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Advances in Brief

Plant-derived Human Papillomavirus 16 E7 Oncoprotein Induces Immune Response and Specific Tumor Protection[1]

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Abstract

Vaccine strategies for treatment of human papillomavirus-induced cervical cancer are based on either the recombinant E7 fusion oncoprotein or E7 CTL peptides. The therapeutic potential of the E7-based vaccine depends on the use of different adjuvants. In this study, we describe for the first time the expression of the human papillomavirus 16 E7 protein in *Nicotiana benthamiana* plant using a potato virus X-derived vector. C57BL/6 mice immunized with E7-containing crude foliar extracts developed both humoral and cell-mediated immune responses and were protected from tumor development after challenge with the E7-expressing C3 tumoral cell line. Our data support the possibility of producing a cost-effective anticancer vaccine in plant with intrinsic adjuvant-like properties.

Introduction

The HPV[4] is considered the causative agent of cervical cancer, a leading cause of cancer-related death among women in developing countries. The products of two early genes, E6 and E7, of the "high-risk" type (mainly HPV16 and 18) are critical factors of tumor development (1). E6 and E7 proteins are constitutively expressed in all of the cervical cancer cells and are considered "tumor-associated antigens," representing appropriate targets for HPV-associated cervical cancer immunotherapy.

Cell-mediated immune response is a critical component for HPV-associated disease development (2). The feasibility of immune intervention in the prevention and treatment of HPV16-induced malignancies was first suggested in a murine E7-expressing tumor model by vaccination with a MHC class I-restricted HPV16 E7 epitope. The peptide-specific CTL response was able to protect vaccinated mice against subsequent challenge with a tumorigenic dose of HPV16-transformed C3 cells (3). Additional studies confirmed that boosting of natural Th1/CTL-type immunity against HPV16 E6 and E7 proteins at an early stage of disease is a promising approach for the prevention and treatment of HPV-associated neoplasia, and now several candidate HPV vaccines are being evaluated in animal models and/or in human clinical trials (reviewed in Ref. 4).

In preclinical models, different E7-based vaccine formulations have been tested including peptides, viral vectors, chimeric virus-like particles, recombinant fusion proteins, and plasmid DNA (4). Vaccines based on recombinant E6 or E7 proteins seem to be more promising because the full-length proteins contain both CD4 and CD8 epitopes and do not require the HLA typing of the receiver. Moreover, protein-based vaccines present less safety concerns for clinical application compared with DNA- or animal virus-based vaccines.

The use of adjuvants and multiple immunizations is generally required for a potent immune response. In the case of E7-based vaccines, the choice of the adjuvant is crucial because it has been shown, in mouse models, that the use of different adjuvants is associated with different therapeutic potential against tumor growth and development (5).

In recent years, plant-based technologies have been used for the production of important biomedical reagents, including vaccine antigens. Plant-derived antigens are able to elicit immune responses, showing several advantages over traditional vaccine technologies, including efficacy, increased safety (no human or animal pathogen is known to infect plants), versatility, stability, and economy. In particular, small plus-sense single-stranded RNA plant viruses have emerged as promising tools, because they can be engineered to rapidly express foreign genes in susceptible host plants, producing larger amounts of proteins compared with those obtained by stable transformation procedures (6).

In this paper, for the first time we provide evidence that: (*a*) the PVX can be used to express the HPV16 E7 protein in the *N. benthamiana* tobacco plant as an unfused protein; (*b*) in a mouse model, the E7-containing crude plant extracts induce both humoral and cell-mediated immune responses without any added adjuvant; (*c*) vaccinated mice are protected from either establishment or progression of tumor after challenge with E7-expressing syngeneic tumor cells.

