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

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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 αMβ2 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). HPV5 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 HPV5 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-2Dβ-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...
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 presented and processed 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 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\(^{\text{d}}\)-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. Furthermore, the adenylate cyclase activity of CyaA was inactivated by inserting the dipeptide leucine-glutamine between codons 188 and 189 within the catalytic site (25). 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\({30-37}\) contains a nine-amino acid–long polypeptide sequence (RAHYNITVF) inserted between codons 224 and 235 of CyaA. To do so, two synthetic oligonucleotides (MWG, Courtabeuf, France), BTP1 (5'-CTA GCC GTG CCC ATT ACA ATA TTG TAA CCT GTG GTA C-3', coding strand) and BTP2 (5'-CAA AGG TTA CAA TAT GTT AGG GGC CGC G-3', noncoding strand) were annealed and ligated into the plTRE5C plasmid digested with NheI and KpnI. CyaA-E7\({30-37}\) contains the entire sequence (98 amino acids) of the HPV16-E7 protein inserted at the same 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 CAC TAC CTG GTT CAG AAC AGA TGC G-3'). The resulting PCR product was digested by NheI and KpnI and ligated into plTRE5 cleaved by NheI and KpnI as described above. CyaA-E7\({30-37}\) 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\({30-37}\) was constructed in two steps. A first DNA fragment encoding amino acid residues 1-29 of HPV16-E7 was PCR-amplified using HPV16-E7 cDNA as target DNA and primers BTP5 (5'-GGG CGC TAG CAT GCA TGG AGA TAC ACC TAC GCA-3') and BTP6 (5'-GGG CAC TAG CAT GCA TGG AGA TAC ACC TAC GCA-3'). A second DNA fragment encoding codons 320-372 of CyaA was PCR-amplified using plTRE5 as target DNA and primers BTP7 (5'-GGG CAC TAG TGA AAG CCA GAT GCT CAC GCG-3') and BTP8 (5'-GGG CAC TAG TGA AAG CCA GAT GCT CAC GCG-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 AgeI and BstBI and inserted between the corresponding sites of plTRE5 to yield plasmid plTRE5-CyaA-E7\({30-37}\). 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 CAT GCA TGG AGA TAC ACC TAC GCA-3') and BTP10 (5'-GGG CGC TAG CAT GCA TGG AGA TAC ACC TAC GCA-3'). The resulting PCR fragment was digested by NheI and KpnI, and ligated into plasmid plTRE5-CyaA-E7\({30-37}\) digested by the same restriction enzymes. All constructions were verified by DNA sequencing (Genome Express, Meylan, France).

All recombinant adenylate cyclases were produced in the E. coli strain BLR (Novagen, Madison, WI) as previously described (25). The recombinant proteins were purified to homogeneity from inclusion bodies by a two-step procedure that includes DEAE-Sepharose and phenyl-Sepharose chromatography (26). 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 CyaA-E7\(\text{E}^{\text{7-49-57}}\)**

The E. coli–optimized cDNA coding for HPV16-E7 protein (GTP Technology) was subcloned into pIEX2-4b vector (Roche Molecular Biochemicals, Meylan, France) between the Ncol and Xhol restrictions sites. The resulting plasmid was then transformed into the E. coli strain BL21DE3 (Novagen). The His-Tag-HPV16-E7 protein was expressed upon induction with 0.5 mmol isopropyl-\(\beta\)-D-thiogalactopyranoside (Euromedex, SouffleWeymersheim, France).
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 phoshphatase 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 × 10^6 TC-1 cells s.c. and were treated by HPV16-E7 recombinant CyaA 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 × 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 E749-57 or E743-77 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 51Cr-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 trypsinated (trypsin-EDTA, Life Technologies) and incubated with 100 μCi of 51Cr 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, Molsheim, 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 E749-57 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 Immunosorbtent 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 with various HPV recombinant CyaAs containing < 100 units of endotoxin per dose. The procedure was introduced in the purification protocol (29) to obtain recombinant proteins containing < 100% of endotoxin per dose. The contamination level by E. coli was below 2.5% (24, 93, and 52 for CyaA-E749-57, -E7Full, and -E730-42, respectively). The presence of the E7 protein in CyaA-E749-57, -E7Full, and -E730-42 was confirmed by Western blotting using a specific monoclonal antibody (Fig. 1C). 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. 1D, 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 E749-57 peptide. Their ability to lyse TC-1 cells was determined 5 days later using a 51Cr 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. 2A). Similar results were obtained when the peptide E749-57 was used for in vitro

**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.
Splenocytes from mice vaccinated with a recombinant CyaA carrying a nonrelevant epitope (OVA257-264) and restimulated in vitro with 1 μg/mL E730-42 peptide yielded a weak nonspecific TC-1 cell lysis (Fig. 2A). The HPV16-E7 protein or its deleted form resulted in a Th1-like profile eliciting 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.

The cell-mediated immune response ex vivo was determined after the restimulation of splenocytes with 1 μg/mL of the purified His-Tag HPV16-E7 protein. As shown in Fig. 2D, immunization with CyaA-E749-57, CyaA-E7Full, and CyaA-E7Δ30-42, respectively, 0.4 μg of each protein, D, immunoblot probed with a mouse anti-E. coli BLR serum. Lanes 1 to 3, CyaA-E749-57, CyaA-E7Full, and CyaA-E7Δ30-42, respectively (10 μg); lanes 4 to 8, E. coli BLR total extract (1, 0.5, 0.25, 0.12, and 0.06 μg, respectively).

