Recombinant Human Papillomavirus Type 16 E7 Protein as a Model Antigen to Study the Vaccine Potential in Control and E7 Transgenic Mice

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Abstract

The early genes E6 and E7 of human papillomavirus type 16 (HPV16) are consistently and exclusively expressed in HPV16-induced cancer lesions and play major roles in the development and maintenance of the malignant phenotype. Because this protein is a good example of a tumor-associated antigen, we have used E7 as a model antigen to test the potential of an experimental vaccine as an immunotherapeutic approach. In this study, we used a murine E7-expressing tumor model (TC1 cells) to assess effects of an E7-based vaccine on tumor growth. We show that vaccination with the E7 protein, formulated in the SmithKline Beecham Biologicals proprietary adjuvants (SBAS 1 and SBAS 2), leads to the rejection of pre-established tumors. Tumor rejection was associated with the induction of a strong systemic T helper 1 response, including CTLs, and the presence of an inflammatory infiltrate within the regressing tumor. Because most identified tumor-associated antigens are self antigens rather than viral antigens, we used E7 transgenic mice to evaluate the E7-based vaccine in conditions where E7 is a self antigen. Transgenic mice, which constitutively and specifically express the E7 HPV16 gene in the thyroid epithelium, rapidly develop thyroid goiters and, after several months, thyroid carcinomas. We show that E7-specific antibodies and CD4 T helper responses can be obtained by vaccinating E7 transgenic mice, although a CTL response was not detected. Despite the absence of measurable CTL responses, vaccination still reduced the growth of pre-established TC1 tumors, although less efficiently than in nontransgenic animals, but was unable to suppress or delay the development of the spontaneous thyroid pathology.

Introduction

The existence of tumor rejection mechanisms has been recognized for several decades. Apart from those viral proteins such as the E6 and E7 proteins from HPV16, which are specifically expressed in cancer cells and involved in the transformation process, most tumor-associated antigens are self proteins or mutated versions of self proteins, which in theory are not recognized by the immune system. However, both types of antigen can occasionally induce detectable immune responses in cancer patients, including antibodies and T-cell responses.

These naturally occurring immune responses are largely ineffective in eliminating tumors. An effective tumor rejection response requires a series of immune amplification phenomena dependent on the intervention of antigen-presenting cells responsible for delivery of a variety of stimulatory signals to T cells, which then release cytokines. Nonspecific effector cells, such as macrophages, NK cells, or granulocytes (eosinophils and neutrophils) can also be recruited and activated by T cell-derived factors.

There are many different experimental vaccine strategies under development for cancer treatment, all aimed at generating a strong cellular immune response targeting identified tumor-associated antigens (reviewed in Ref. 5). We have chosen a protein-based vaccine approach that has the advantage of being applicable to individuals with various HLA backgrounds and to present both CD4 and CD8 epitopes to the immune system. Protein antigens are well-characterized and well-defined molecules with good safety profiles, but they are poor immunogens per se, and as a consequence, they need to be mixed with effective adjuvants. Indeed, the expected role of the adjuvant should be to deliver the protein through the correct antigen presentation pathway, lead to class I and class II presentation, and redirect and eventually enhance a natural immune response to overcome the tolerance to self protein. SmithKline Beecham Biologicals have developed two adjuvant systems containing the immunostimulators MPL (6) and QS21 (7) in a liposomal formulation (SBAS 1) or in an oil-in-water emulsion (SBAS 2). The latter has already been shown to improve both humoral and cellular immune responses to malaria antigens and to provide protection against malaria challenge in humans.

HPV16 E6 and E7 proteins are expressed by HPV-infected cells and are involved in the development of HPV-induced lesions and cervical cancer. Both proteins are tumor-specific antigens and good potential model antigens to evaluate experimental vaccine strategies for cancer immunotherapy. Most tumor antigens identified thus far are self

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3 The abbreviations used are: HPV16, human papillomavirus type 16; NK, natural killer; Tg, transgenic.
proteins, some of which are not expressed exclusively in the
tumor but are also expressed at low levels in certain normal
tissues. It is important therefore to determine whether an
effective antitumor immune response induced by a vaccine
can also induce autoimmunity to the normal organs expressing
background levels of the same antigen.