Materials and Methods

Construction of a PVX-based Expression Vector. The HPV16 E7 open reading frame was PCR-amplified from the plasmid E7-pGEX-4T1 (7) using the forward primer E7dir (5′ GGCCATCGATTCTAGACATGC*ATG*-GAGATACACCTACATTG 3′; the added *ClaI* restriction site is underlined and the E7 initiation translation codon is in italics) and the reverse primer E7rev (5′GGCCGTCGACCCCGGG*TTA*TGGTTTCTGAGAACAGATGGG 3′; the added *SalI* site is underlined and the stop codon is in italics). The PCR products were cloned in the polylinker site of the pPVX201 vector (PVXwt; Ref. 8) obtaining the plasmid pPVXE7 shown in Fig. 1*A*. Sequence authenticity was confirmed by sequencing.

Infection of Plants and Preparation of Soluble Protein Extracts from Leaves. Two leaves of 4-week-old *N. benthamiana* plants were dusted lightly with carborundum powder and inoculated with 10 μg of either pPVXE7 or pPVXwt plasmids, diluted in 100-μl bidistilled water. The inoculation was accomplished by gentle rubbing to spread the inoculum and additionally to abrade the surface. Plants were grown under 16-h daylight at 22°C and observed daily for infection signs. Symptomatic leaves were harvested and

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[4] The abbreviations used are: HPV, human papillomavirus; PVX, potato virus X; *Nb-PVXwt*, extracts from *Nicotiana benthamiana* leaves infected with PVX wild-type; *Nb-PVXE7*, extracts from *Nicotiana benthamiana* leaves infected with PVXE7; His-E7, histidine-E7 fusion protein; DTH, delayed-type hypersensitivity; ELISPOT, enzyme-linked immunospot; HRP, horseradish peroxidase.

