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E7 Oncoprotein of Human Papillomavirus Type 16 Expressed Constitutively in the Epidermis Induced or Sustained after Immunization with E7 Protein

K. HERD,[1] G. J. P. FERNANDO, L. A. DUNN, I. H. FRAZER, P. LAMBERT,* and R. W. TINDLE[2]

*Centre for Immunology and Cancer Research, University of Queensland, Princess Alexandra Hospital, Brisbane Qld 4102 Australia; and *McCardle Laboratory for Cancer Research, University of Wisconsin Medical School, Madison, Wisconsin 53706*

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A line of FVB (H-2^q) mice transgenic for the E6/E7 open reading frames of Human Papillomavirus type 16 driven from the α-A crystallin promoter expresses E7 mRNA in lens and skin epithelium. E7 protein is detectable in adult skin, coinciding with the development of inflammatory skin disease, which progresses to papillomata and squamous carcinomata in some mice. By examining the outcome of parenteral immunization with E7 protein, we sought to determine whether endogenous expression of E7 in skin had induced a preexisting immune outcome, i.e., specific immunity or tolerance, or whether the mice remain naive ("ignorant") to E7. Our data show that the antibody response to defined E7 B-epitopes, the proliferative response to Th epitopes, and the delayed-type hypersensitivity (DTH) response to whole E7 did not differ between groups of young and old E6/E7 transgenic mice (likely having different degrees of lifetime exposure to E7 protein) or between E6/E7-transgenic and nontransgenic parental strain control mice. Although an E7-specific CTL response could not be induced in the H-2^q background of these mice, incorporation of a D^b allele into the genome allowed comparison of D^b-restricted CTL responses in E6/E7 transgenic and nontransgenic mice. Experiments indicated that the E7-immunization-induced CTL response did not differ significantly between E6/E7 transgenic and nontransgenic mice. We interpret these results to indicate that in spite of expression of E7 protein in adult skin, E6/E7 transgenic mice remain immunologically naive (ignorant) of E7 epitopes presented by immunization.

INTRODUCTION

Human Papillomavirus type 16 (HPV 16) is tropic for human anogenital epithelium and is associated with the development of premalignant and invasive squamous disease (Gissmann, 1992; Koutsky and Kiviat, 1993). In experimental systems, the products of the E6 and E7 viral open reading frames (ORFs) are oncoproteins (Straight *et al.,* 1995; McIntyre *et al.,* 1996).

A line of mice (denoted #19) transgenic for the HPV16 E6 and E7 ORFs driven from the αA crystallin promoter develops a skin pathology, including hair loss, dermal thickening, papilloma development, and eventual squamous cell carcinomata associated with increased levels of E6 and E7 mRNAs (Lambert *et al.,* 1993). E7 protein was detected in normal and diseased skin in older mice (>28 weeks) and was highest in skin tumors (Frazer *et al.,* 1995). No E7 protein was detected in skin samples from young (7–13 week old) mice (limit of assay sensitivity 0.05 ng E7/mg cellular protein; Selvey *et al.* 1994). Expression of E7 protein in the skin increases with age and is limited to localized proliferating poorly differentiated keratinocytes.

Earlier work has shown that older #19 mice with skin disease, but not younger mice with phenotypically normal skin, develop an antibody response to E7 and that immunization with E7 results in an antibody response to the protein in #19 and FVB mice (Frazer *et al.,* 1995). The former observation is probably a result of E7 protein from damaged keratinocytes being taken up by "professional" antigen presenting cells (Langerhan's cells, dendritic cells) and presented to the immune system. Prior to the onset of inflammatory skin disease any presentation of E7 protein is by keratinocytes (KC). In noninflamed skin, KCs express at the cell surface MHC class 1 but not class II molecules, and do not express the B7.1 (CD80) second signal molecule (Williams *et al.,* 1994). While endogenous peptide may be presented to the immune system via class 1 in the absence of appropriate second signalling, the outcome may be tolerogenic rather than immunogenic (Bal *et al.,* 1990). A further determinant of immune outcome is the level of expression of antigen in KCs; below a threshold of 100 molecules per cell, no response (i.e., neither tolerance or immunity, but "ignorance") is likely (Miller and Flavell, 1994).

This presentation is likely to model that which occurs in HPV16 +ve cervical carcinoma patients where the immune system is naive to E7 protein prior to HPV 16

[1] Present address: Sir Albert Sakzewski Virus Research Centre, Royal Children's Hospital, Herston Road, Herston Qld 4029 Australia.

[2] To whom correspondence and reprint requests should be addressed at present address: Sir Albert Sakzewski Virus Research Centre, Royal Children's Hospital, Herston Road, Herston Qld 4029, Australia. Fax: +61-7-3253-1401. E-mail: r.tindle@mailbox.uq.edu.au.

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infection, and professional antigen presentation is only likely to occur following rupture of the basement membrane and subepidermal tissue invasion by transformed cells.

In this study we sought to determine whether constitutive expression of E7 protein in skin in #19 mice had induced E7-directed immunity or tolerance, or whether the mice remain naive (ignorant) to E7. We did this by examining antibody production, the generation of T-proliferative, delayed-type hypersensitivity (DTH), and T-cytotoxic (CTL) responses in #19 mice of various ages immunized with E7 protein in comparison with E7 immunized nontransgenic (FVB) controls. We present evidence to show: (1) antibody titers to major E7 B-epitopes elicited in response to E7 immunization did not differ significantly among groups of #19 mice from 6–32 weeks of age or between #19 mice and FVB nontransgenic controls. In addition, antibody titers were not significantly different between #19 mice of similar age with and without skin disease. (2) T-proliferative and DTH responses were similar among #19 mice of varying ages and between #19 and similarly aged FVB control mice. (3) #19 and FVB mice failed to make a CTL response to E7 immunogen, suggesting that no E7 CTL epitope recognised in the context of MHC haplotype H-2D^q and K^q exists. However, both #19 and FVB mice made transgenic for H-2D^b and immunized with E7 protein displayed CTL responses directed to a D^b-restricted E7 CTL epitope.

These data indicate that #19 mice, including those of an age where E7 protein in skin is demonstrable, but no skin disease has developed, and those with overt skin disease, remain naive to the B- and T-cell epitopes presented to the immune system as a result of E7 immunization.

The results have implications for (1) the presentation to the immune system of any non-self-antigen (e.g., viral, tumor, or "altered-self") expressed in skin keratinocytes and (2) specific immune response development in recipients of E7-based therapeutic vaccines for E7 expressing HPV-associated cervical carcinoma.

