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Immunological Ignorance of an E7-Encoded Cytolytic T-Lymphocyte Epitope in Transgenic Mice Expressing the E7 and E6 Oncogenes of Human Papillomavirus Type 16

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Certain human papillomaviruses (HPV) have been implicated in the etiology of cervical malignancies, and the E7 and E6 gene products of HPV type 16 are frequently expressed in these lesions. However, cytolytic T-lymphocyte (CTL)-mediated responses to HPV are rarely detectable in patients with cervical cancer. To examine whether the T-cell response is deficient during the HPV-induced transformation, we produced lines of transgenic (Tg) mice that expressed the E6 and E7 oncogenes in keratinized epithelia. The mice developed severe hypertrophy of all keratinized epithelia, but no malignancies were observed. Although epithelial cells from Tg mice could present at least an E7-encoded CTL epitope (E7 49-57), CTLs from these mice were neither primed to nor made tolerant of this epitope. No quantitative or qualitative differences were seen in the CTL responses of the Tg mice compared to those of their littermates following immunization with the peptide E7 49-57. Immunization of Tg mice with the E7 49-57 peptide protected them against a subcutaneous challenge with tumor cells expressing a transfected E7 gene, yet the skin was unaffected, although the cultured skin epithelial cells from Tg mice expressed E7. Our results suggest that the Tg mice were immunologically ignorant of HPV oncoproteins with respect to a CTL response and that a similar type of ignorance may explain why HPV-associated cervical cancer cells can escape immunological destruction.

Human papillomavirus types 16 and 18 (HPV-16 and HPV-18) are implicated in the induction and progression of cervical carcinoma (16, 47). The E6 and E7 oncoproteins of these viruses are frequently detected in the lesions and play a crucial role in both transformation and maintenance of the malignant phenotype in cell culture (26). Therefore, these proteins are potential tumor-specific targets for immunotherapy, and tumor destruction by a T-cell-mediated immune response to E6 and E7 protein has been demonstrated both in animal models and in humans (6, 8, 9, 12, 14, 39). A dysfunction of the T-cell response thus may facilitate the progression of HPV-associated cancers. In fact, several studies have demonstrated that there is little or no cytolytic T-lymphocyte (CTL) response to the E7 gene product of HPV-16 in cervical cancer patients (12, 39), although a humoral response and a $CD4^+$ T-cell response to HPV-associated proteins can be readily detected in many of them (44). The mechanism for the T-cell unresponsiveness is unclear.

An analysis using transgenic (Tg) mice expressing HPV oncoproteins under the control of epithelial cell-specific promoters may provide insight into the role of HPV in the neoplastic transformation. Tg mice expressing HPV-16 genes via the keratin-14 promoter were shown to develop cutaneous dysplasia and papillomatosis, but these lesions did not progress to invasive neoplasms in many strains of mice (2, 5) unless the mice were given estrogens, potential cofactors for the tumorigenesis of cervical cancer (3). Similar results were observed in Tg mice expressing HPV-18 E6 and E7 genes via the keratin-1 promoter (18). These findings are in agreement with the clinical observation that infection of the human cervical squamous epithelium by high-risk HPV does not usually lead to malignancy unless some cofactor is introduced together with the E6 and E7 oncogenes. Recent reports also highlighted the role of genetic predisposition in HPV-associated tumorigenesis since in transgenic mice expressing the oncogenes of HPV-16 in an FVB background malignant progression occurred (11, 22). Ectopic expression of these HPV-16 oncogenes with various gene promoters including αA crystallin (19, 31), β-actin (1), and the mouse mammary tumor virus long terminal repeat (30) also induced locally invasive tumors. The occurrence of tumors appears to be correlated with high levels of expression in these oncoproteins (1, 19, 30, 31).

Tg mice expressing HPV transgenes provide a valuable model for exploring the immune response to HPV during the process of transformation. In a lineage of Tg mice expressing the E6 and E7 oncogenes of HPV-16, no antibodies to the E7 protein were detected unless a locally invasive tumor developed (15); CTL-mediated immunity was not investigated in that study (15). To investigate the immune response during HPV-16-associated transformation, we have generated Tg mice expressing the entire open reading frames of the E6 and E7 oncogenes of HPV-16 under the control of the keratin-14 promoter. While no cancer was observed, the mice developed progressive cutaneous lesions, including papillomatosis and dysplasia. We report here that a major CTL epitope within the E7 protein was ignored by the immune system of the Tg mice and that CTLs recognizing this epitope could be generated after immunization with the peptide.

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MATERIALS AND METHODS

Mouse strains and generation of K14E6E7 Tg lineages. C57BL/6 × C3H/HeN mice and female C57BL/6 mice, 6 to 10 weeks old, were bought from Charles

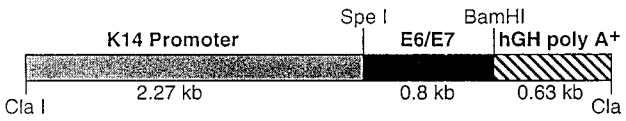

FIG. 1. Schemetic representation of the K14E6/E7 transgene construct. A DNA fragment containing the overlapping open reading frames of the HPV-16 E6 and E7 genes was amplified by PCR with primers (sense, GAGAACTGCA ATGTTTCAGG; antisense, TTATGGATTCTGAGAACAGAT) and cloned into PCR II vector (Invitrogen, San Diego, Calif.). The E6 and E7 gene sequences were verified by DNA sequencing. The insert was then digested with *Spe*I and *Bam*HI and subcloned into pK14pA. pK14pA is a pBSSK-based plasmid containing a 2.27-kb fragment of the mouse keratin-14 (K14) gene promoter and a 0.63-kb portion of the hGH gene 3′ untranslated region with the poly(A) signal sequence. The transcriptional unit of the transgene (*Cla*I fragment) was prepared and used for injection.