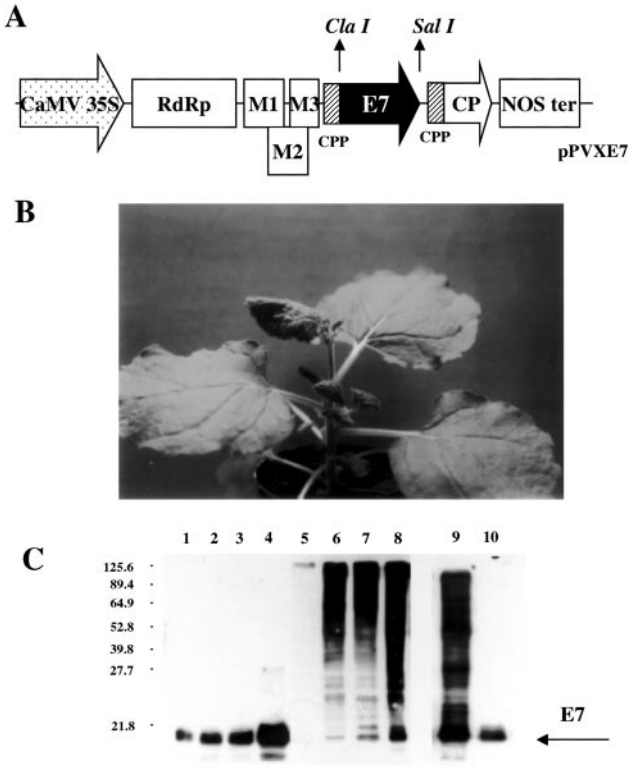

Fig. 1. Expression of the HPV16 oncoprotein E7 in *N. benthamiana* plant. *A*, schematic representation of the PVX-derived plasmid used for the expression of the E7 gene in plant (*pPVX-E7*). In this vector the full-length viral cDNA is inserted between the constitutive 35S promoter derived from the Cauliflower Mosaic virus (*CaMV 35S*) and the transcription terminator (*NOS ter*) from the nopaline synthase gene of *Agrobacterium tumefaciens* necessary for the regulation of the viral genome on infection with plasmid DNA. Features represented are as follows: viral replicase gene (*RdRp*); triple gene block encoding protein for cell-to-cell movement (*M1–3*); viral coat protein gene necessary for encapsidation of viral RNA (*CP*); coat protein promoter (*CPP*); and HPV 16 oncoprotein gene E7 (*E7*). *Cla I* and *Sal I* restriction enzyme sites for directional cloning are indicated. The drawings are not done to scale. *B*, *N. benthamiana* apical leaves with infection symptoms 7 days after inoculation with pPVXE7 plasmid. *C*, detection of the E7 protein in plant extracts. Samples of purified recombinant His-E7 protein and of *Nb-PVXE7* 7 days after inoculation were examined by immunoblot using the anti His-E7 mouse polyclonal antibody. *Lanes 1–4*, 13 ng, 26 ng, 40 ng, and 67 ng of recombinant His-E7 protein, respectively. *Lane 5*, 20 μg of total soluble proteins of *Nb-PVXwt*. *Lanes 6–8*, 5, 10, 20 μg, respectively, of total soluble proteins of *Nb-PVXE7*. *Lane 9*, total soluble protein (20 μg) containing 40 μg of purified His-E7. The sample was incubated 1 h at 4°C before loading. *Lane 10*, 40 μg of purified His-E7 added to total soluble protein extract (20 μg) from uninfected *N. benthamiana* leaves, boiled 10′. The sample was incubated 1 h at 4°C before loading.

stored in liquid nitrogen until use. Crude plant extracts were prepared by grinding the foliar tissue to a fine powder in liquid nitrogen. The powder was resuspended and homogenized in PBS (1 ml/0.3 grams of fresh leaves) containing protease inhibitors (Complete, EDTA free; Roche Diagnostics, Monza, Italy). Tissue homogenates were centrifuged at 4°C, 12,000 × *g*, for 10 min. The supernatant was transferred to a fresh tube and kept on ice (or at 4°C) until use. Total soluble protein content was estimated by the Bradford assay (Bio-Rad Inc., Segrate, Italy).

Homogenized tissues of infected plant were also used to inoculate *N. benthamiana* plants to propagate the infectious recombinant PVX particles.

Detection and Quantification of E7 Protein in PVX-infected Plants. Total soluble proteins (5–10-20 μg/lane) were separated by 12% SDS-PAGE and analyzed by Western blot performed using a 1:1000 dilution of a mouse polyclonal serum raised against the His-E7 protein produced in *Escherichia coli* using a recombinant pQ-30 vector (Qiagen Spa, Milan, Italy). The immunocomplexes were revealed using the enhanced chemiluminescence system (Amersham Pharmacia Biotech Italia, Cologno Monzese, Italy). The amount of E7 protein in the plant extracts was estimated by a quantitative triple antibody sandwich ELISA. Plant extracts (100 μl/well) were added to microtiter plates coated with an anti-E7 rabbit polyclonal antibody (9). The captured E7 protein was detected with a 1:1000 dilution of the anti-His-E7 mouse polyclonal antibody, followed by incubation with an HRP-conjugated goat antimouse IgG antibody (ICN Immunobiologicals, Costa Mesa, CA). Enzymatic activity was measured by adding 2,2 azino-di-3-ethylbenz-thiazoline sulphonate substrate, and the absorbance of the samples was read at 450 nm on an ELISA microtiter plate reader. Known amounts of purified His-E7 protein diluted in *N. benthamiana* plant extracts were used as a standard.