MATERIALS AND METHODS

Mice

A line of mice, α A cry HPV 16 E6 E7. FVB, designated #19, are homozygous for a HPV 16 E6/E7 ORF transgene driven off an α-A crystallin promoter (Lambert *et al.,* 1993). #19 and FVB parental strain mice (both H-2^q), and C57 BL (H-2^b) mice were held under specific pathogen-free conditions. Genetic purity was checked by isoenzyme analysis. FVB mice transgenic for H-2D^b (Azoulay *et al.,* 1994) were obtained from Dr. M. Brahic (Institut Pasteur, Paris). #19/D^b mice were obtained by crossing FVB/D^b with #19 mice. F_1 offspring were obligate heterozygotes for the E7 transgene and express E7 protein in skin. They were selected for the D^b transgene by PBMC surface expression of D^b in FACS analysis using antibody 28-8-6S (Ozato and Sachs, 1981).

Immunization for antibody

Groups of three to four #19 mice of ages 6, 11, 15, 23, 27, and 32 weeks of age, and nontransgenic FVB control mice aged 6 weeks were immunized intraperitoneally (ip) (first immunization) and subcutaneously (sc) at the base of the tail (second and third immunizations) with 50 μg HPV 16 E7 as a glutathione *S*-transferase fusion protein (E7.GST) + 10 μg Quil A adjuvant (Alonso de Velasco *et al.,* 1994) in saline, on Days 0, 21, and 98. Serum samples were prepared from retro-orbital plexus blood on Days 0, 31, 98, and 105.

Antibody ELISA

Sera were reacted at 37° for 45 min at a range of dilutions in blocking buffer (PBS +5% skim milk + 0.1% Tween 20) with microtiter plate-bound E7 peptides 101, 104, and 106 (Fig. 1), which contain the strong B epitopes EYMLD, IDGP, and QAEPD, respectively (Tindle *et al.,* 1990). Antibody binding was visualized in an ELISA reaction using HRPO-linked Rabbit anti-mouse Ig (Silenus Pty, Australia) plus ABTS (2, 2^1-azinobis (3-ethyl benzthiazoline) sulphonic acid) substrate as detecting reagents as described (Tindle *et al.,* 1990). Reactions were quantified as optical density at 490 n*M* on a Titertek plate reader. Positive controls included monoclonal antibodies 8F, 4F, and 6D specific for epitopes EYMLD, IDGP, and QAEPD, respectively (Tindle *et al.,* 1990).

Delayed-type hypersensitivity

Mice were immunized on Day 0 with 50 μg E7.GST + 10 μg Quil A or 50 μg GST + 10 μg Quil A in 100 μl saline sc at the base of the tail. Four hundred nanograms of Pertussigen (Commonwealth Serum Laboratories Ltd.) in 0.2 ml saline were injected intravenously (iv) immediately after immunization (de Moerloose *et al.,* 1986). Seven days later, mice under anesthesia were challenged in the pinna of the ear with 10 μg E7.GST (right ear) and 10 μg GST (left ear) in a ca 10-μl volume. At 24 and 48 hr the thickness of each ear was measured at the site of injection with a spring-loaded micrometer. The results are presented as ear thickness at 48 hr $\pm$ SD (ear thicknesses at 24 and 48 hr did not differ significantly). A positive control group of mice was immunized with 50 μg bovine serum albumin (BSA) + 10 μg Quil A and challenged with 60 μg BSA.

T-proliferative response

Groups of mice were immunized subcutaneously at the base of the tail with 50 μg E7.GST protein, or a mixture of E7 peptides 103 and 106 (50 μg of each) emulsified in Freund's complete adjuvant (FCA, Difco, RA 137). Both these peptides contain strong T-proliferative

epitopes active in the context of H-2^q. Eight to ten days later, mononuclear cells (LNC) were collected from inguinal and periaortic lymph nodes and set up in culture in 96-well microtiter plates (Nunc) with peptides 103 or 106, or with irrelevant peptide at 10 and 2 μg/ml as described (Tindle *et al.,* 1991). Assay groups were done in triplicate. Four days later wells were pulsed with [^{3}H]thymidine (100 μCi) overnight. Cells were harvested onto filter mats and bound radioactivity was quantified by Beta-plate (Wallac) scintillation counting. A positive control recall antigen was purified protein derivative (PPD) of tuberculin. Results were expressed as a stimulation index (SI) = mean cpm from wells with experimental peptide divided by mean cpm from wells with irrelevant peptide.

Cytotoxic T-cell responses

Induction of CTL and CTL assay. Mice were injected intradermally (id) in each of the hind footpads with 50 μl PBS containing 50 μg E7.GST protein or 106 peptide plus 10 μg Quil A. Four days later, popliteal and inguinal lymph nodes were excised and LNC were cultured at 3–5 $\times$ 10^6 cells per 2 ml plus 30 U/ml recombinant human IL-2 (Gibco BRL) in a 24-well plate. After 4 days in culture, LNC were harvested, washed, and set up in microtiter well culture with 5 $\times$ 10^3/well ^{51}Cr-labeled target cells (below) at effector cell: target cell ratios of 40:1 and 4:1. Supernatants were harvested 4.5 hr later and ^{51}Cr release was quantified by γ-counting. Results are expressed as percentage of specific cytotoxicity = $a - b$, where a = (^{51}Cr release in experimental well minus background)/(detergent mediated total ^{51}Cr release minus background) $\times$ 100%; b = (^{51}Cr release from targets without E7 peptide or E7 gene minus background)/(total release $-$ background) $\times$ 100%.

Target cells. Target cells (10^6) in 100 μl supplemented DMEM + 10% FBS were labeled for 60 min with 100 μCi^{51}Cr (Na chromate; Amersham), and washed twice using FBS underlay. One hundred microliters containing 5 $\times$ 10^3 cells were dispersed into microwells. In the case of peptide pulsed cells, microwells contained 20 μl of predispensed peptide in tissue culture medium to give a final concentration of 10 μM. Plates were incubated for 1 hr at 37° to allow target cells to become "sensitized" by peptide-MHC class I interaction, before addition of effector cells. Controls for the assay included targets without effectors ("background release"), nonsensitized targets, and in some assays, target cells with "irrelevant" effector cells.

Cell lines from E7 transgenic mouse epithelium

Cell lines T93 and 1022.16 were derived from αA cry HPV16 E6/E7 transgenic mice. T93 was derived from a spontaneous epithelial tumor and 1022.16 was derived from a tumor that arose in a FVB mouse injected with cells established in tissue culture from αA cry HPV16 E6/E7 transgenic mouse lens tissue (Griep *et al.,* 1993). The cells were maintained in RPMI medium, supplemented with glutamine, pyruvate, HEPES buffer, and 10% FBS. The cells grew as monolayers with a life span of ca. 10 passages. For purposes of CTL assays, 10^4 cells were seeded in microtiter trays the day before assay. Individual wells were labeled with 1 μCi ^{51}Cr *in situ* and washed prior to assay.