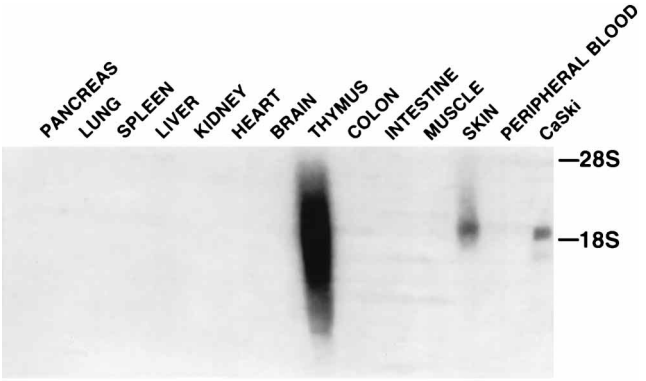

FIG. 2. Northern blot analysis of transgene expression. Organs were isolated from a 4-month-old Tg mouse and were frozen immediately in liquid nitrogen, after which the frozen organs were smashed. The total RNA from each organ was extracted, separated on a 1% agarose-formaldehyde gel with 10 μg of RNA in each lane, transferred to a nitrocellulose membrane, and hybridized to a ^{32}P-labeled E7 fragment from pCDM8/E7 (8). The same quantity of total RNA from the human cervical carcinoma line CaSki, which contains integrated HPV-16 (42), was used as a control.

River Breeding Laboratory (Wilmington, Mass.). Male p53-deficient mice, 6 weeks old, were purchased from Genpharm International (Los Altos, Calif.)

For generation of Tg mice, the HPV-16 E6 and E7 open reading frames were amplified by PCR from HPV-16 DNA (8, 9) and inserted into pK14pA at the unique *Spe*I-*Bam*HI sites. This vector contains 2.27-kb 5′ transcriptional regulatory elements of the human keratin-14 promoter upstream of the *Spe*I site and 0.63-kb human growth hormone (hGH) polyadenylation sequences downstream of the *Bam*HI site (Fig. 1). A 3.7-kb *Cla*I fragment containing the transcription unit was injected into a one-cell embryo from an F_1 (C57BL/6 × C3H/HeN) mouse. Transgenic mice were determined by dot blot and PCR analysis of tail DNA. Thirty founder mice were obtained, and one of them (line 4) was further backcrossed with C57BL/6 mice to the fifth and sixth generations, with which all of the experiments described in this report were carried out. Mononuclear cells from peripheral blood samples from the mice were stained with specific monoclonal antibodies (MAbs) to H-2 (Pharmingen, San Diego, Calif.) and were demonstrated to be homozygous for *H-2^b*.

Assays for gene expression. Dot blot analysis of tail DNA of Tg mice for detection of the transgene was performed as described previously (25) by using a probe from the hGH sequence. The use of reverse transcriptase-coupled PCR for analysis of HPV-16 E6 and E7 gene expression has also been described previously (8). For Northern blot analysis (9), snap-frozen organs were prepared from Tg mice and total RNA was extracted, separated on a 1% agarose-formaldehyde gel with 10 μg of RNA per lane, transferred to a nitrocellulose membrane, and hybridized to a ^{32}P-labeled E7 fragment from pCDM8/E7.

Cell lines. An EL4 lymphoma of C57BL/6 origin was purchased from the American Type Culture Collection (Rockville, Md.). The RMA-S line, which is deficient in the TAP-2 peptide transporter (32) was a gift from H. G. Ljunggren (Karolinska Institute Medical School, Stockholm, Sweden). The method used for transduction of EL4 cells with a recombinant retrovirus containing the E7 gene of HPV-16 has been described elsewhere (10). G418-resistant clones obtained by limiting dilution were screened by reverse transcriptase-coupled PCR for the HPV-16 E7 message, and clone EL4E7, which stably expresses the E7 gene, was selected for further experiments. The EL4E7 clone was also transfected with a vector containing the B7-1 (CD80) gene (7), and stable transfectant EL4E7B7 was obtained and used for in vitro T-cell stimulation as described below. The CaSki line (from the American Type Culture Collection) was derived from an HPV-16-positive squamous cell carcinoma of the human cervix (42).