We describe here the use of E7 Tg animals as a way to mimic this situation. This system provides a unique opportunity to study the impact of the E7 transgene expression on: (a) the ability of these animals to mount an immune response upon vaccinations with the E7 protein in the presence of adjuvants and compare this with normal mice; (b) the ability of the induced immune response to stimulate autoimmune thyroiditis; and (c) the ability of E7 Tg animals to reject an E7-expressing tumor either s.c. implanted in mice or spontaneously developing in these animals as a consequence of the oncogenic potential of E7.

Materials and Methods

Molecular Biology

Construction of Expression Plasmid. The genomic E7 sequence (10) was amplified from the HPV16 full-length genome (obtained from Deutsches Krebsforschungszentrum, Heidelberg, Germany) and subcloned into a plasmid containing the first 109 amino acids of Hemophilus influenzae protein D (PD-E7). An affinity polyhistidine tail was added to the COOH terminus to simplify purification. The plasmid containing the E7 transgene transcrip-
tion is driven by the thyroglobulin gene promoter, which is specifically and exclusively in the thyroid epithelial cells. E7 expression is too low to be detected by immunohistochemistry but can be shown by Northern blot analysis (11). Although weak, a consequence of the constitutive E7 expression in the thyroid cell is induced proliferation, and mice rapidly develop goiters, followed at the age of ~1 year by the development of thyroid carcinoma in all of the animals (11). Tg animals are heterozygotes for the HPV E7 transgene. Nontransgenic litter-
mates were used as controls in these experiments. All animals were maintained in pathogen-free conditions.

Tumor Cell Line. TC1 tumor cells were kindly provided by T. C. Wu (Johns Hopkins University, Baltimore, MD). They were generated from primary lung cells of C57BL/6 mice by the successive transfer of HPV16 E6 and E7 genes and an activated ras oncogene as described previously (12). The EL4 tumor cell line was obtained from American Type Culture Collection (TIB-39).

Cells were grown in RPMI 1640 (BioWhittaker) containing 10% FCS and additives [2 mm L-glutamine, 1% antibiotics (10,000 units/ml penicillin and 10,000 μg/ml streptomycin), 1% nonessential amino acid 100X, 1% sodium pyruvate (Life Tech-
nologies, Inc., St. Louis, MO), 50 μM 2-mercaptoethanol] and maintained at 37°C in humidified air containing 7% CO2.

In Vivo Tumor Regression Experiments. TC1 cells (passage <8) were used in all experiments after being trypsinized and washed in serum-free medium before injection. Nontransgenic or Tg C57BL/6 mice received s.c. 5 x 10^5 or 10^6 TC1 cells in 200 μl medium in the flank. Mice were monitored twice weekly for evidence of tumor growth. Any apparent tumors were measured (longest width and length) and expressed as their area (product of these two measurements) in mm^2.

On days 7 and 14 after TC1 challenge, mice were vaccinated in the footpad with 5 μg of PD-E7 protein either alone or in one of the adjuvant formulations. Animals showing evidence of illness or distress during the tumor growth period were sacrificed immediately. All experiments were repeated at least twice. Representative experiments are shown.

Immunological Read-Out

In Vitro Lymphoproliferation. Lymphoproliferation was performed on spleens and/or popliteal lymph node pools. Cells (2 x 10^5) were plated in 96-well plates. After 72 h of in vitro restimulation with different amounts of PD-E7 (0.1, 1, or 10 μg/ml), fresh medium containing 1 μCi of [3H]thymi-
dine (5 Ci/mmol; Amersham) was added for an additional 16 h, after which cells were harvested onto filter plates, and the incorporated radioactivity was counted in a beta counter. Results are expressed in cpm (mean of triplicate wells) or as stimulation index (mean cpm in cultures with antigen/mean cpm in cultures without antigen).

CTL Assay. Spleen cells (2 x 10^7) were cocultured with 2 x 10^6 irradiated (18,000 rads) TC1 cells for 5–7 days. Target cells used to assess cytotoxicity were either TC1 cells loaded with 51Cr (DuPont NEN; 37 MBq/ml) for 1 h at 37°C or EL4 cells loaded with the E7-derived peptide (AAag_s7) compared with EL4.

Two thousand target cells were added to each well of 96-well plates (Maxisorb Immuno-plate; Nunc, Roskilde, Den-
mark) with 100:1 being the highest E:T ratio. Target cells in medium alone or in 1.5% Triton X-100 were used to measure spontaneous or maximal 51Cr release, respectively. The 51Cr released into the supernatant was counted after 4 h incubation at 37°C in 7% CO2. Data are expressed as (experimental release – spontaneous release)/(maximal release – spontaneous release) × 100.