Retroviral transfection

Retroviruses recombinant for HPV16 E7 (PA317 E7) and HPV16 E6 (PA317 E6) were obtained from Dr. D. Galloway (Fred Hutchinson Cancer Center, Seattle). Cell lines were infected and selected in 500 μg/ml Geneticin as described (Demers *et al.,* 1994). The presence of the E7 DNA in infectants was confirmed by PCR of total cellular DNA using primer pairs yielding full-length E7. The PCR product was dot-blotted onto nitrocellulose and probed by nucleic acid hybridization using a ^{32}P-end-labeled oligonucleotide spanning E7 bases 689–755. Negative controls for E7 included cellular DNA and nontransfected cells. Following appropriate stringency washes, blots were subject to autoradiography using X-omat film (Kodak).

Hybridoma CTL target cells expressing H-2^q and H-2D^b

Spleen cells from C57-BL6 (H-2^b) $\times$ FVB (H-2^q) F1 hybrid mice were fused with Sp_20 myeloma cells using standard polyethylene glycol (PEG)-mediated procedures (Campbell, 1980) and selection in HAT medium. Twelve hybridomas were cloned twice by limiting dilution. All screened positive for cell-surface expression of H-2D^b, D^q, and K^q using monoclonal antibodies 28-8-6S, KH117, and KH114 (Pharmingen), respectively, in standard FACS analysis. Two hybridomas were selected for infection with Retrovirus E7 (above). One infected hybridoma containing the E7 gene and denoted ICI-2C-Ret E7 was selected as a target cell for H-2D^b and H-2^q-restricted CTL lysis.

RESULTS

Antibody response following E7 immunization in #19 mice of varying ages

At Day 31 after immunizations with E7.GST on Days 0 and 14, the sera of #19 mice of all age groups tested displayed high titer antibody to peptides 101, 104, and 106 (Fig. 1) containing the three major E7 epitopes EYMLD, IDGP, and QAEPD, respectively (Fig. 2). (The titer varied among antibodies to the three peptides being highest for peptide 101 and lowest for peptide 106; optical density readings at serum dilutions 1/12,800 (peptide 101), 1/3,200 (peptide 104), and 1/1,600 (peptide 106) were used to compare groups.) In all age groups the antibody response declined by Day 98, but was recalled by a third immunization on Day 98 (measured as eleva-

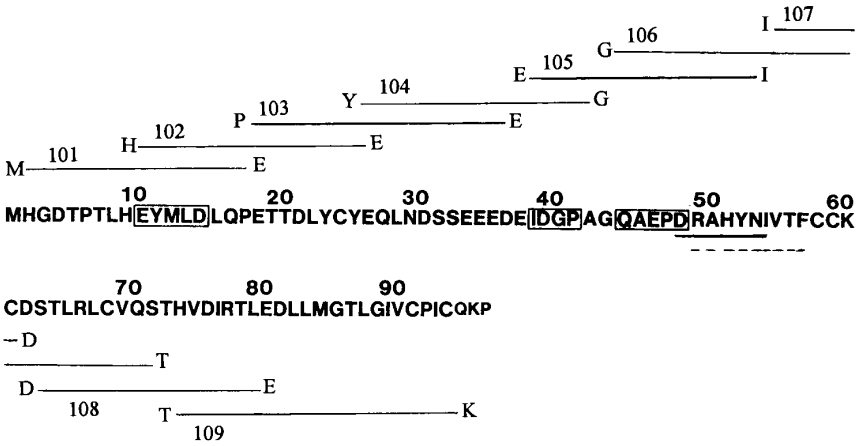

FIG. 1. Overlapping peptides spanning the HPV16 E7 protein. (Peptides were synthesized with unblocked ends in our laboratory using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on an Applied Biosystems 431A peptide synthesiser. The HPLC profile, amino acid composition, toxicity and mitogenicity of all peptides was checked.) Linear B-epitopes (Tindle *et al.,* 1990) are boxed. An immunodominant T-helper epitope (Tindle *et al.,* 1991) is underlined (solid). A D^b-restricted CTL epitope (Feltkamp *et al.,* 1993) is underlined (dashed).

tion of OD by Day 105). This pattern of response was similar to that displayed by FVB nontransgenic parental mice. The sera of E7-immunized #19 and FVB mice did not react with peptides spanning other regions of the E7 molecule (data not shown). The sera of #19 mice immunized with adjuvant + saline alone gave negligible OD readings.

Antibody response following E7 immunization in #19 mice with and without skin disease

The timing of the spontaneous onset of skin disease among individual #19 mice is variable. In the above experiment, some mice in the older age groups already displayed skin disease at the time of first immunization or had developed it by the time of the first bleed (Day 31 post-first-immunization). Others remained disease free at Day 31. Among mice from the younger age groups (and those from the older age groups which had not developed skin disease at Day 31), many had developed skin disease by Day 105 post-first-immunization while others had not. We thus analyzed the data from the above experiment to compare serum antibody response in mice with and without skin disease at Day 31 and in mice with and without skin disease at Day 105 (Table 1).

Read-outs using sera from #19 mice presenting with skin disease at Day 31 did not differ significantly from those of #19 mice without skin disease at Day 31 in the magnitude of the serum antibody response following immunization with E7 against peptides 101 and 104 measured on Day 31, and against peptides 101, 104, and 106 measured on Day 105 (students *t* test). A marginally significant difference was observed in reactivity to peptide 106 on Day 31 ($t = 3.602$, $P = 0.0024$). #19 mice presenting with skin disease at Day 105 did not differ significantly from mice without skin disease at Day 105 in the magnitude of the serum antibody response against the three peptides measured on Day 105.

Delayed-type hypersensitivity response to HPV16 E7

Three groups of #19 mice aged 13, 28, and 37 weeks, and age-matched FVB controls were immunized with E7.GST fusion protein in Quil A adjuvant and 7 days later challenged intradermally in the ear-lobe with E7.GST fusion protein (left ear) and GST alone (right ear). A DTH response was measured by ear thickness increment at 48 hr. #19 mice of all ages showed augmented ear thickness in the E7.GST challenged ear compared to the GST challenged ear, indicating an E7 specific component to the response. No significant differences in thickness increment in the E7.GST challenged ears were observed between the three age groups of #19 mice, nor between #19 mice and FVB mice of corresponding age (Fig. 3).

T proliferative response

LNC from groups of FVB mice immunized with overlapping peptides spanning the entire E7 molecule (Fig. 1) proliferated when recalled *in vitro* with peptides 103 and 106 but not with the other peptides (data not shown). Thus within the context of peptide immunization, peptides 103 and 106 contain major proliferative epitopes recognized in the context of $H\text{-}2^q$. LNC from FVB mice immunized with E7.GST protein proliferated *in vitro* in response to 103 but not 106 or other peptides of the series. (Stimulation index of 2.9I at 10 μg/ml *in vitro* recall peptide 103).