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. 2D, immunization with CyaA 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 levels obtained after immunization with CyaA-E7Full and CyaA-E7Δ30-42 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 E730-42 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-2D^b-restricted T cell epitopes, are much higher than those obtained with CyaA-E749-57 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-2b tumorigenic cell line expressing HPV16-E6 and E7 proteins (TC-1 cells). In this model, tumor rejection is mediated by E749-57-specific CD8+ T cells (24, 25, 40, 41). Thus, 5 × 10^5 TC-1 cells were injected s.c. in the right flank of C57BL/6 mice and 50 μg of CyaA-E749-57, -E7Full or -E7Δ30-42 were injected i.v. to mice 1, 5, or 10 days later. Figure 3A 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. 3A, c, d, e, insets). Control animals developed tumors of a size > 1,000 mm^3 within a maximum of 49 days (Fig. 3A, 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. 3A, 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. 3A, f). CyaA-E7Δ30-42 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).

We tested another injection route of clinical interest: hence, 10 μg of CyaA-E7Δ30-42 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-E7Δ30-42 (Fig. 3B, a, b). This therapeutic immunization resulted in a 100% survival at 90 days of the CyaA-E7Δ30-42-treated mice, whereas the survival medians of untreated and mock-treated animals were 30 and 32 days, respectively (Fig. 3B, 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-E7Δ30-42 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-E7Δ30-42 to that of HPV16-E7 43-47 peptide supplemented with CpG ODN 1826 (42). Mice were therefore injected s.c. with 5 × 10^5 TC-1 cells and treated therapeutically 10 and 17 days later via the i.v. route with 10 μg of CyaA-E7Δ30-42 or 10 μg of HPV16-E7 43-47 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-E7Δ30-42 were slightly better but not statistically different from those obtained with HPV16-E7 43-47 peptide mixed with CpG ODN 1826. Of note, this result was obtained using 50 times more HPV16-E7 43-47 peptide than CyaA-E7Δ30-42 on a molar basis. When used alone, the peptide HPV16-E7 43-47 had no effect on TC-1 tumor growth.

Prior Immunity to CyaA Vector Marginally Affects the Therapeutic Efficacy of CyaA-E7Δ30-42. 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 × 10^5 TC-1 cells. Therapeutic treatment with two i.d. injections at a 7-day interval of 10 μg CyaA-E7Δ30-42 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. 5A). We then compared the ability of CyaA-E7Δ30-42 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<sub>30-42</sub> remained tumor-free throughout the experiment (Fig. 5B). Only one animal in the day 30 immune mice group, and two in the day 90 immune mice group developed tumors (Fig. 5B, b, d, f). In contrast, 100% of mock-treated animals developed tumors and were sacrificed (Fig. 5B, 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<sub>30-42</sub>-treated mice (Fig. 5B, 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<sub>49-57</sub>-Specific CD8<sup>+</sup> 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<sub>43-77</sub> peptide. Their ability to lyse TC-1 cells was then determined by a 51Cr release assay. As shown in Fig. 6A, specific CTL responses to HPV16-E7<sub>43-77</sub> 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<sup>4</sup> 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. 6B). 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<sub>full</sub> and CyaA-E7<sub>30-42</sub> displayed a higher level of protection than those vaccinated with CyaA-E7<sub>49-57</sub>. 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<sup>+</sup> and CD8<sup>+</sup> 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-2Db-restricted CTL epitope. We suggested that cognate T cell help provided by CyaA carrying the full E7 epitopes to the MHC class II and I presentation pathways of dendritic cells. In experimental and artificial tumor murine models, we showed 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-2Db-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

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**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<sup>5</sup> 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<sub>49-57</sub> (c), CyaA-E7<sub>full</sub> (d), or CyaA-E7<sub>30-42</sub> (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<sup>3</sup> 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<sub>49-57</sub> (●), CyaA-E7<sub>full</sub> (★). 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<sub>30-42</sub> (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<sub>30-42</sub> (★).
delivery of the HPV16-E7 H-2D\(^b\) CTL epitope by CyaA required a fully functional class I presentation pathway as CyaA-E7\(_{30-42}\) 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\(_{30-42}\)-specific CD8\(^+\) T cells, we observed that the frequencies of HPV16-E7-specific IFN-\(\gamma\)-secreting T cells induced by CyaA-E7\(_{full}\) and CyaA-E7\(_{30-42}\) were superior to those induced by CyaA-E7\(_{49-57}\). These observations indicate by the efficient CTL responses obtained in MHC class II-/- mice indicating that optimal CTL priming was independent of the presence of CD4\(^+\) T cells as CyaA-E7\(_{30-42}\) immunization 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 \(\mu\)g 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 against 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 \(\mu\)g CyaA-cysOVA (a, c, e) or 10 \(\mu\)g CyaA-E7\(_{30-42}\) (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.

Using the i.d. route, we compared CyaA-E7\(_{30-42}\) immunization to that of peptide HPV16-E7\(_{43-77}\) given with CpG ODN 1826 (42). Results highlighted the potency of CyaA-mediated immunization because it compared favorably to a therapeutic scheme based on
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 HPV16-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 \textit{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\(_{330-42}\), 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.

Acknowledgments


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References


Tumor Therapy Using Recombinant Adenylate Cyclase

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