA on the ability of CyaA-E7$_{\Delta 30\text{-}42}$ to induce TC-1 tumor rejection. *A,* C57BL/6 mice were either left untreated or immunized at day –90 or day –30, with two injections i.d. at a 7-day interval of 10 μg of CyaAE5. At day −1, animals were bled and sera were individually assessed by ELISA for the presence of anti-CyaAE5 IgGs. Results are expressed as individual antibody titers calculated by linear regression analysis plotting dilution versus A_{492}. Horizontal bars represent the median response of each group. *B,* untreated (*a*, *b*), day −30 CyaAE5-immunized (*c*, *d*), and day −90 CyaAE5-immunized (*e*, *f*) animals were grafted s.c. on day 0 with 5 $\times$ 10^4 TC-1 tumor cells and were treated on days +10 and +17, with one i.d. injection of 10 μg CyaA-cysOVA (*a*, *c*, *e*), or 10 μg CyaA-E7 $_{\Delta 30\text{-}42}$ (*b*, *d*, *f*). *Insets* (*b*, *d*, *f*) are close-ups of the 0- to 35-day period to show that all animals had palpable tumors at the time vaccination was given. Each curve represents the tumor growth in a single animal. Mice were killed when the tumor sizes were > 1,000 mm^3 or whenever the sanitary status of the animals commanded. *Top right,* number of sacrificed animals versus the total number of animals included.

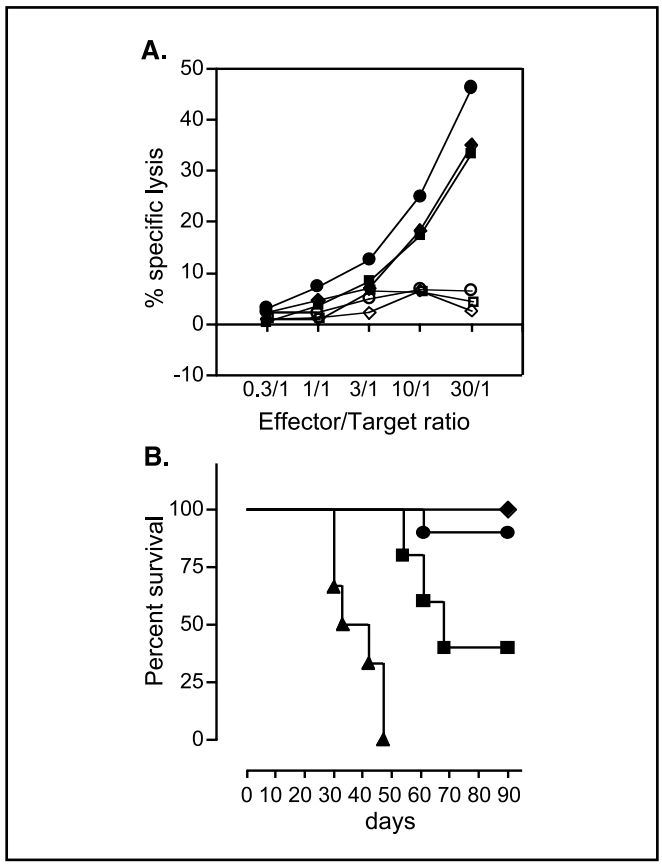

Figure 6. Persistence of functional HPV16-E7$_{49-57}$-specific CD8$^+$ T cells in mice treated with recombinant HPV16-E7 CyaAs. *A,* C57BL/6 mice immunized with CyaA-E7$_{49-57}$ (□, ■), CyaA-E7$_{Full}$ (◇, ◆), or CyaA-E7$_{\Delta30-42}$ (○, ●) and surviving from TC-1 grafts in the therapeutic set of experiments were sacrificed at day +90 and splenocytes were stimulated *in vitro* for 5 days with 1 μg/mL of the HPV16-E7$_{43-77}$ peptide in the presence of irradiated syngeneic splenocytes. Target lysis (TC-1, *closed symbols*; EL4, *open symbols*) were evaluated by ^{51}Cr release. The data represent the median percentage of the specific lysis values (n = 6). *B,* surviving C57BL/6 mice from TC-1 grafts in the therapeutic set of experiments were re-grafted s.c. at day +100 with 5×10^4 TC-1 cells (day of graft taken as 0). The survival curves of age-matched untreated mice (n = 6, ▲) or immunized animals with CyaA-E7$_{49-57}$ (n = 5, ■), CyaA-E7$_{Full}$ (n = 4, ◆), or CyaA-E7$_{\Delta30-42}$ (n = 10, ●) are shown. In all cases, the survival of recombinant HPV16-E7 CyaA-treated animals are significantly increased compared with that of untreated mice ($P < 0.05$).

the injection of 2.5 nmol of peptide supplemented with the potent adjuvant CpG ODN 1826.

In this study, we also assessed the effect of prior immunity to CyaA on the ability of this vector to further induce immunity to a foreign given antigen. Strong immunity to CyaA marginally affected the ability of CyaA-E7$_{\Delta30-42}$ to trigger rejection of established TC-1 tumors. Thus, we showed that immunity to CyaA does not neutralize the effects of subsequent vaccinations with E7-containing constructs. This is of importance in a clinical setting in which patients might be preimmune to *B. pertussis* CyaA due to prior whooping cough disease or vaccination and will, most probably, receive multiple injections of HPV16-E7-containing constructs.

Upon re-challenge with TC-1 cells, surviving mice immunized with HPV16-E7 recombinant CyaAs were selectively protected. This was correlated with the presence of HPV16-E7$_{49-57}$ CD8$^+$ T cells among the splenocytes of these animals. The better survival rate of mice immunized with recombinant CyaAs containing Th epitopes might indicate that providing cognate T cell help also results in an efficient recall of HPV16-E7$_{49-57}$ CD8$^+$ T cells. In this respect, it has been proposed that CD4$^+$ T cells, through CD40L, may imprint a unique molecular signature on effector CD8$^+$ T cells, endowing them with improved cytotoxic activity (47). In this experimental setting, it is unlikely that TC-1 re-challenge may have boosted HVP16-E7$_{49-57}$ CD8$^+$ T cells as Daemen et al. (48) showed that well-established TC-1 tumors are unable to either prime CTL responses, or to potentiate immune response already induced by vaccination.

In a validated preclinical model, we have shown that CyaA is an efficient vector to induce regression of established tumors as well as to provide protection against tumorigenic challenge over a long period of time. Although the TC-1 model is poorly related to *in situ* HPV-associated malignancies, it is presently the experimental system of reference to evaluate and compare novel immunotherapeutic approaches (14). CyaA-based immunotherapy precludes the need to select HLA-restricted epitopes as full proteins can be inserted, and avoids the use of plasmids or viral vectors that may contain potentially oncogenic HPV DNA sequences. We obtained best results with CyaA-E7$_{\Delta30-42}$, which was designed to avoid limitations in efficacy of CyaA catalytic domain translocation. This construction includes all the HPV16-E7 HLA class I and II epitopes described in the literature (49–51) and will therefore be selected as a candidate vaccine. Our present results indicate that potent antitumor immunity can be obtained by i.d. immunization using low doses of recombinant CyaAs carrying the HPV16-E7 protein. The fact that recombinant CyaAs harboring human melanoma epitopes have been shown to be efficiently processed by human dendritic cells to activate epitope-specific CTL clones (27), is also a strong argument in favor of their efficacy in humans.

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