To evaluate whether E7 protein expressed in skin in #19 mice engendered an endogenous T proliferative response, LNC cells from groups of #19 mice of various ages (and FVB mice of similar ages as controls) not immunized with E7 or E7 peptide were challenged *in vitro* with peptide 103. No proliferative response to peptide 103 was observed in any of the age-groups of #19 mice (Fig. 4.1).

To evaluate whether E7-peptide immunized #19 mice

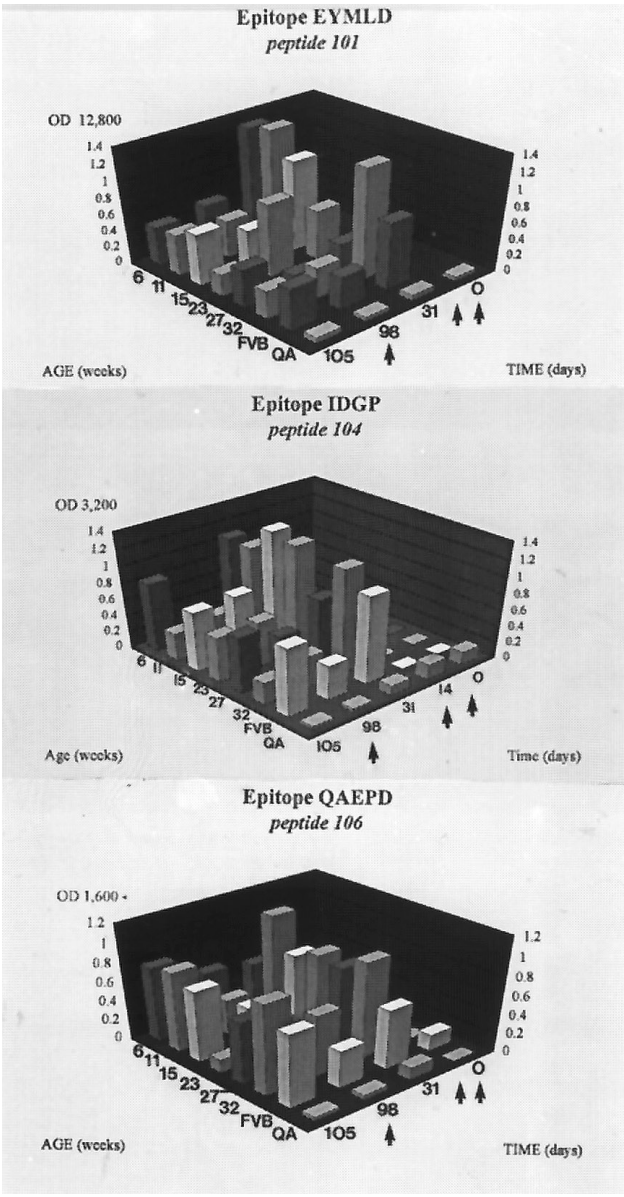

FIG. 2. ELISA reactivity of sera from #19 mice of varying ages as indicated and FVB mice of 6 weeks of age, immunized with E7.GST protein + Quil A (arrows indicate times of immunization) against three HPV16 E7 peptides, each containing a major B-epitope. QA denotes #19 mice (6 weeks old) immunized with Quil A alone.

would mount T-proliferative response to peptides 103 and 106 comparable to those in nontransgenic FVB mice, and whether there might be age-related differences among #19 mice, groups of #19 mice, and FVB controls of various ages were immunized with a 103/106 peptide mix, and 8–10 days later, LNC were challenged *in vitro* with peptides 103 or 106 or irrelevant control peptide (Fig. 4.2). No significant differences in proliferative responses to peptides 103 were detected among groups of #19 mice of 7, 32, and 46 weeks of age. Similarly no significant differences in LNC proliferation were detected between #19 mice and that of nearest-age FVB controls. Similar observations were made for 106 peptide.

Cytotoxic T-cell (CTL) response

In preliminary experiments it was determined that E7.GST-immunized FVB mice failed to kill E7-DNA containing cell lines T93 and 1022.16 established from α-A cry HPV16 E6/E7 transgenic mice, although these lines were killed in allo-CTL reactions and were, therefore, in principal susceptible to effector-mediated lysis in ^{51}Cr release assays (data not shown). In further experiments we augmented the amount of E7 protein in these cells to levels detectable in an E7 protein capture assay (Selvey *et al.,* 1994) by infection with recombinant Retrovirus E7. The resultant T93.Ret E7 and 1022.16.Ret E7 cells expressed 48.5 ng E7/mg and 76.7 ng E7/mg total cellular protein, respectively. These E7 expressing cells were not killed by effectors from E7 immunized H-2^q mice. T93 and 1022.16 cells pulsed with overlapping peptides spanning the entire E7 molecule were not killed by effectors from E7 immunized H-2^q mice or H-2^q mice immunized with groups of these peptides (not shown). All of these experiments contained a positive control arm to confirm the efficacy of the immunization and experimental procedures used: E7-expressing or E7 peptide pulsed H-2^b targets (EL4.C2 (Tindle *et al.,* 1995) and EL4 cells, respectively), were killed by effectors from E7.GST immunized H-2^b (C57 BL6) mice or effectors from H-2^b mice immunized with peptide 106 containing the H-2D^b restricted CTL epitope RAHYNIVTF.

We inferred from these experiments that HPV16 E7 protein does not contain a CTL epitope recognized in the context of H-2^q. To investigate this further we constructed cells expressing both H-2^q and H-2D^b restriction elements and E7 protein, as follows. B-cell hybridomas were derived from spleen cells of H-2^q $\times$ H-2^b (FVB $\times$ C57 BL) F1 hybrid mice and were infected with Retrovirus E7. Hybridoma ICI-2C-Ret E7, demonstrably expressing H-2D^q, H-2K^q, and H-2D^b at the cell surface, and containing the E7 gene (Fig. 5), and IC-2C (not infected with Retrovirus E7) were used as targets for CTL assays. ICI-2C-Ret E7 was specifically killed by effector cells from E7.GST immunized H-2^b (C57 BL6), but not H-2^q (FVB) mice (Figs. 6A and 6B). In addition, FVB H-2^q effector cells failed to kill ICI-2C pulsed with any of the peptides covering the entire E7 molecule, while control H-2^b effectors killed ICI-2C pulsed with peptide 106 containing the H-2^b-restricted CTL epitope RAHYNIVTF (percentage of specific lysis at 40:1 target: effector ratio = 23.5 $\pm$ 0.7% (ICI-2C + peptide 106), 11.1 $\pm$ 0.9% (ICI-2C + irrelevant peptide).

These results suggest most strongly that the H-2^q background of FVB and #19 mice does not permit the recognition of a CTL epitope in E7 protein.