Immunization of Mice with Plant-derived Extracts and Detection of Anti-E7 IgGs. Female C57BL/6 mice (Charles River, Como, Italy) were maintained in specific pathogen-free conditions and used at 4–8 weeks of age. Institutional animal use guidelines were followed in all of the experiments. Groups of 10 mice were injected s.c. on days 0, 15, 30, and 45. Each mouse was inoculated with 500 μl of the following immunogens containing ~1 mg of total proteins: extracts from pPVXE7 infected *N. benthamiana* leaves containing 0.5 μg of E7 protein (*NbPVXE7*); purified His-E7 protein (0.5 μg) plus the adjuvant QuilA (Ref. 10; 10 μg/mouse, kindly provided by G. Fernando, University of Queenland, Princess Alexandra Hospital, Brisbane, Australia; *His-E7+QuilA*); and extracts from pPVXwt-infected *N. benthamiana* leaves (*Nb-PVXwt*). Each experiment was repeated at least three times. No plant extract-related toxicity was noticed in any of the vaccinated mice. Serum samples were collected from immunized mice 1 week after the second and fourth booster. At each point, the sera from mice of the same group were pooled and analyzed for the presence of E7-specific antibodies by ELISA. Microtiter plates were coated with 200 ng/well of His-E7 protein in bicarbonate buffer [50 mM $NaHCO_3$ (pH 9.6)]. Sera were diluted 1:50 and 1:100 in PBS containing 1% BSA, and then added to the coated wells. The different classes of IgGs were detected by a HRP-conjugated goat antimouse IgG (H+L; ICN Immunobiologicals) diluted 1:1000; HRP-conjugated goat antimouse IgG1, $IgG2_b$, IgG3; and by a HPR-conjugated rabbit antimouse $IgG2_a$ (ICN Immunobiologicals). The immunocomplexes were revealed as already described.

Spontaneous DTH to E7 Protein. DTH to E7 protein was assayed essentially as described by Dunn *et al.* (11). Briefly, 5 μg of His-E7 protein diluted in PBS were injected intradermally in one ear of vaccinated mice. Ear thickness was assessed 48 and 96 h after challenge using a microcaliper. The ears of a few mice remained unchallenged as a control. Ear swelling was reported as the difference in thickness between the challenged and the unchallenged control ear.

ELISPOT Assay for IFN-γ-secreting Cells. HPV16 E7-specific T-cell precursors were detected by ELISPOT, as described by Miyahira *et al.* (12). One week after the last booster, animals were sacrificed and spleens removed. Single cell suspension of splenocytes (2×10^5 cells/well), harvested from each group of vaccinated mice, was added to microtiter wells coated with a rat antimouse IFN-γ antibody (clone R4–6A2, 8 μg/ml; PharMingen, San Diego, CA) along with interleukin 2 (50 units/ml; Sigma-Aldrich Italia, Milan, Italy). Samples were incubated at 37°C for 24–48 h with 10 μg/ml of a peptide corresponding to the E7-specific $H\text{-}2D^b$ CTL epitope (amino acids 49–57, RAHYNIVTF; Ref. 3). Plates were incubated with biotinylated anti-IFN-γ antibody (clone XMG1.2, 2 μg/ml; PharMingen). Avidin-HRP (2.5 μg/ml; Sigma Aldrich) was then added, and the cell spots were stained by adding 0.22 μm filtered 3,3′-diaminobenzidine/peroxidase substrate (Sigma Fast; Sigma-Aldrich) for 15 min. The spots were counted using a dissecting microscope.

***In Vivo* Tumor Protection Experiments.** Mice vaccinated with the different E7-containing preparations were challenged, 2 weeks after the last booster, with the E7-expressing C3 tumor cells (5×10^5 cells/mouse) by s.c. injection on the flank (3). Tumor growth was monitored by palpation twice a week. Tumor volume was calculated as length × width^2 × 0.5. Ten unvaccinated mice received the same amount of C3 cells for a natural tumor-growth control.

Results

Expression of E7 Protein in Plants. The complete E7 open reading frame was cloned in a PVX-based vector under the control of a duplicated PVX coat protein subgenomic promoter (Fig. 1*A*). To test E7 expression, *N. benthamiana* plants were mock-infected, or infected with either pPVXE7 or pPVXwt DNA plasmids. Mock-infected plants showed no symptoms, whereas mild leaf deformation and mottling, typical signs of infection, were observed on the inoculated leaves infected with either pPVXE7 or pPVXwt 4–5 days after inoculation.

The infection spread systemically to apical leaves where symptoms generally appeared 7 days after inoculation (Fig. 1*B*).