Serology. The presence of anti-E7 antibody was measured by ELISA using a recombinant nonfused E7 protein (provided by Dr. A. Bollen; Service de Genetique Applique, Université Libre de Bruxelles, Gosselies, Belgium) as coating antigen. Antigen was adsorbed overnight at 4°C in 96-well microtiter plates. Serial dilutions of sera were added to the E7-coated plates and incubated for 90 min at 37°C. After a washing step, biotin-conjugated antimouse IgG1, IgG2a, or IgG2b or total IgG (Amersham, Buckinghamshire, United Kingdom) was added, followed by the streptavidin-biotinylated peroxidase complex (Amersham) and subsequently tetramethylbenzidine. The reaction was stopped with 4 N H2SO4 and read at 450 nm. Midpoint dilutions were calculated using SoftmaxPro (using a four-parameter equation).

Immunohistochemistry. Tumor samples were frozen either in isopentane to perform immunohistochemistry or fixed in paraformaldehyde for classical histology. Cryosections of tumors were stained with antibodies specific for CD4, CD8, NK (Ab PK136), and MAC1 + cells (Serotec, Oxford, United Kingdom). Briefly, sections were air dried, fixed in acetone containing 0.3% H2O2 for 10 min, rinsed, and then fixed in paraformaldehyde for 15 min. Sections were then saturated with PBS containing 0.5% BSA and 5% normal rabbit serum for 30 min at room temperature. The first antibody was added and incubated overnight at 4°C. After a wash, the rabbit antirat immunoglobulin Biot was added for 30 min. After a washing step, streptavidin-horseradish peroxidase (Zymed Lab, San Francisco, CA) was added for an additional 30 min. Sections were washed and incubated for 10 min with diaminobenzidine before counterstaining with hematoxylin and dehydration. All these steps were performed at room temperature.

Results

E7-expressing Tumors Are Rejected in Mice Immunized with E7 Recombinant Protein Formulations. To evaluate the therapeutic potential of protein-based vaccines, we used the rate of tumor growth in the TC1 tumor model (12), after the injection of the recombinant fusion protein PD-E7 (5 μg), mixed with the SBAS 1 and SBAS 2 adjuvant systems, in the footpad. The results of a representative experiment are presented in Fig. 1 showing the evolution of the mean tumor size in groups of four to five animals over 38 days. Injection of 5 × 105 TC1 cells gave rise to a progressively growing tumor in 100% of the nonvaccinated animals as well as in those groups of mice vaccinated with the PD-E7 protein or either of the adjuvants alone. As shown in the figure, curves for the groups of nonvaccinated animals, or those given adjuvants alone, end before the 38 days because of the deaths of most of these animals. Interestingly, although PD-E7 alone did not result in elimination or retardation or tumor growth, all of the animals in this group survived through 38 days. In groups of mice that received PD-E7 formulated in SBAS 1 or SBAS 2, the mean tumor size remained very low, reflecting a marked slowing in the rate of tumor growth, particularly after the second vaccine dose on day 14; by the end of the surveillance period, 80–100% of the mice in groups given PD-E7 in an adjuvant completely rejected their tumors.

These tumor-free animals were shown to resist a second challenge with TC1 cells when applied 2 months later in the opposite flank, remaining tumor free for at least another 2 months. Increasing the number of doses from two to four or increasing the antigen dose from 5 μg to 15 or 40 μg further increased the therapeutic potential of the vaccines. However, if the first vaccination was delayed until day 14 after the tumor challenge, complete rejection could not be observed, although the tumor growth still appeared to be slowed (data not shown).

To demonstrate the specificity of the therapeutic effect observed with the HPV16 PD-E7 protein, mice were immunized according to the same experimental protocol with 5 μg of purified recombinant E7 protein from HPV18 (both in the presence of SBAS 2). Although the E7 proteins from HPV18 and HPV16 share some degree of homology (46% identity in the amino acid sequence) and induced similar immune responses, no effect on tumor rejection was observed with the HPV18 E7 (Fig. 2), and the mortality rate after 21 days was similar to the control group. This indicates that the antitumor immune response is specific for the HPV16-derived molecule and is able to discriminate between closely related proteins.