Thus, in order to investigate CTL responses in #19 mice induced by immunization with E7, we introduced the D^b restriction element of the known E7 CTL epitope RAHYNIVTF into the H-2^q background of #19 mice, by crossing to the F1 generation with a line of FVB mice transgenic for H-2D^b. The resultant mice were denoted

TABLE 1

Antibody Response to HPV16E7 Peptides in E7 Immunized #19 Mice with and without Skin Disease: Serum ELISA Reactivity vs E7 Peptides

	Day post first immunization					
	Day 31 peptide			Day 105 peptide		
	101[a]	104[b]	106[c]	101[a]	104[b]	106[c]
Without skin disease[d] ($n = 14$)	0.8 ± 0.14	0.31 ± 0.29	0.17 ± 0.19	0.85 ± 0.21	0.40 ± 0.41	0.17 ± 0.07
With skin disease[d] ($n = 4$)	0.97 ± 0.50	0.61 ± 0.41	0.72 ± 0.48	0.86 ± 0.54	0.32 ± 0.31	0.29 ± 0.38
	ns	ns	$t = 3.602$ $P = 0.0024$	ns	ns	ns
Without skin disease[e] ($n = 3$)				0.55 ± 0.28	0.14 ± 0.08	0.11 ± 0.07
With skin disease[e] ($n = 5$)[f]				0.43 ± 0.21	0.15 ± 0.11	0.24 ± 0.2
				ns	ns	ns

[a] OD 280 at 1:12,800 dilution, mean ± standard deviation.
[b] At 1:3,200 dilution.
[c] At 1:1,600 dilution. [Note: These dilutions are end titers reflecting the strength of the responses to epitopes contained in peptides 101, 104, and 106 in H-2^q mice.]
[d] At Day 31 post first immunization.
[e] At Day 105 post first immunization.
[f] Note that the group of mice presenting with skin disease at Day 105 includes those mice which already developed skin disease by Day 31.

#19/D^b. Effector cells from E7 immunized #19/D^b mice specifically killed EL4 (H-2^b) cells pulsed with peptide 106 containing the RAHYNIVTF CTL epitope, but not EL4 cells pulsed with irrelevant peptide (Fig. 6C). The effector cells also killed D^b targets expressing the E7 gene (1C1-2C-Ret-E7 cells) (Fig. 6D). Results demonstrating a positive E7-directed CTL response of similar magnitude in E7.GST immunized FVB/D^b (H-2^q, D^b) but not FVB (H-2^q) mice were obtained. (not shown).

DISCUSSION

The E7 oncoprotein of HPV 16 transforms KCs of anogenital epithelium resulting in anogenital cancer. E7 pro-

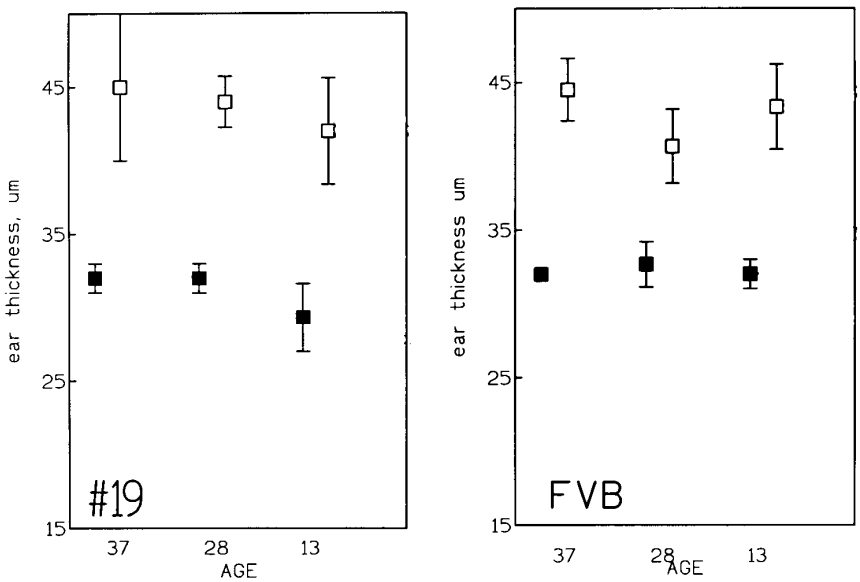

FIG. 3. DTH response evoked by intradermal ear challenge in HPV16 E7.GST-immunized #19 and FVB mice of various ages. Results are expressed as ear-thickness (μm) ± SD. Open symbols, mice challenged with E7.GST. Closed symbols, mice challenged with GST. Mean ear thickness of GST immunized #19 mice challenged with E7.GST was 35.7 ± 8.15 μm. (Ears were measured at 24 and 48 hr. Only 48-hr data are shown.)