Mouse keratinocytes from female mice were cultured as previously described (21). Briefly, shaved ventral skin was removed from euthanized mice and placed in cold phosphate-buffered saline. Debris and connective tissue on the dermal side were removed, and the skin was fragmented into pieces of 100 to 500 mm^2 and incubated overnight with Dispase (Sigma, St. Louis, Mo.) at 10 mg/ml in Hanks balanced salt solution at 4°C. Subsequently, the epidermis was detached from the underlying dermis and was further digested in 0.05% (wt/vol) trypsin–phosphate-buffered saline for 15 min at 37°C to obtain a single-cell suspension. Cells were then plated onto 60-mm^2 petri dishes in keratinocyte culture medium SMF (Life Technologies, Gaithersburg, Md.) supplemented with recombinant epithelial cell growth factor and bovine pituitary extracts according to the manufacturer's instructions. Thymic epithelial lines were derived from the plastic-adherent cells from the thymuses of Tg mice and cultured in Iscove's modified Dulbecco's medium with 10% fetal calf serum. The adherent cells were cloned by limiting dilution in flat-bottom 96-well plates.

Peptide synthesis and assay. Peptides were synthesized on a Gilson multiple peptide synthesizer, model AMS422, with 9-fluorenylmethoxycarbonyl amino acids (17). The assembled peptides were cleaved from the resin by a reaction with trifluoroacetic acid-water-thioanisole-ethanedithiol (100:5:5:2.5) for 2 h. Following precipitation from cold ether, the peptides were dissolved in formic acid, diluted with water, and lyophilized. Purification of peptides was achieved by reverse-phase high-pressure liquid chromatography with a gradient of acetonitrile in 0.1% trifluoroacetic acid. The peptides were characterized by molecular weight determination on a Bio-ion 20 mass spectrometer. The binding potentials of peptides to major histocompatibility complex (MHC) class I molecules were determined in an RMA-S cell assay (14) by flow-cytometric measuring of the mean intensity of fluorescence with a FACScan fluorescence-activated cell sorter (Becton Dickinson, Mountain View, Calif.) upon staining with fluorescein isothiocyanate-labeled anti-H-2D^b MAbs (Pharmingen). The results were expressed as the ratio of the intensity of fluorescence to the background fluorescence intensity for RMA-S cells without peptide.

CTL induction, cloning, and assay. Female mice were injected subcutaneously (s.c.) with 100 μg of the E7 49-57 peptide (RAHYNIVTF) (14) emulsified in incomplete Freund's adjuvant (IFA), and 3 to 4 weeks later spleen cell suspensions were prepared and cocultured with irradiated (10,000 rads) EL4E7 cells (see above) in 24-well plates (Costar, Cambridge, Mass.) for 5 days. CTLs were cloned by limiting dilution. For long-term propagation of CTL lines and clones, the cells were restimulated every 7 to 16 days with 10^5 irradiated (10,000 rads) EL4E7 cells together with irradiated (3,000 rads) syngeneic spleen cells in the presence of 5 U of recombinant interleukin-2 (Cetus Co., Emeryville, Calif.). The cytolytic activities of bulk-cultured cells from spleens or lymph nodes, CTL lines, and clones were measured by a standard 4-h ^{51}Cr release assay (29).

Skin grafting. Tail skin was obtained from 3- to 5-month-old female Tg mice which had been backcrossed five times with C57BL/6 mice. It was grafted onto skin of the upper left side of the backs of sex-matched littermates as described previously (41). Since skin from the tail has less hair and a different color, it can be easily distinguished from the surrounding skin. Bandages were removed on day 7, and the persistence of the graft was checked twice a week thereafter for a later follow-up period of 2 months.

Peptide immunization. Mice were injected s.c. with 100 μg of the E7 49-57 peptide emulsified in IFA. Mice injected with the influenza virus NP peptide (ASNENMETM) (13) emulsified in IFA or injected with IFA alone were used as controls. Fourteen to 21 days later, 5×10^4 EL4E7 or EL4 cells were injected into the shaved left flanks of mice. Mice were scored twice weekly for tumor growth and were euthanized when their tumors reached a mean diameter of 15 mm. Tumor-free animals were monitored for 20 weeks after challenge.

RESULTS

Expression of the E6 and E7 oncogenes of HPV-16 in keratinized epithelium induced progressive hyperplasia and thymic hypertrophia. To test the immunological response to HPV-16 oncogenes during the process of transformation, we targeted the expression of the E6 and E7 genes to the epithelium, a natural host for HPV infection (47), by using the keratin-14 promoter (Fig. 1). RNA isolated from different tissues was examined for the expression of the transgenes by Northern blot analysis. E7-specific RNA was detected in the skin and thymus but not in other tissues, including that of the pancreas, lung, spleen, liver, kidney, heart, brain, colon, intestine, and muscle, or in peripheral blood (Fig. 2).

By 10 to 14 days of age, the majority of the Tg mice exhibited a characteristic phenotype: sparse, matted, and curled fur with flaky and wrinkled skin. This phenotype correlated with the