Vaccination Using the PD-E7 Protein in Adjuvants Induces Both Humoral and T Helper 1-Type Cellular Immune Responses Including CTLs. The lymphoproliferative responses, presented as stimulation indices, obtained in the different groups of mice after a 72-h in vitro restimulation with 1 and 10 μg of E7 protein are shown in Fig. 3. No significant
proliferative response was observed with spleen cells from mice given PD-E7 or adjuvants alone, but a high lymphoproliferation was obtained in the groups of mice given PD-E7 in the presence of SBAS 1 or SBAS 2, indicating that T cells have been efficiently primed in vivo by these vaccine formulations.

Two weeks after the last vaccination, spleen cells were restimulated in vitro with irradiated TC1 cells to determine cytolytic activity. After a 7-day stimulation period, a CTL response (20–50% of specific lysis) was detectable, but only in mice that had received the PD-E7 in SBAS 1 or SBAS 2. When mice received the protein or the adjuvant alone, no lysis was observed. When tumor-free and tumor-positive animals were analyzed separately at 2 weeks after dose 2, there was a trend to have a slightly higher CTL response in the tumor-free mice (Fig. 4).

**Humoral Response: Isotypic Profile of Anti-E7 Responses.** Anti-E7 antibody responses, total IgG, and isotypes (IgG1, IgG2a, and IgG2b) were measured by ELISA using the E7 protein as a coating antigen, as described in “Materials and Methods.” Table 1 shows the response 2 weeks after the second vaccination in the pooled samples from each of the experimental groups. The weak antibody response induced after two doses of PD-E7 alone was strongly increased in animals that received the protein with adjuvant. The strongest response was obtained with the SBAS 2 adjuvant.

A mixed antibody response was triggered by the protein with adjuvant; the levels of all IgG subclasses tested (IgG1,
IgG2a, and IgG2b) were higher in these mice than in mice that received injections of the antigen or the adjuvants alone. The predominant E7-specific antibody subclass was clearly IgG2b (80–90% of the total IgG), which in C57BL/6 mice corresponds to the induction of a T helper 1 type of immune response (13).

Vaccination with PD-E7 and Adjuvant Induces Massive Infiltration of the Tumor with T Lymphocytes and Inflammatory Cells. To observe what occurs at the tumor site, pieces of tumors (two samples/group) were taken and analyzed for the presence of different cell populations. Cryosections of regressing tumors were stained with the appropriate antibodies to detect inflammatory and T cells. In the groups of mice that received the PD-E7 protein in SBAS 1 or SBAS 2, there was evidence of necrotic areas and a massive infiltration that was immunologically positive for the presence of many CD8-, NK-, MAC1-, CD86-, MHC class II-, and some CD4-positive cells (Fig. 5). There was no evidence of such infiltration in tumor samples from animals given PBS, the PD-E7 antigen, or the adjuvants alone.

Therapeutic Potential of Vaccination with PD-E7 and Adjuvants in E7 Tg Mice. The E7 antigen in normal mice is an exogenous viral antigen. However, most tumor-associated antigens discovered thus far are self antigens resulting from either genetic alteration or the specific reactivation of a gene in tumors, or from genes expressed at low levels in normal tissue becoming overexpressed in tumors. To address this situation, we investigated the effect of PD-E7 vaccination in E7 Tg mice in which expression of the E7 transgene in the thyroid is believed to induce a certain level of tolerance.

To assess the impact of the E7 transgene expression, we initially studied the ability of such animals to mount an immune response upon vaccination with the PD-E7 protein in the presence of adjuvants and then analyzed the ability of the E7 Tg animals to reject a pre-established E7-expressing tumor either implanted s.c. or spontaneously developing in the thyroid as a consequence of the E7 expression.

E7 Tg Animals Reject Pre-established E7-expressing Tumors Less Efficiently Than Control Mice. Tg and littermate control mice received 10⁶ TC1 tumor cells s.c. in the flank and were then immunized following the same protocol as described previously, i.e., two injections of either PBS or 5 μg of PDE7 formulated in SBAS 1 or SBAS 2, respectively, on days 7 and 14. Tumor growth was measured over the following 4–5 weeks, and the immune response was analyzed 2–3 weeks after the second vaccination. The s.c. injection of TC1 cells gave rise to progressively growing tumors in 100% of the E7 Tg animals. Growth of the tumors in PBS-vaccinated E7 Tg mice appeared slightly more aggressive than in nontransgenic control animals, which could suggest a priming by the tumor in the controls, although control mice died earlier (Fig. 6). In E7 Tg mice receiving PD-E7 with adjuvants, the mean tumor size was clearly decreased (by 40–50%), and survival was obviously improved, but the antitumor effect was less than in their similarly vaccinated nontransgenic littermates, where 80–100% resection was observed.