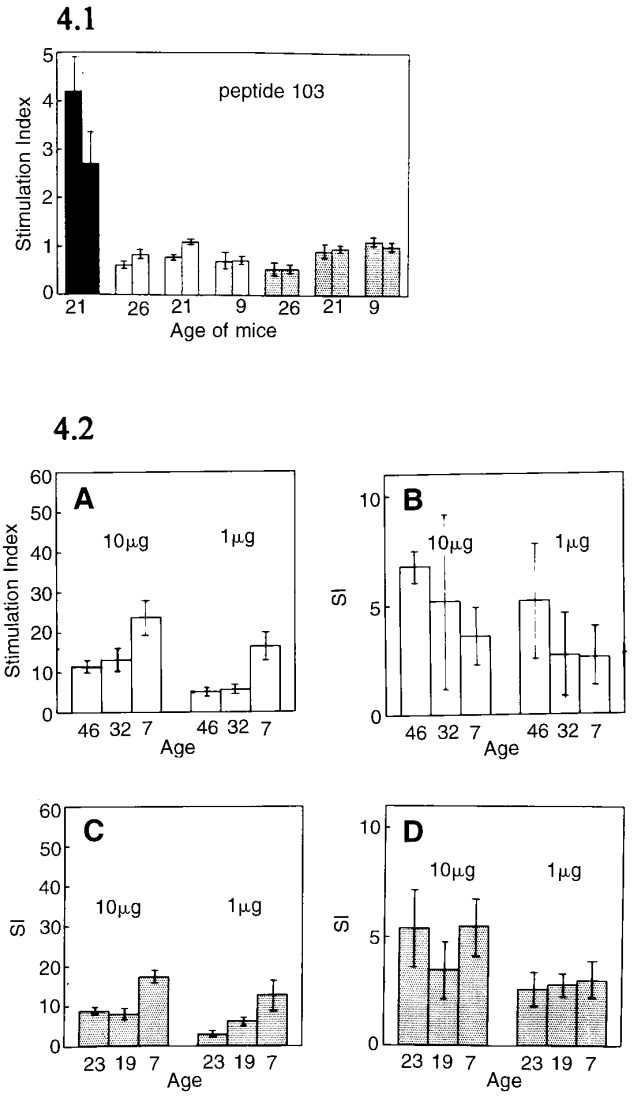

FIG. 4. T-proliferative responses. (4.1) LNC from mice #19 and FVB mice of various ages immunized with saline:Freund's complete adjuvant (FCA) were cultured for 4 days in the presence of 10 μg/ml (left bar of a pair) or 1 μg/ml (right bar of a pair) of peptide 103. Proliferation was quantified by [^{3}H]thymidine incorporation. Results are expressed as a stimulation index (see Materials and Methods). Open bars, FVB mice; stippled bars, #19 mice; solid bars, FVB mice which had been immunized with 100 μg of a 50/50 mix (w:w) of peptides 103 and 106 in FCA and recalled with 10 μg/ml peptide 103 (positive control). Medium and irrelevant peptide controls were in the range 7,000–18,000 counts per minute (cpm) and 11,500–22,000 cpm, respectively; PPD positive controls were in the range 340,000–428,000 cpm. (4.2) LNC from #19 mice (open bars) and FVB mice (stippled bars) immunized with 100 μg of a 50:50 (w:w) mix of peptides 103 and 106 in FCA were cultured for 4 days in the presence of 10 or 1 μg/ml of peptide 103 or 106. Proliferation was quantified by [^{3}H]thymidine incorporation. (A) LNC from 103/106 immunized #19 mice recalled with peptide 106; (B) LNC from 103/106 immunized #19 mice recalled with peptide 103; (C) LNC from 103/106 immunized FVB mice recalled with peptide 106; (D) LNC from 103/106 immunized FVB mice recalled with peptide 103. Results are expressed as stimulation index $\pm$ standard deviation. Medium alone and irrelevant-peptide negative controls gave counts in the range 1,200–5,000 cpm and 2,600–8,200 cpm, respectively. PPD-positive controls gave counts in the range 260,000–357,000 cpm. #19 and FVB mice immunized with adjuvant alone gave stimulation indices <2, after LNC challenge with peptides 103 or 106.

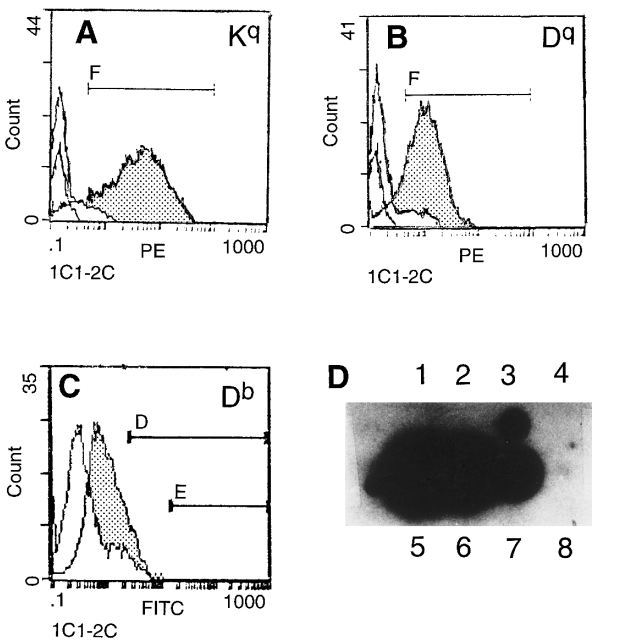

FIG. 5. A B-cell hybridoma, IC1-2C, derived from (H-2^b $\times$ H-2^q) F_1 hybrid spleen cells expresses at the cell surface (A) K^q; (B) D^q; and (C) D^b (shaded); and (D) when infected with rRetrovirus E7, contains E7 DNA: In (A) and (B), cells were reacted with biotinylated anti-mouse K^q and D^q MAbs and visualized in FACs analysis using Streptavidin-phycoerythrin (Caltag). In (C), cells were reacted with anti-D^b murine Mab (IgG$_{2a}$) and visualized in FACs analysis with FITC-anti-mouse IgG$_{2a}$ (Silenus). Negative controls included ICI-2C cells stained with irrelevant antibody, and also, irrelevant cells (H-2^d Sp$_2$0 mouse myeloma) stained with anti-K^q, D^q, and D^b Mabs. (D) DNA from hybridomas ICI-2C and 2B4, and ICI-2C, infected with rRetrovirus-E7 or E6, and E7-containing plasmid controls were amplified in PCR with E7 primers, dot-blotted onto nitrocellulose, and probed with a ^{32}P-labeled E7 oligonucleotide probe. (1) ICI-2C; (2) ICI-2C Ret E6; (3) ICI-2C Ret E7; (4) 2B4; (5) pHPV16 0.02 μg; (6) pJ4omega E7; (7) pHVP16 0.02 μg; (8) No DNA.

tein exerts its effect by combining with and abrogating the cell-cycle regulatory effect of the retinoblastoma protein (RB) (Slebos *et al.,* 1994). A corollary is that E7 protein must persist if cells are to remain transformed (von Knebel Doeberitz *et al.,* 1994). E7 protein thus functions as a tumor-associated antigen to which therapeutic vaccine strategies may be directed. That the immune response, particularly if augmented by vaccination, might serve to control disease is indicated by worsening outcomes of HPV infection in constitutively or iatrogenically immunosuppressed individuals (see Tindle and Frazer, 1994 for review), and by the control by E7 immunization of E7 containing tumour growth in animal tumor challenge models (Chen *et al.,* 1992; Feltkamp *et al.,* 1993; Tindle *et al.,* 1995).

Papillomavirus infection presents a natural model for the study of immune activation by, and immune recognition of, epithelial cells presenting non-self-antigen. HPVs are obligatory epitheliotropic (Pfister, 1984). Unlike other known epitheliotropic viruses (herpes simplex, pox viruses), HPVs do not kill the KCs which they infect: rather