To examine whether the E7 protein accumulated in infected plants, total soluble protein extracts were prepared from apical leaves and subjected to immunoblotting. A product of the expected molecular mass (~17 kDa) was identified in the *Nb-PVXE7* extracts, whereas no proteins were detected in *Nb-PVXwt* extracts (Fig. 1*C*). Besides the signal corresponding to the E7 protein, a smear corresponding to products with higher molecular mass was observed on the blot. These aggregates were resistant to SDS treatment and boiling. Reconstituted samples, containing *N. benthamiana* extracts and purified His-E7, showed a pattern similar to that of *Nb-PVXE7* extracts. However if the *N. benthamiana* extracts were extensively heat-treated (100°C/10 min) before the addition of the His-E7 protein, only a single band corresponding to the monomeric form of the E7 protein was present on the blot (Fig. 1*C*).

Successful infection and E7 expression were obtained after at least five reinfection cycles, demonstrating the stability of the expression vector. Animal immunization was performed using extracts from plants at the second or third infection passage.

The amount of the E7 protein in the extracts was estimated by quantitative ELISA to be approximately 3–4 μg/g fresh leaf.

E7 Expressed in Plants Induces an Immune Response. In preliminary experiments we established that the *Nb-PVXwt* had no toxic effect on mice. Groups of 10 C57BL/6 mice were immunized several times, at 2-week intervals, by s.c. administration of plant extracts, some containing the E7 protein and others without it. A group of mice was immunized with His-E7+QuilA, known to be a vaccine preparation, which is effective in inducing immune responses.

Whereas the *His-E7+QuilA* preparation induced a high titer of specific IgGs after the second booster, *Nb-PVXE7* reached the same titer after the fourth booster. The other preparations showed a very low IgG induction (data not shown).

Although it is impossible to come to unequivocal conclusions regarding the type of T-helper responses, the mouse isotypic profile is often used as a marker of the induced immune response type. Therefore, we examined the different IgG isotypes (IgG1, IgG_{2a}, IgG_{2b}, and IgG3) in the sera taken after the last booster. As clearly shown in Fig. 2, the IgG subtype profiles are quite different. The *HisE7+QuilA* profile with high levels of IgG_{2a} is suggestive of a Th1 response as already reported for a vaccine preparation containing a GST-E7 fusion protein together with QuilA (13). On the other hand, the *Nb-PVXE7* profile shows a stimulation of all of the isotypes including high levels of IgG3, indicating that both Th1 and Th2 responses are induced in these animals.

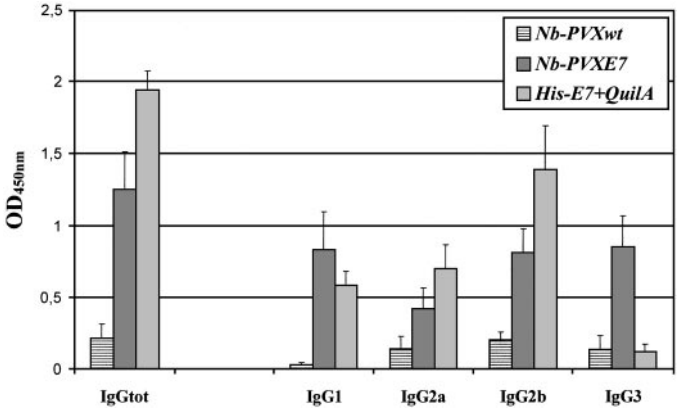

Fig. 2. Total E7-specific IgGs and IgG isotypes in sera of vaccinated mice. Sera from mice immunized with either crude *Nb-PVXE7* or *His-E7+QuilA*, or crude *Nb-PVXwt* were subjected to different ELISAs using His-E7 as a coating antigen and anti-IgGs or isotype-specific secondary antibodies; *bars*, ± SE.