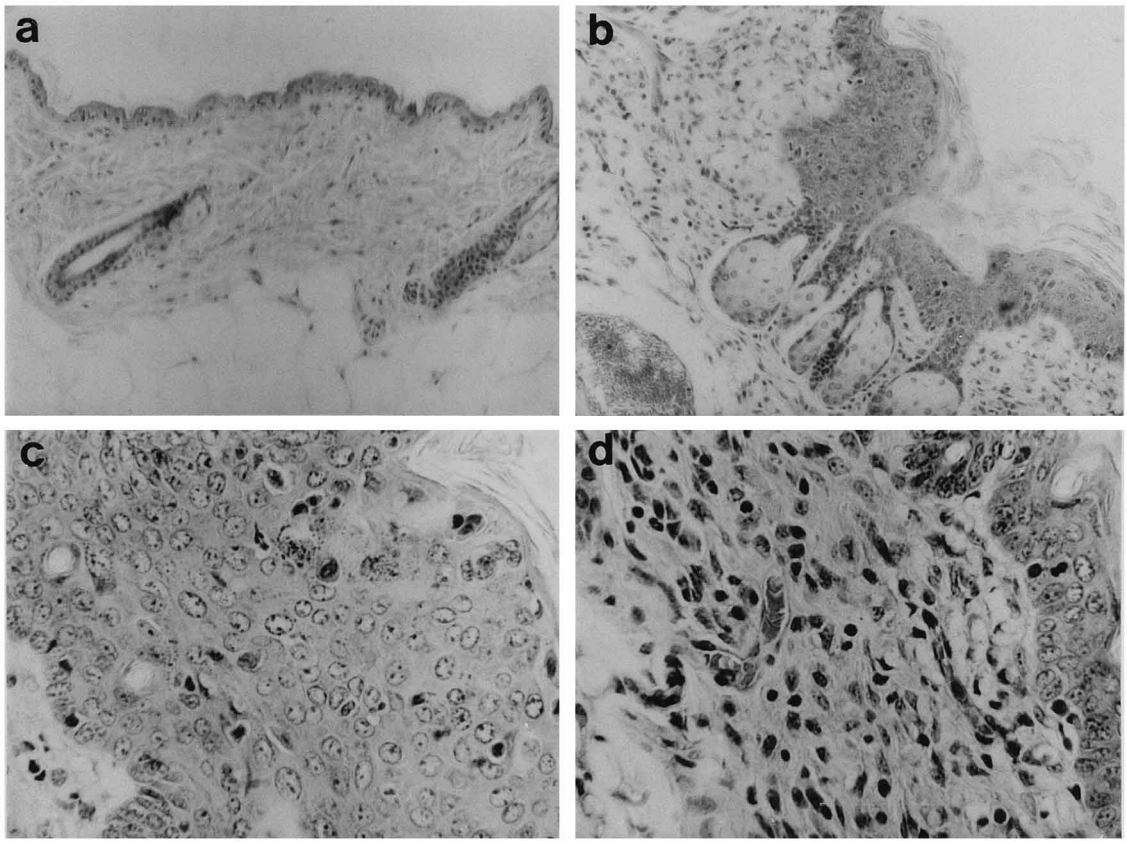

FIG. 3. Histopathology of K14E6/E7 Tg skin. Representative sections are shown from the skin of the back of a normal mouse (a) and from back skin of a 6-month-old hemizygous Tg mouse with morphologically detectable abnormality (b). Larger magnifications (×1,350) of the epidermis (c) and dermis (d) of the Tg mouse skin shown in panel b are also shown.

expression of the E6 and E7 transgenes as determined by dot blot and PCR analysis, with 100% of the mice with the phenotype being positive in these assays. As the mice grew older (>6 months) other skin lesions appeared such as an excess of keratinaceus debris which filled their ears and indicated a progressive hyperplasia of the epithelium. A histological examination of Tg mice at 3 months of age showed a thicker skin (Fig. 3b and c) than that of normal mice (Fig. 3a), along with acanthosis, hyperkeratosis, and moderate multifocal ulcerations. In older mice (>4 months) the accumulation of a few inflammatory cells in the skin was observed (Fig. 3d). No malignancies were apparent in any lineages of the mice over an observation period of up to 1.5 years.

The sizes of the thymuses of newborn Tg mice were normal but they progressively increased after 3 months of age. A histopathologic examination disclosed an enlarged thymus with preservation of the tissue architecture and no indication of malignancy. When the mice were approximately 4 months of age, the enlarged thymus caused respiratory difficulties in some cases due to compression of the thorax. Lymphocyte subsets from Tg mice were analyzed with fluorescence-labeled MAbs to T-cell markers CD3, CD4, and CD8 and to B-cell marker B220. Thymocytes from the Tg mice revealed normal proportions of the different subpopulations, although the absolute number of cells was dramatically increased, up to 30- to 100-fold compared to normal mice of the same age (data not shown).

A gross examination of the peripheral lymphoid organs revealed enlarged lymph nodes but normal-sized spleens, an observation which was in agreement with the numbers of cells in the respective organs; the numbers of cells in the lymph nodes of Tg mice were approximately five times higher than those in the lymph nodes of littermates. The numbers of spleen cells, the proportions of CD4 and CD8 cells, and the T-cell receptor Vβ repertoires remained normal (data not shown).

Tg mice did not develop a spontaneous CTL response to E7, but such a response could be induced following immunization with an E7-encoded peptide. To determine whether Tg mice harbored CTLs specific for transgene products, we examined the CTL reactivities of bulk-cultured cells from both spleens and lymph nodes. Lymphocytes were prepared from Tg mice of different ages and were stimulated in vitro by cocultivation with EL4E7B7 cells, which express both E7 and B7-1 genes; this method has been shown to recall a very weak CTL response (10). No CTL activity was detected against E7-expressing cells in vitro (Fig. 4) in either young (Fig. 4A and C) or old (Fig. 4B and D) mice even when the bulk-cultured lymphoid cells had been restimulated twice (data not shown). The CTL response was normal to allogeneic ($H\text{-}2^d$) MHC antigens presented by the P815 tumor (Fig. 4), suggesting that there is no general immunosuppression in the Tg mice. Our results thus demonstrated that the transgene-encoded E7 protein was not immunogenic.