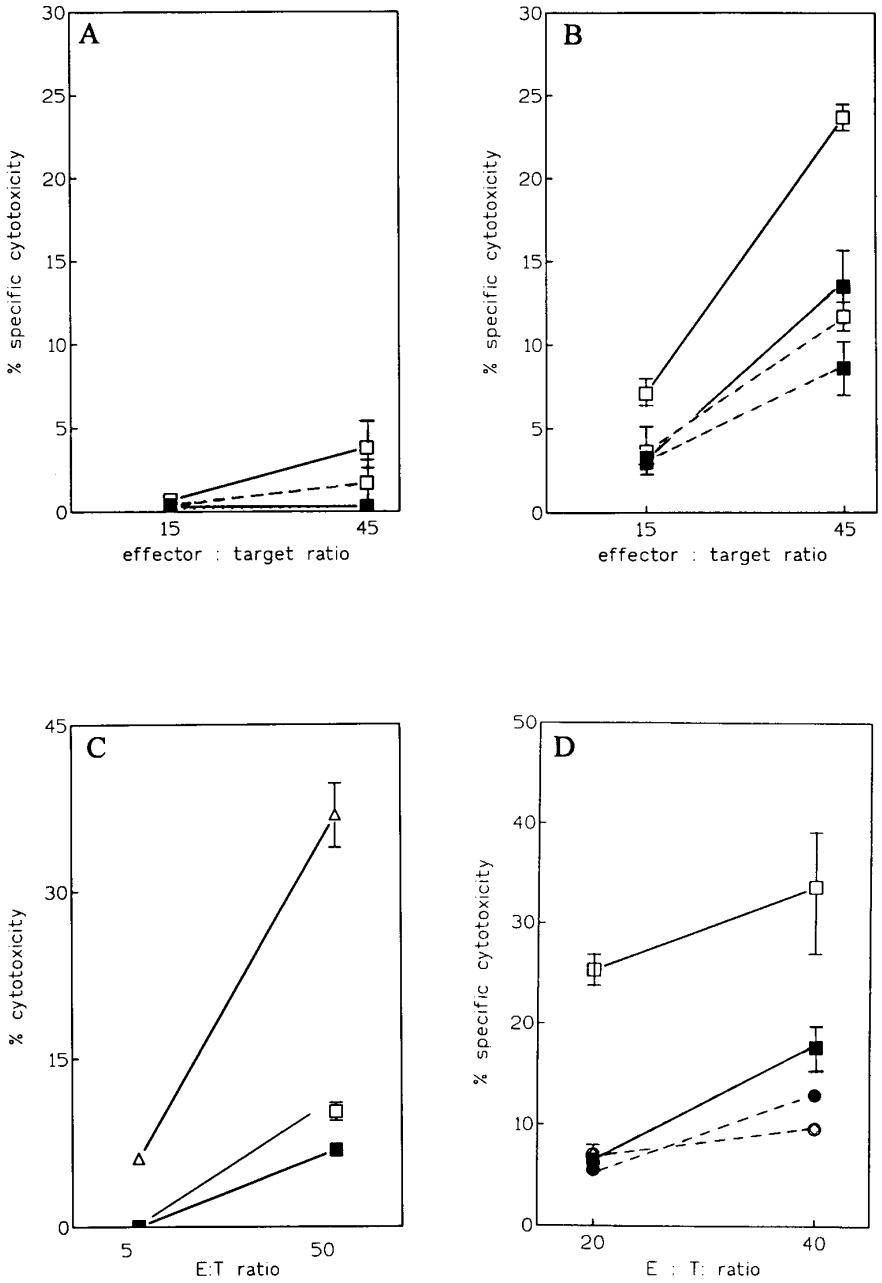

FIG. 6. CTL lysis of targets measured by ^{51}Cr-release assay. CTL from (A) FVB (H-2^q) mice, (B) C57 BL (H-2^b) mice immunized with E7.GST + Quil A (solid line) or Quil A alone (dashed line) versus B-hybridoma ICI-2C (K^{q+}, D^{q+}, D^{b+}) (closed squares) or ICI-2C-Ret E7 (open squares) targets. (C) CTL from #19/D^b (H-2^q, D^b) mice immunized with E7.GST + Quil A versus EL4 (D^{b+}) targets pulsed with irrelevant peptide (closed square), or EL4 pulsed with peptide 106 containing the D^b-restricted CTL epitope (open triangle), or transfected with E7 gene (EL4.C2) (open square). (D) CTL from #19/D^b mice immunized with E7.GST + Quil A (solid line) or Quil A alone (dashed line) versus ICI-2C hybridoma (closed symbols) or ICI-2C-Ret E7 (open symbols) targets. Results are expressed as means ± SD of triplicate samples.

the infected KCs mature and are desquamated, complete with viral particles. As a result there is little or no extracellular release of viral proteins for presentation to the immune system by MHC class 2 molecules on professional APCs (Langerhans cells). Furthermore, no inflammatory response occurs in HPV-infected epithelium thus obviating cytokine mediated induction of MHC class 2 molecules on the surface of infected KCs, and there may be no induced secondary signals from KCs or Th cells for cytotoxic T cell (CTL) activation.

#19 mice transgenic for the HPV16 E7 ORF provide a means to investigate E7 antigen presentation to the immune system. In these mice, there is no expression (RT-PCR) of E7 in the thymus (Lambert *et al.,* 1993) and therefore, the mice escape central tolerization to E7 protein (Frazer *et al.,* 1995). E7 protein is first detected in noninflamed, "normal" skin, and this precedes the development of inflammatory skin disease (beginning at ca. 12 weeks of age) in which E7 protein expression is highest, and invasive squamous tumors.

It is pertinent to examine the outcome of E7 presentation to the immune system prior to inflammatory skin disease since this sheds light on the efficacy or otherwise of KCs to induce a (negative or positive) immune response, with implications for any non-MHC antigen presented through skin as a result of infectious agents, tumorigenesis, or the appearance of autoantigens. The chronology of E7 protein putative exposure to the immune system in #19 mice has parallels with that which occurs in progressing HPV16-associated lesions of human cervical epithelium. Here, E7 is expressed in an intact noninflamed epithelium, prior to progression to preneoplastic and neoplastic lesions which coincide [at least in 20–60% of patients (Jochmus-Kudielka *et al.,* 1989; Garenstroom *et al.,* 1994)] with the appearance of E7-specific serum antibody.

In the present study, we compared the outcome of immunization with E7 protein in young #19 mice at an age at which E7 protein could not be detected in skin, with older #19 mice displaying E7-associated skin phenotype, and with syngeneic nontransgenic FVB control mice. We hypothesized that the outcome of the E7 protein immunization would reflect the immunological status, i.e., tolerant, versus immune versus "naive" of the mice with regard to E7.

We have previously shown that E7 immunized #19 mice exhibited E7-specific serum antibody responses (Frazer *et al.,* 1995). In this paper we show that irrespective of age (and therefore presumably degree of life-time exposure to endogenous E7 protein) and skin phenotype (normal vs diseased), E7-immunized #19 mice show E7-directed antibody responses, DTH responses, and T-proliferative responses of similar magnitude and, in the case of antibody, of similar chronology. Furthermore, no consistent differences were detected between these responses and those exhibited by identically immunized FVB nontransgenic control mice. No responses to the B- and T-epitopes we examined were seen in unimmunized #19 mice. These data argue that the immune system of #19 mice prior to immunization with E7 protein, has not encountered, i.e., is naive to, the E7 B- and T-epitopes involved in these responses. The epitopes constitute the major epitopes recognized by B- and T-repertoires of mice of a variety of MHC haplotypes (Tindle *et al.,* 1991).

One would not expect #19 mice to be centrally tolerant to E7 protein since it is not expressed perinatally in the thymus nor at any immunologically accessible site. In this study we found no evidence for the acquisition of peripheral tolerance to E7 protein in #19 mice with age as measured by specific antibody production, DTH, and T-proliferation.