E7 Expressed in Plants Stimulates E7-specific Cytotoxic T-Cell Immune Responses. Because $CD8^+$ cytotoxic T cells have a recognized role as effectors in anticancer responses, the induction of E7-specific $CD8^+$ T cells was investigated in the vaccinated animals of each group by ELISPOT. Splenocytes were activated by an E7-specific CTL epitope, and IFN-γ-secreting cells were visualized as spots by an anti-IFN-γ monoclonal antibody. The results obtained from the different vaccinated mice are reported in Fig. 3. The highest score in the spot number was obtained in the mice vaccinated with *Nb-PVXE7*. Very low levels of E7-specific $CD8^+$ cells were found in mice vaccinated with the *His-E7+QuilA* preparation. No spots were detected in both unvaccinated and *Nb-PVXwt* vaccinated mice.

Plant-derived E7 Antigen Induces DTH to E7 Protein. Protection against the development of HPV-associated disease is thought to be primarily associated with the cellular arm of the immune system. To evaluate the activation of this arm of the immune system, we investigated the DTH to the HPV16 E7 protein in the vaccinated mice. DTH responses, thought to represent an antigen-specific cytokine-mediated inflammation, particularly involving Th1-type cytokines, have been reported previously in mice immunized with E7 (11).

Groups of five mice were vaccinated with either *Nb-PVXE7* or *His-E7+QuilA* preparations. A control group was injected with *Nb-PVXwt* After E7 challenge, the ear swelling was measured and recorded as the average of the difference between challenged and control ear (mm ear thickening $\times 10^{-2} \pm$ SE). A positive DTH response, peaking at 48 h, specific to the E7 protein, was induced in mice vaccinated with *Nb-PVXE7* with an ear thickening of 22 ± 3 mm $\times 10^{-2}$. This response was comparable with that induced by the His-E7+QuilA preparation (18 ± 2 mm $\times 10^{-2}$). The E7 challenge of nonimmunized or *Nb-PVXwt* -immunized mice produced no significant ear swelling (2 ± 1 mm $\times 10^{-2}$), excluding any inflammatory effect of the challenge antigen.

Plant-derived E7 Vaccine Protects Mice against HPV16 E7-expressing Tumors. To determine whether the cellular immune response to the E7 protein resulted in antitumor activity, vaccinated mice were challenged with C3 cells, an embryonic mouse cell line expressing HPV16 proteins including E7 (3). Plant-derived vaccine elicited a tumor protection in ~40% of the animals. The same data were obtained in the mice vaccinated with a comparable amount (0.5 μg/mouse) of His-E7 protein plus QuilA (Fig. 4*A*). This antitumor activity appeared to be long lasting as the tumor-free animals rechallenged with C3 cells in the opposite flank to the first inoculation remained tumor-free for up to 4 weeks. Interestingly, in the group of animals vaccinated with the E7-containing foliar extracts, the remaining 60% of the animals showed delayed tumor growth and a marked reduction in tumor burden (Fig. 4*B*). This effect on tumor growth was not detected in mice vaccinated with *His-E7+QuilA* preparation.

Discussion

There is now abundant experimental evidence indicating the need for vaccines against viral and/or tumor antigens, which are able to stimulate the cell-mediated immunity carried out by $CD8^+$ CTL (14). Treatment of HPV-associated diseases will benefit from therapies that boost natural immune-mediated tumor defense mechanism and that focus the immune response on the relevant tumor antigens.

Several vaccine strategies against HPV-induced cervical cancer are now being evaluated in clinical trials (4). The approach based on the delivery of HPV tumor-associated antigens, E7 and E6, as full-length proteins, has the advantage that the proteins contain potentially immunogenic epitopes for every MHC haplotype.