The fact that the E7 gene of HPV was expressed in epithelial cells of the thymuses and in epidermal keratinocytes from the Tg mice raised the question of whether E6- and E7-specific CTL clones had been made tolerant or had been deleted. To address this issue, Tg mice and their littermates were immu-

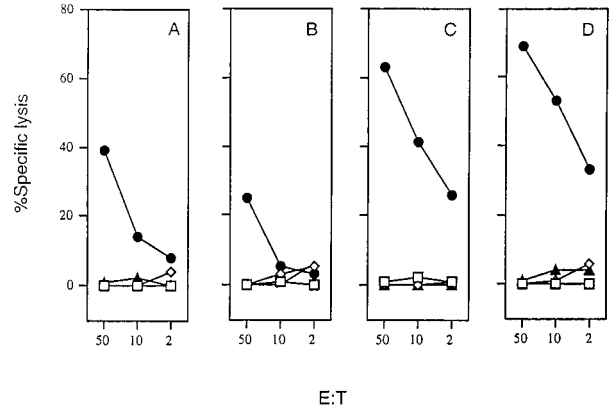

FIG. 4. CTLs from Tg mice are not primed to E7. Cell suspensions from lymph nodes (A and B) and spleens (C and D) from 1-month-old (A and C) and 2-month-old (B and D) Tg mice were prepared at 5×10^6/ml and incubated with gamma-irradiated (10,000 rads) EL4E7B7 or B7$^+$ P815 (*H-2^d*) cells (10) for 5 days. The cytolytic activities of the bulk-cultured cells were tested against target cells in a standard 4-h ^{51}Cr release assay. □, in vitro stimulation, EL4E7B7; target, EL4; ◇, in vitro stimulation, EL4E7B7; target, EL4E7; ▲, in vitro stimulation, P815B7; target, EL4; ●, in vitro stimulation, P815B7; target, P815.

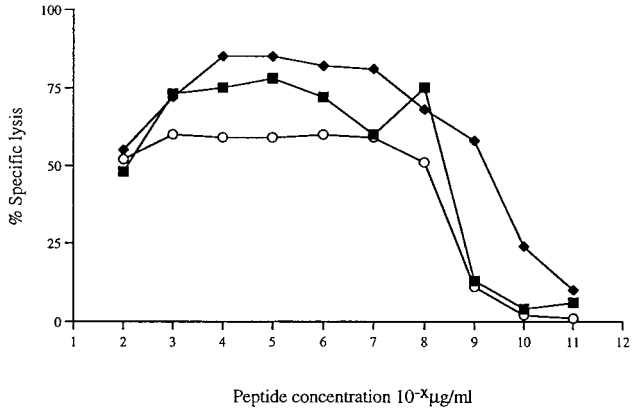

FIG. 6. Peptide concentration requirements of anti-E7 CTL clones derived from Tg and non-Tg mice. Standard 4-h ^{51}Cr release assay with the anti-E7 49-57 CTL clones from a Tg mouse (9d and 39d) and a non-Tg littermate (4C) against RMA-S cells pulsed with the E7 49-57 peptide. ■, CTL clone 9d from Tg mouse; ◆, CTL clone 39d from Tg mouse; ○, CTL clone 4C from non-TG mouse.

nized s.c. with the E7 49-57 peptide emulsified in IFA. Three weeks later, single-cell suspensions were harvested from their spleens and stimulated once in vitro with irradiated EL4E7 cells, after which they were tested for CTL activity in a standard ^{51}Cr release assay. The results showed that anti-E7 CTLs could be generated in the Tg mice with an activity comparable to that of lymphocytes from control mice (Fig. 5). Furthermore, CTL clones generated from the Tg mice could lyse E7 49-57 peptide-pulsed RMA-S cells within a range of peptide concentrations comparable to that seen with CTL clones from non-Tg mice (Fig. 6).

A minimal 8- or 9-mer peptide may contain several CTL epitopes and may be recognized by distinct CTL clones (40). Tolerance by E7 49-57-specific CTL clones may, therefore, be selective to some, but not to all, epitopes contained in the E7 49-57 peptide. To test this possibility, several E7 49-57-specific