E7 Tg Animals Develop an Antibody Response and Lymphoproliferative Response against the E7 Transgene upon Vaccination, But CTLs Are Not Detectable. E7 Tg mice did not develop a spontaneous immune response against the E7 protein alone, i.e., there were no detectable E7 antibodies, no E7-specific lymphoproliferation, and no CTL responses. In contrast, after two doses of PD-E7, E7 Tg mice developed an E7-specific antibody response and a good E7-specific lymphoproliferation after in vitro restimulation of spleen cells with the protein. The lymphoproliferation was slightly lower than in the controls (not shown), and although the total IgG response was similar, there was a different isotypic profile, with predominance of the IgG1 subclass, suggestive of the induction of a more T helper 2 response (Table 2).

No toxicity or signs of autoimmune could be detected in these animals. The major difference between nontransgenic and E7 Tg mice was the absence of a detectable E7-specific CTL response in Tg mice (Fig. 7).

Because the growth of the E7-expressing tumor TC1 seemed to be controlled in E7 Tg mice after vaccination, we were interested to observe whether the immune response triggered by the vaccines could affect the thyroid gland, which also displays a low level expression of E7 gene. In particular, was there induction of autoimmune thyroiditis and thyroid infiltration and was the appearance of the thyroid pathology (goiter and thyroid tumors) delayed? Thyroids were taken from mice 3–4 weeks or 3 months after the last immunization with PD-E7 in either SBAS 1 or SBAS 2, or when animals were 1 year of age. Samples were embedded in paraffin for morphological analysis, and cryosections were used to look for the presence of necrosis, signs of inflammation, or T-cell infiltration.

The morphological analysis showed the presence of a marked enlargement of the thyroid (goiter) with flat epithelial cells, attributable to accumulation of colloid in the follicles. No major differences were observed between vaccinated and nonvaccinated animals. No inflammatory infiltration was seen, except for an increased number of macrophages and polymorphonuclear cells in the vaccinated groups. Furthermore, there was no delay in the development of the thyroid goiter, and no inflammatory infiltrate of T cells could be observed.

Discussion

The use of protein-based vaccines is one of the many different approaches under investigation for the immunotherapeutic treatment of cancer. However, when using this approach, two important factors must be considered: (a) there is the choice of which of the many tumor-associated antigens now identified offer the best potential targets for immunotherapy (reviewed in Ref. 14). Some are only present in tumors but others are either not fully tumor specific or are members of protein families, which may be closely related to normal proteins; and (b) soluble proteins by themselves are poorly immunogenic and need to be modified or formulated with a strong adjuvant to increase their immunogenicity. In the therapeutic setting, and especially in the case of cancer, an adjuvant should first allow the breakdown of the immune tolerance to self tumor antigens and then induce a T helper 1 type of immune response and target the effector cells to the tumor. The adjuvant should also bypass or revert any possible local immune biases induced by the tumor.

In this study, we investigated the ability of a vaccine based on the purified recombinant E7 protein (PD-E7) from HPV16 formulated with SmithKline Beecham Biologicals’ proprietary...
adjuvants, SBAS 1 and SBAS 2. The E7 protein of HPV16 represents an interesting model of a tumor-specific antigen (15) because the expression of this oncogenic early protein is necessary for the maintenance of the transformed state of the HPV-infected cells and is conserved in HPV-induced preneoplastic lesions and cancer (16). Both vaccine formulations in-

Fig. 5 Histology of TC1 tumors from mice treated with PD-E7 with (A, C, E, and G) or without (B, D, F, and H) SBAS 1. Samples were stained for the presence of CD4 (A and B), CD8 (C and D), NK (E and F), or Mac-1 (G and H) cells.
Recombinant HPV16 E7 Protein

Fig. 6  Mean tumor size in E7 Tg mice and nontransgenic littermates as controls after vaccination with PD-E7 formulated with SBAS 1 or SBAS 2 as shown. Numbers shown from day 25 represent the number of tumor-free animals/number of surviving animals. Bars, SD.

duced the regression of s.c., pre-established E7-expressing TC1 tumors (12) associated with the induction of an E7-specific, systemic, antitumor immune response, including CD4+ T helper cells, cytolytic T cells, and antibodies. In view of the predominantly IgG2 antibody response, the presence of CTLs, and the release of IFN-γ by the cells (not shown), it can be concluded that a T helper 1 type of immune response has been triggered by these vaccinations.