Although HPV16 E6 mRNA occurs in adult epithelium in #19 mice (Lambert *et al.,* 1993) E6 protein has not been detected. This may represent a lack of appropriately sensitive immunodetection reagents and assay, rather than absence of specific protein translation. However, spontaneous antibody response to E6 protein in #19 mice cannot be detected (R. Azoury, unpublished). Further experiments in our laboratory indicate that #19 mice appropriately immunized with E6 fusion protein make E6-specific antibody, T-proliferative, and DTH responses in the same manner as identically immunized nontransgenic controls (R. Azoury, unpublished). Thus, even if E6 protein is produced in the skin, #19 mice remain positively immunologically responsive to the epitopes presented by E6 immunization. This parallels the E7 responsiveness of E7-immunized #19 mice.

No CTL response measurable against E7-containing H-2^q target cells could be induced in #19 mice or FVB mice immunized with whole E7 protein or any of a series of generously overlapping peptides spanning the entire E7 molecule, using experimental conditions which routinely induced a good CTL response in C57 (H-2^b) mice. This observation suggests that there is no CTL epitope in E7 protein recognized by mouse CTL precursors in the context of H-2K^q and D^q. Two pieces of data add weight to this surmise; (1) (hybridoma) target cells shown to coexpress both H-2^q and H-2^b at the cell surface, were killed by effectors from H-2^b E7-immunized mice, but not from H-2^q E7-immunized mice when these target cells were made to express whole E7 (by Retrovirus E7 transfection), or were pulsed with E7-peptides, and (2) LNC from E7-immunized #19 or FVB mice made cotransgenic for H-2D^b specifically killed the H-2^b, H-2^q expressing target cells (but not targets expressing H-2^q alone) pulsed with a peptide containing the D^b-restricted CTL epitope. This latter observation suggests that exposure to E7 expressed in skin had not rendered anergic E7-directed CTL precursors in #19/D^b mice (which constitutively express both D^b and E7.) (It also obviates the possibility of a constitutive inability of #19 and FVB to mount CTL responses as an explanation for the lack of CTL response to E7 in these mice).

We report that immunization of #19 mice (with or without skin disease) produces an antibody response directed to the N′ terminus of the E7 molecule. (Note in addition that antibody titers were highest against the most N′ terminal peptide (101) and lowest against the least N′ terminal peptide (106)). We have earlier reported that unimmunized #19 mice with skin disease (but not unimmunized #19 without skin disease) spontaneously develop antibody to the C′ terminal end of the E7 molecule (Frazer *et al.,* 1995). We hypothesize that E7 protein expressed in the skin is not perceived by the immune system until the development of inflammatory skin disease when (a) upregulation of MHC class 2 on keratinocytes may occur as a result of inflammation (Gaspari and Katz, 1995) allowing presentation to CD4 T-cells of processed endogenous protein including E7, and (b) E7 is liberated from ruptured keratinocytes, and may be taken up by local "professional" antigen presenting cells (LCs).

This surmise is reinforced by the observation that #19 mice which are heterozygous for the E7 gene and which

do not acquire skin disease (see below), although they express E7 protein in skin, do not develop a spontaneous antibody response to endogenous E7 (R. Tindle, unpublished).

Reactive T-cells in the periphery may ignore cognate antigen if (1) antigen bearing cells are sequestered at a site anatomically impervious to T-cells; (2) antigen bearing cells express few or no MHC or costimulatory molecules necessary to activate T-cells [e.g., B7.1 (Williams *et al.,* 1994)] or; (3) reactive T-cells are peripherally deleted or anergized by interaction with antigen bearing cells. A number of transgenic models address these issues, and while the determinants of outcome (i.e., naivety, tolerance, or immunity) are not fully understood, it is clear that timing and level of expression and availability of costimulatory signals are important parameters. Thus, deletional B and/or T tolerance ensues from high level of transgenic protein expression in multiple tissues, whereas naivety or developing immunity is a likely outcome where transgene expression is lower grade and tissue specific (reviewed in Miller and Flavell, 1994), as it is in the case of E7 and #19 mice.

The inability of H-2^q mice to mount an E7-directed CTL response, reported here, is consistent with the finding that depletion of $CD8^+$ cells fails to alter the onset of skin disease in these mice (Hilditch *et al.,* submitted). An active CTL response is coincident with rejection of skin allografts (reviewed in Strom *et al.,* 1996) and tumors (Kast *et al.,* 1989; Toes *et al.,* 1994), including syngeneic H-2^b tumors expressing E7 as a tumor-associated antigen. In preliminary experiments in which groups of young #19 mice were immunized twice with E7 fusion protein or irrelevant protein in Quil A or Algammulin adjuvants, or were unimmunized, no between-group differences in timing of appearance or severity of skin disease were noted (G. Fernando, unpublished). While more extensive experiments are necessary, these findings suggest that antibody or DTH may not have a major role in control of skin disease onset or progression. It may be surmised that an absence of an inducible E7-specific CTL response on a H-2^q background contributes to the development of E7-mediated skin disease and tumors in #19 mice. Skin disease and tumors occur in #19 mice homozygous for E7, but not in #19 mice heterozygous for E7 (Lambert *et al.,* 1993). This may represent a gene dosage effect. #19/D^b F1 hybrid mice used in our CTL induction experiments were necessarily heterozygous for E7 and therefore cannot be used for skin disease experiments. We are presently deriving a line of #19 mice homozygous for E7 which also carries the D^b transgene encoding the CTL restriction element. The question whether E7-directed CTL play a role in control of skin disease onset or tumors will be amenable to probing by appropriate E7 immunization of these mice.

In summary, our data in #19 transgenic mice indicate that (1) E7 expression in phenotypically normal or in diseased skin does not serve to activate or render anergic B- and Th-cells specific for epitopes presented by E7 immunization, (2) Th and B cells are activated by immunization with E7 protein, (3) E7 expression in normal skin does not functionally abrogate the recall of E7-specific Th or T_{DTH} cells, or of memory B cells. (4) There is an absence of an E7-directed CTL response restricted through H-2^q. Incorporation of a suitable restriction element (D^b) into the genome allows a CTL response directed to a D^b restricted CTL epitope to be induced by immunization in both #19/D^b and FVB/D^b mice. That such a response can be engendered in #19/D^b mice indicates that low levels of E7 expression in phenotypically normal skin does not serve to render CTL precursors anergic.

The inference drawn in this paper that E7 expressed in skin keratinocytes may not be presented to the afferent arm of the immune system in such a way as to induce a (positive or negative) response receives support from further work in our laboratory showing that FVB mice fail to reject E7 expressing skin grafts from #19 mice (L. Dunn, submitted).

The studies reported here are relevant to the immunological outcome of any non-MHC, non-self-protein expressed in epithelial cells, with implications for viral infection, tumor development, and autoimmunity. There is also relevance to the development of tumor vaccines, in particular to the application of E7-based vaccines for HPV E7-expressing cervical carcinoma.

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