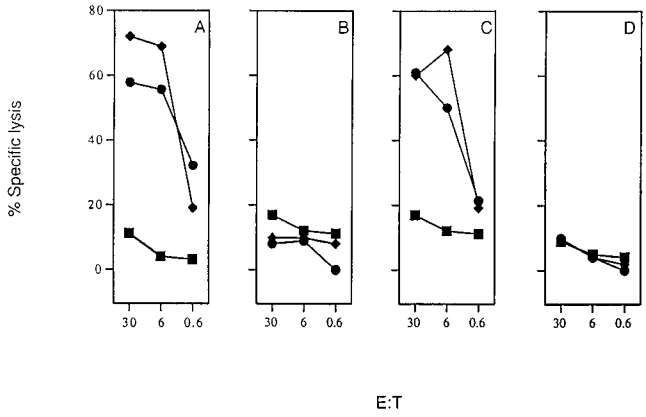

FIG. 5. CTLs from Tg mice do not become tolerant of E7. Two Tg mice (A and B) and two non-Tg littermates (C and D) were immunized with the E7 49-57 peptide emulsified in IFA (A and C) or with IFA alone (B and D). Three weeks later, their spleens were removed, and splenocytes at 5×10^6/ml were cultured for 5 days with irradiated EL4E7 at 10^5/ml. The cytolytic activity of the bulk-cultured cells was tested against EL4 (■), E7-transfected EL4 (●), and EL4 cells pulsed with the E7 49-57 peptide (◆) at 10 μg/ml in a standard 4-h ^{51}Cr release assay.

CTL clones were generated from Tg mice and their non-Tg littermates, after which the cytolytic activities of the clones were tested on RMA-S cells loaded with a series of E7 49-57 peptides which had sequential substitutions of each position with alanine. The same peptide variants were also tested for their ability to stabilize *H-2D^b* expression on RMA-S cells. The results from experiments with four clones from control mice and three clones from Tg mice are summarized in Table 1. The substitution of alanine at position 5 eliminated the binding of all peptides, whereas substitutions at other positions did not, indicating that asparagine in position 5 is critical for the binding of the E7-encoded epitopes to H-2D^b. Substitution at position 4 or 8 abolished the lysis of RMA-S cells by all clones tested. These substitutions did not affect the binding to MHC class I molecules, which indicates that residues at position 4 and 8 are involved in T-cell receptor recognition. Replacement at position 6 with alanine eliminated CTL recognition for some clones but not for others. No significant differences in epitope recognition by CTL clones were observed between Tg and control mice. Our results indicate that there is no obvious clonal deletion of CTL activity against E7 in this model. Therefore, the transgene products appear to be "ignored" by the T lymphocytes such that a CTL response is dependent on prior immunization.

Epithelial cells derived from Tg mice are killed by E7-specific CTLs. The ignorance that characterizes the CTL response against the E7 49-57 peptides in Tg mice may be due to a low level of expression of the transgene preventing recognition by the CTL. To test this possibility, several cell lines were established from the epidermises of both Tg mice and their littermates. The lines from Tg mice grew significantly faster (threefold) than lines from the skin of littermates (Fig. 7), and they lost contact inhibition (data not shown), suggesting that the cells from the Tg mice may have become immortalized. Adherent epithelial cell lines from the thymuses of the Tg animals with phenotypes similar to those of the lines established from the skin were also established.

We then examined whether anti-E7 CTL clones could lyse cultured epithelial cells from Tg mice. CTL clone 18c lysed epithelial cells derived from the skin and thymuses of Tg mice but failed to lyse epithelial cells cultured from a syngeneic p53−/− mouse (Fig. 8A) or from a non-Tg littermate (data not shown). The cytolytic activity of 18c was shown to be specific

TABLE 1. Recognition of epitopes in the E7 49-57 peptide by CTL clones derived from Tg mice and non-Tg littermates

Position of Ala substitution[a]	MHC binding (fold)[b]	Assay results[c] for:						
		Non-Tg mouse CTL clone:				Tg mouse CTL clone:		
		4C	1N	9N	10C	9d	27d	39d
1	3.0	−	−	−	−	−	−	−
2 (wild type)	2.8	−	−	−	−	−	−	−
3	2.3	−	−	−	−	−	−	−
4	2.9	++	+++	+++	+++	+++	+++	+++
5	1.1	++	+++	+++	+++	+++	+++	+++
6	2.2	+++	−	−	+++	−	−	++
7	2.5	−	−	−	++	−	−	−
8	2.6	+	+++	++	+++	++	++	++
9	2.4	−	+	−	+	−	−	−

[a] Peptides were synthesized with a sequential substitution by alanine at each position of the E7 49-57 peptide RAHYNIVTF.
[b] Fluorescence-activated cell sorter analysis of *H-2D*^{b} surface expression on RMA-S cells preincubated with 10 μg of each peptide per ml as described in Materials and Methods. Data are displayed as the fold increase over the mean intensity of fluorescence of cells without incubation with peptides.
[c] CTL clones obtained from Tg and non-Tg mice immunized with E7 49-57 in IFA were tested in 4-h ^{51}Cr release assays against RMA-S cells which were prepulsed with peptides (10 ng/ml) having substitutions at the indicated positions. The effector-to-target cell ratio was 5:1, and the percent specific lysis was greater than 50% against the wild-type peptide (position 2) in every case. The results are summaries of three experiments. −, no inhibition; +, <25% inhibition; ++, 25 to 50% inhibition; and +++, >50% inhibition of recognition compared to that of the nominal E7 49-57 peptide.

for E7 since EL4 cells transfected with E7 were lysed, whereas untransfected wild-type EL4 was not unless pulsed with E7 49-57 peptide (Fig. 5B). We conclude that cultured epithelial cells from Tg mice could present an E7-encoded antigen through the MHC class I pathway.