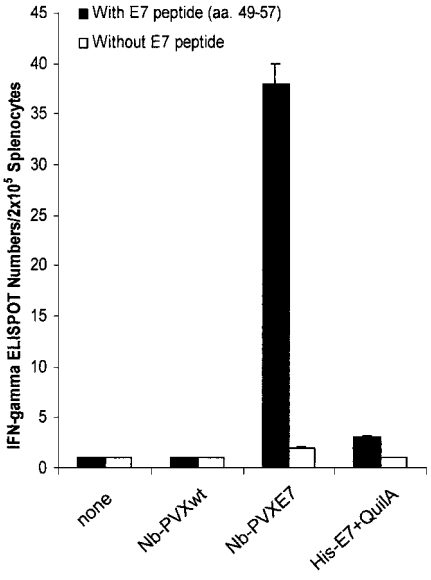

Fig. 3. ELISPOT analysis of splenocytes of vaccinated mice. C57BL/6 mice were vaccinated with *Nb-PVXwt*, *Nb-PVXE7*, *HisE7+QuilA*, or not vaccinated. Splenocytes were recovered from sacrificed animals after the last booster and were stimulated with specific CTL E7 peptide (amino acids 49–57). The number of IFN-γ producing E7-specific T-cell precursors was determined using an anti-IFN-γ antibody. The number of spots was recorded manually by two different readers and expressed as mean per 2×10^5 splenocytes. *Solid and open columns* refer to activated and unactivated cells, respectively. Results shown here are from one representative experiment of three performed; *bars*, ± SE.

E7 is a short-lived multifunctional protein interacting with several proteins in animal and human cells (15). Attempts to produce large amounts of sequence-authentic, nonfused E7 protein by baculovirus or *E. coli* expression systems have been unsuccessful because of the low yield and rapid degradation (16). In this study, we report the expression of the HPV16 E7 protein in *N. benthamiana* tobacco plants using a vector derived from the virus PVX, already used to express soluble proteins (8, 17). No modifications were introduced in the E7 gene, and the E7 protein was produced in both inoculated and systemic leaves. After homogenization in PBS without detergent and centrifugation, the E7 protein was found in the aqueous supernatant suggesting that it is produced as a soluble protein. No loss of E7 expression was observed after several plant passages, indicating that the PVX-E7 recombinant virus is quite stable and that this system could be useful for large-scale preparation of the E7 protein.

The presence of high molecular weight aggregates in the foliar extracts might indicate that the E7 protein forms stable complexes with plant components, possibly plant proteins. Recently, plant proteins homologous of the human retinoblastoma-related proteins have been described, reinforcing the hypothesis of a possible interaction between E7 and plant proteins (18). The complex formation might stabilize the protein preventing degradation by endogenous peptidases. The presence of stable macro-aggregates of E7 in crude plant extracts, resembling the complexes that can be formed in immunogen preparations with adjuvants, prompted us to explore the possibility that the E7-containing foliar extracts could be *per se* an antigen with adjuvant-like activity. It is well known that plant cell components possess adjuvant potential, and the adjuvant QuilA, proved to be active in this system, is of plant origin (10).

In animal models it has been clearly established that the presence of at least HPV E7-specific CD4/CD8 lymphocytes correlates with the protection against challenge with a HPV E7-expressing tumor (3, 5). Our results are consistent with the induction of both humoral and cell-mediated immunity in mice immunized with *Nb-PVXE7*. The presence of anti-E7 specific IgGs in the serum of vaccinated mice indicates that E7-containing foliar extracts are able to induce a humoral response. However, this humoral response is lower than that obtained with the recombinant *His-E7+QuilA* preparation. The isotypic profile of specific IgG antibodies clearly indicates that in the mice immunized with the *Nb-PVXE7* preparation both Th1 and Th2 responses may be present, whereas in mice vaccinated with *His-E7+QuilA* the response seems prevalently of Th1 type. This result shows that the *Nb-PVXE7* is a promising vaccine preparation because the simultaneous induction of both Th1 and Th2 responses has been shown to be necessary for a good antitumor immunity (19).