These data show that the combination of MPL and QS21, presented either in liposomes (SBAS 1) or an oil-in-water emulsion (SBAS 2), have efficiently delivered the soluble antigen into both the cytotoxic and endosomal pathways of antigen processing, as demonstrated for other adjuvant systems (16–18), to induce CTL responses and tumor rejection. The precise mechanisms by which these adjuvants function are not fully understood, but it is clear that they have direct effects on antigen-presenting cells (6, 7, 19). Moreover, immunohistochemistry performed on tumor tissue indicates that only tumors regressing after vaccination with PD-E7 with adjuvants are massively infiltrated by CD8-, MAC1-, NK-, CD80-, CD86-, MHC class II-positive cells and some CD4 T lymphocytes. Because no such infiltration was seen in control tumors or tumors from animals receiving PD-E7 or adjuvant alone, this indicates a clear homing of effector cells induced by the vaccine and the adjuvant.

Furthermore, it is important to note that the immune system is able to distinguish between two proteins that display homology in 46% of their amino acid sequences (E7 from HPV16 and HPV18), because some tumor antigens, which are potential targets for immunotherapy, are closely related to normal proteins or belong to families of related proteins with different patterns of expression.

Our data are in accordance with data obtained by others showing the potential of the E7 antigen as target for cancer immunotherapy. Either when integrated in a vaccinia virus (12, 16) or used as a purified protein in adjuvant like MF59 (16), Provac (17) or immunostimulating complexes (18). E7-derived peptides have also been shown to have protective potential against E7 HPV16-expressing tumors either by themselves (20) or when pulsed on dendritic cells (21).

E7 is a good model tumor antigen, but it is a viral protein and is thus exogenous for the tumor-bearing host (either mouse or human). To more closely approach the reality of most tumor associated-antigens being self proteins and to address the question of tolerance, mice Tg for the E7 HPV16 open reading frame (E7 Tg mice) were used. These mice, which express the E7 antigen in the thyroid epithelial cells, rapidly develop goiters, and after several months, thyroid carcinoma (11).

In E7 Tg animals, the antigen encoded by the transgene is considered as a self antigen to which tolerance could have developed. No expression is supposed to occur in the thymus, and no central tolerance is believed to develop (11). Indeed, different Tg mouse models have been generated for the HPV16 E7 protein, with the transgene expression driven from different promoters (aA-crystallin, keratin 10, or keratin 14) in epithelial tissues. The anatomical consequences (hair loss, papilloma development, hyperplasia of the epidermis, squamous carcinoma, and inflammatory skin lesions) and the associated immunological sequelae of the transgene expression are variable from one model to another (22–24). The reported data are somewhat confusing, generally describing central or peripheral tolerance (25, 26) or specific CTL tolerance (27, 28), because of the E7 transgene expression in peripheral epithelia. On the other hand, some authors have also reported immunological ignorance for antigen strictly located outside of the immune system but expressed in normal cells of peripheral organs in general (29) and in E7 Tg mice (30).

Although vaccination with the protein with adjuvants in E7 Tg mice induced a cellular response (CD4+ T cells) and a humoral response, the therapeutic effect was less important than in nontransgenic mice, i.e., 40–50% tumor rejection rather than 80–100%. This appears to be attributable to, at least in part, the difficulty of E7 Tg mice to mount an E7-specific CTL response or to the fact that E7 Tg mice seemed to develop more of a T helper 2 type of immune response upon vaccination than the control mice (more IgG1, less IFN-γ, no CTL). The observation of antitumor activity in the absence of measurable E7-specific CTLs could mean that CTLs have been induced but not at a detectable level, or that CTL-independent mechanisms contribute to tumor rejection. The frequency of CTL precursors could be very low in these E7 Tg animals so that two vaccinations and only one in vitro restimulation may not be sufficient to display them. Also, cells other than CD8 T cells may play a role in the tumor rejection. This is suggested by the presence of more macrophages and polyinnuclear cells in the tumors of vaccinated E7 Tg mice. This is also supported by results obtained in other murine tumor models, where there is evidence for an important role of CD4 T helper cells or cytokine released by T helper cells regulating macrophages, natural killer cells, eosinophils, and antibody-dependent pathways in antitumor immunity (31–34).