Lack of a CTL response to E7 in Tg mice is tissue selective. To examine whether the failure of CTLs to cause tissue destruction in vivo was due to some protection mechanisms operating in the skin epithelium, we immunized Tg mice with the E7 49-57 peptide and subsequently challenged the mice s.c. with a lethal dose of E7EL4 tumor cells. Four of five immunized Tg mice rejected the EL4E7 tumor cells (Fig. 9), a result similar to that seen for non-Tg mice (data not shown). Mice that were challenged with E7-negative EL4 tumor cells and mice that were immunized with a control peptide from influenza virus nuclear protein developed progressive growing tumors (Fig. 9), indicating that the tumor rejection observed was specific for the E7 epitope. Fifteen Tg mice immunized with the E7 49-57 peptide were observed over 3 months to assess whether there was any aggravation of the mild cutaneous lesions described above. No signs of enhanced inflammation were detected either by weekly macroscopic inspections or by microscopic examination of biopsies taken from the dorsal skin at different times (data not shown). A histopathologic examination of skin samples from the immunized Tg mice did not demonstrate any enhanced inflammation or tissue lesions (data not shown).

To further examine the immunogenicity of Tg skin, we transplanted skin from the tails of the Tg mice onto the backs of sex-matched non-Tg littermates. The skin was accepted by the non-Tg littermates even when these had been preimmunized with E7 49-57 peptide prior to the transplantation. In contrast, transplantation of an allogeneic skin graft from DBA/2 mice led to a complete rejection in less than 3 weeks (34a).

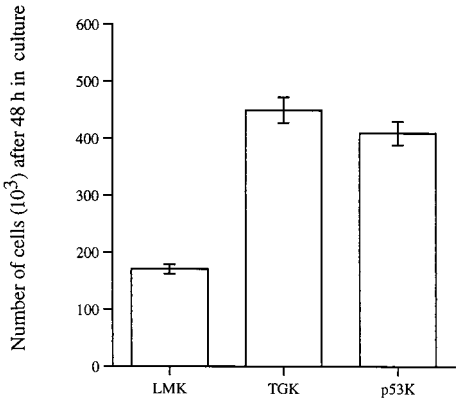

FIG. 7. Growth of epithelial cells from the skin of a Tg mouse and its non-Tg littermate. Number of cells recovered from 10^5 keratinocytes obtained from a 7-month-old Tg mouse (TGK), a non-Tg littermate (LMK), and a p53-deficient mouse (p53K) after 2-day culture in SMF medium as counted in a hemocytometer chamber. Results show the means and the standard errors of the means of three different experiments.

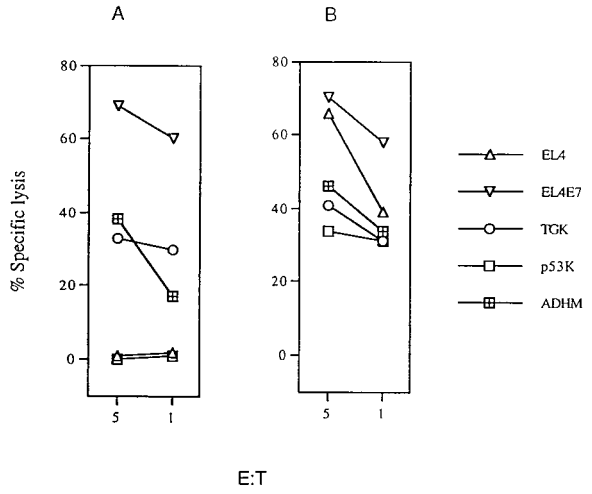

FIG. 8. In vitro lysis of epithelial cells from Tg mice by an E7-specific CTL clone. (A) Percent specific lysis in standard 4-h Cr release assays displayed by anti-E7 CTL clone 18c against the indicated target cells. (B) Similar experiment to that shown in panel A, but one in which target cells were pulsed with 100 ng of synthetic peptide E7 49-57 per ml during the assay. TGK, epithelial cells derived from the skin of Tg mice; ADHM, epithelial cells derived from the thymuses of Tg mice; p53K, epithelial cells cultured from a p53−/− syngeneic mouse.

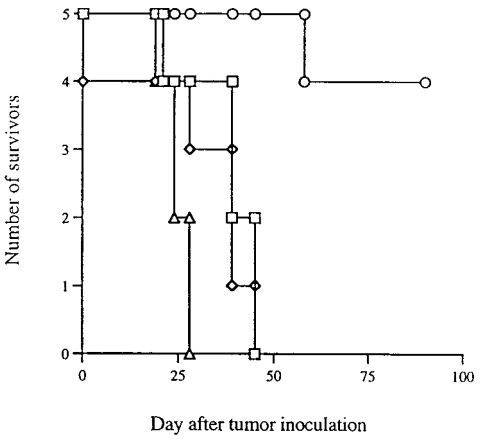

FIG. 9. Rejection of an s.c. challenge of the EL4E7 tumor by Tg mice immunized with the E7 49-57 peptide. Mice were immunized s.c. with 100 μg of the indicated peptides emusified in IFA 15 days prior to the challenge with 5×10^4 EL4E7 or EL4 cells as shown. Mice were monitored for 3 months and were euthanized when tumors reached 1.5 to 2.0 cm (mean diameter). □, no immunization, challenge with EL4E7; ◇, immunization with influenza virus nuclear peptide, challenge with EL4E7; ○, immunization with E7 49-57, challenge with EL4E7; △, immunization with E7 49-57, challenge with EL4.