In mice vaccinated with E7-containing foliar extracts, the DTH and ELISPOT data are consistent with the induction of an anti-E7-specific cell-mediated immune response. The mice vaccinated with either E7-containing foliar extracts or the *His-E7+QuilA* preparation showed a similar pattern of tumor protection; 40% of mice were tumor-free 60 days after challenge with the E7-expressing C3 cells, whereas the control mice and mice vaccinated with the foliar extracts from pPVXwt-infected plants, were all tumor affected 7 days after the challenge. These data clearly demonstrate that E7 plant extracts are able to induce tumor protection and that there are no unspecific antitumor effects mediated by the plant extract on its own.

The tumors induced in both groups of mice vaccinated with E7-containing preparations were reduced in volume compared with the tumors present in both *Nb-PVXwt* and untreated animals. The reduction was more evident on *Nb-PVX E7*-vaccinated mice, in which the tumor volume at day 60 was the 15% of that found in untreated mice.

It has been reported that recombinant E7 protein purified from *E. coli* and yeast is able to induced a Th1 response in vaccinated mice only when injected with adjuvants, like QuilA, conferring a protection

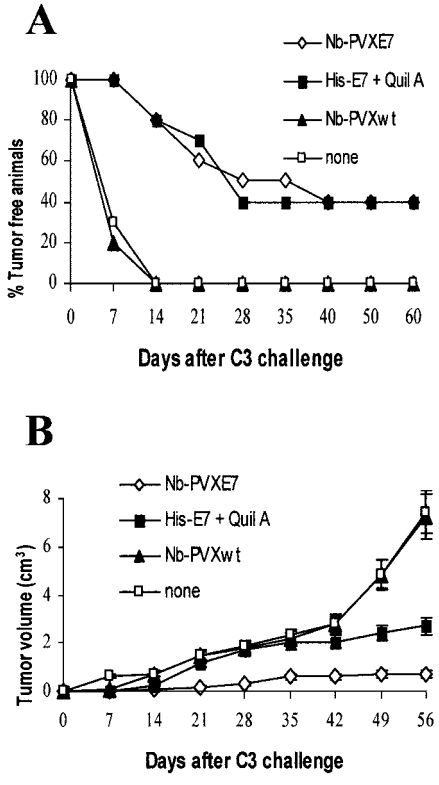

Fig. 4. Mouse protection against C3-induced tumor. *A*, mice were vaccinated on day 0, 15, 30, and 45 with *Nb-PVXwt*, *Nb-PVXE7*, *HisE7+QuilA*, or not vaccinated. Two weeks after the last booster the different groups of mice (10/group) were challenged s.c. with 5×10^5 C3 cells per mouse. The presence of the tumor was monitored by palpation twice a week. *B*, the dimensions of the developed tumors was recorded by a caliper; the tumor volume was calculated as width^2 × length × 0.5 and expressed as mean; *bars*, ± SE.

close to 100% after challenge with E7-expressing tumor cells (20). In these experiments, the amounts of E7 protein was higher than in our preparations (10–50 μg *versus* 0.5 μg). It is reasonable to assume that a higher protection in animals could be achieved by increasing the E7 expression level in plant. On the other hand these data confirm the adjuvant-like effect of the plant extracts, showing that the dose of antigen necessary to acquire tumor protection can be decreased.

Our results suggest that both B- and T-cell epitopes are presented on the E7 protein expressed in *N. benthamiana* plants, as the E7-containing foliar extract not only induces a humoral but also a cell-mediated immune response, and that the plant extract acts *per se* as a potent adjuvant. One explanation of this function may be the peculiar ability of the E7 protein in interacting with plant cell components, producing macro-aggregates able to induce strong Th1 immune response. It would be of interest to known if the adjuvant-like properties can be applied to other antigens. Studies on the stimulation of dendritic cell by E7 plant extracts as well as experiments with other plant-expressed antigens would clarify this issue.

In conclusion, the reported data support the idea to produce a reliable antitumor vaccine in plants using a new and low-cost procedure.

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