It is important to note also that vaccinated E7 Tg mice, which partially reject the TC1 tumor implanted s.c., or which are protected against a TC1 tumor challenge applied after vaccination (not shown), do not show any signs of autoimmunity. No
Table 2  Isotypic profile of the humoral anti-E7 response in pooled sera taken from E7 Tg mice and their nontransgenic littermates (Cont) 2 weeks after the second vaccine dose

IgG and the different IgG antibody isotypes were determined by ELISA and expressed in ELISA units/ml (EL.U/ml).

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<td>1,840 19,168</td>
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Fig. 7  CTL responses in the E7 Tg and nontransgenic control animals shown in Fig. 6 after vaccination with PBS or PD-E7 formulated with SBAS 1.

sign of T-cell infiltration within the thyroid gland expressing the E7 antigen could be seen, although antibodies and T helper cell responses were induced in these animals. No delay in the appearance of thyroid goiters, nodules, or thyroid cancer could be obtained in older animals.

Many reasons can be proposed to explain the absence of autoimmunity or the lack of impact on the tumor development in the thyroids of E7 Tg mice: (a) of course, there could be a poor quality CTL response induced, including the absence of a high avidity CD8 population induced by the vaccine because of peripheral or central tolerance; (b) low-avidity CTLs specific for a self epitope could be sufficient to protect mice against a tumor challenge but are not sufficient to induce tumor rejection (35). It could also be that the presence of effective CTL is transient or that the adjuvants used are not strong enough to bypass the tolerance; and (c) the effector cells may not be able to reach the tumor. Willinsky and Blankenstein (36) described recently how injection of adenoviruses expressing interleukin 7 and B7.1 are able to get rid of small, rapidly growing, transplanted tumors in mice but not nontransplantable tumors growing more slowly. One of the reasons identified was that although CTLs were induced, they did not home to the tumor.

Other parameters have to be taken into account, such as the differences in the level of expression of E7 between transduced TC1 cells which express high levels of E7 protein and mRNA, and the thyroid gland. In the latter, E7 mRNA could be detected by Northern blot analysis (11), but the protein was not detectable by immunohistochemistry. Although preactivated immune cells have been shown to recognize epitopes expressed at very low density on tumor cells, it has been shown that a threshold expression level of the antigen is required to obtain lysis of cell lines by CTL clones (37). The low level of E7 expression could be below the threshold level needed to be seen by the immune system.

Tumors are also known to develop mechanisms to avoid the immune surveillance. They may locally suppress T-cell responses by the expression of Fas-L or by the local secretion of immunosuppressive molecules (interleukin 10, transforming growth factor-β, and prostaglandin E2; Ref. 34). Thyroid epithelial cells may produce this kind of molecule. Another important parameter is the nature of the antigen itself, because not all antigens are good tumor rejection antigens (14). In this case, E7 from HPV16 has largely been shown to be a good tumor rejection antigen (38), but it is an oncogenic protein that could participate in the development of a local, immunologically unfavorable microenvironment, because it has been shown recently to have immunosuppressive and proangiogenic activities (39).

Our data provide evidence that a vaccine composed of a recombinant protein mixed in an adjuvant is an efficient strategy to induce an antitumor immune response able to get rid of pre-established tumors. A certain degree of control of tumor growth, but not total rejection, can also be achieved toward TC1 tumors implanted in Tg animals for which the target antigen is a self antigen, but there was no impact on the development of the thyroid pathology that spontaneously develops slowly (over 1 year) in the thyroid of these animals. Furthermore, we found no evidence of autoimmunity.

These data are in agreement with preliminary data obtained in Phase I clinical trials that indicate that whatever the immunotherapeutic strategy evaluated and whatever the target antigen used, no evidence of autoimmunity and no adverse events have been seen thus far, although some objective clinical responses could be obtained (5, 40–42). The only manifestation of auto-
immunity was the development of vitiligo upon vaccination with some melanoma differentiation antigens (43). In animal models also, only rare cases of autoimmunity have been reported after vaccination with pulsed dendritic cells (44), after a viral infection (45), or with DNA immunization (46).

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References


Recombinant Human Papillomavirus Type 16 E7 Protein as a Model Antigen to Study the Vaccine Potential in Control and E7 Transgenic Mice

Catherine M. Gérard, Nathalie Baudson, Kirsty Kraemer, et al.

Clin Cancer Res 2001;7:838s-847s.

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