DISCUSSION

Although there is ample evidence that a CTL response can be elicited against transforming gene products in both animal and human cancers (6, 8, 9, 12, 14, 39), it is unclear whether such a response exists during the early stages of tumorigenesis. We demonstrate here that mice Tg for the E6 and E7 oncogenes of HPV-16 do not mount any detectable CTL response against E7, although the expression of the transforming genes in the Tg mice results in a progressive phenotype. Furthermore, the Tg mice did not mount any detectable immune response to the HPV-16-encoded antigen in their skin. Our results suggest that the E6 and E7 oncoproteins are ignored by the immune system at a point of time when they are inducing a premalignant state.

The tissue-specific keratin-14 promoter was chosen to direct the expression of the E6 and E7 oncogenes to the basal layer of the epidermis (35). Transgene expression was detected in the skin samples from the Tg mice by Northern blotting (Fig. 4). Furthermore, RNA encoded by the transgenes was isolated from the thymus and could be detected in epithelial cell cultures of this organ. The cultivated thymic epithelial cells could be specifically lysed by anti-E7 CTL, as could keratinocytes from the skin of Tg mice. This demonstrated that cultured epithelial cells of Tg mice can present the E7 epitope in a manner recognizable by activated CTLs in vitro.

Expression of MHC class I-restricted antigens on epithelial cells of the thymus may delete specific T-cell precursors (negative selection) and induce tolerance (38). In this study, however, the expression of the E7 transgene in the thymus seemed to have no detectable effect on the generation of CTLs recognizing an epitope encoded by the transgene (Table 1; Fig. 6). This can have several explanations. It has been speculated that T-cell precursors that have a great affinity for antigens are deleted in the thymus, whereas those with less avidity can escape to the periphery (24). Several recent studies also suggest that CTL precursors for specific antigens are not deleted if the respective antigens are expressed at low levels in the thymus (4). It has been demonstrated that targeted expression of an alloantigen under the keratin-14 promoter in the thymic cortical but not the medullary epithelial cells exclusively induces positive selection of T-cell precursors without clonal deletion (32), while ample evidence indicates the role of the thymic medullary epithelium in negative selection (23). We have also found that the expression of the E6 and E7 oncogenes is restricted to thymic epithelial cells with a cortical but not a medullary phenotype (13a).

Specific CTL activities generated in Tg mice immunized with the E7 49-57 peptide were comparable to those observed in their nontransgenic littermates (Fig. 5; Table 1). Equal concentrations of the E7 peptide were needed to sensitize target cells for lysis by the CTL clones derived from Tg and non-Tg mice (Fig. 6), and the amino acid requirements for recognition by the T cells were overlapping (Table 1). The affinities of anti-E7 CTLs from both Tg and non-Tg mice were comparable, and thymic expression of the E7 gene did not impair the T-cell repertoire specific for this particular epitope. Our results thus indicate that there is CTL ignorance (36, 37, 46) but no central or peripheral tolerance of E7-encoded antigen in the Tg mice. Tg mice immunized with the E7 49-57 peptide mounted an E7-specific response against challenge with $E7^+$ EL4 tumor cells (Fig. 9) but not against skin grafts from Tg mice (34a). The latter findings are in contrast to the observation that skin epithelial cells from the Tg mice are lysed by E7-specific CTLs in vitro (Fig. 8) and suggest that the lack of a CTL response to E7 in Tg mice involves failure in both the induction and effector phases. A similar conclusion has been reached by using a different experimental system, in which transfer of T cells specific for the Env antigen of Friend murine leukemia virus could eliminate leukemia cells expressing the same antigen in *env*-containing Tg mice while leaving the normal tissue undamaged, although these tissues also expressed the env transgene (27).

It is unclear how the immunological ignorance of E6- and E7-encoded antigens in the Tg mice is maintained. It has been shown that localized inflammation in the skin of the transgenic mice expressing HPV oncoproteins correlated with the level of anti-E7 antibody (15). However, we can detect neither antibodies (6a) nor the CTLs directed against E7 (Fig. 4) in these transgenic mice despite the moderate inflammatory cell infiltrations that occurred in skin (Fig. 3). In addition, we have recently demonstrated that there is not a detectable CTL response in the lymph nodes of the transgenic mice even after the injection of complete Freund's adjuvant or vaccinia virus to induce strong inflammatory responses (34a). It is tempting to speculate that the level of expression of the E7 gene in the epidermis may be too low to induce an immune response and even too low to render the epidermis as sensitive CTL targets in vivo. Lack of proper costimulation may be also considered as a contributing factor since it has been shown that costimulation provided by molecules of the B7 family and/or other accessory molecules, such as CD48, increased CTL response to otherwise silent tumor antigens (7, 10, 29, 33) and epithelia do not usually express these costimulatory molecules (26). Coexpression of B7-1 with viral antigen (43, 45) or MHC class II molecules (20) can break ignorance and lead to autoimmunity. Nevertheless, our studies demonstrate that a foreign oncogene product can be ignored by the immune system even when the early stage of oncogenic transformation is taking place.

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