Mice Expressing the E7 Oncogene of HPV16 in Epithelium Show Central Tolerance, and Evidence of Peripheral Anergising Tolerance, to E7-Encoded Cytotoxic T-Lymphocyte Epitopes

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In order to derive mice which expressed both the E7 open reading frame transgene of human papillomavirus type 16 in skin and MHC class 1 restriction elements for several E7-encoded cytotoxic T-lymphocyte (CTL) epitopes, K14.HPV16E7 mice which express E7 in basal keratinocytes were crossed to the F1 generation with A2.1 Kb transgenic mice which express the MHC binding cleft domains of human HLA A*0201, and murine H-2Kb. F1 mice (denoted K14E7×A2.1) expressed E7 in the thymus at least as early as 2–5 days before birth. Immunisation of FVB×A2.1 control mice (transgenic for HLA A*0201 and H-2Kb but not for E7), with two HLA A*0201-restricted epitopes of E7 and one H-2Kb-restricted CTL epitope of E7, gave strong primary CTL responses recognising epitope-pulsed or constitutively E7-expressing syngeneic target cells. In contrast, in immunised K14E7×A2.1 mice, the CTL responses to the H-2Kb epitope and one of the HLA A*0201 CTL epitopes of E7 were strongly down-regulated, and to the other HLA A*0201 epitope, completely abolished, as demonstrated by percentage specific killing by bulk splenocyte cultures in cytotoxicity assays, and by CTL precursor frequency analysis. In thymus-transplanted bone marrow radiation chimeras in which the immune system of K14E7×A2.1 mice was replaced by a FVB×A2.1 immune system, specific immunisation did not result in reemergence of strong E7-directed CTL responses. In agreement with these in vitro findings, specific immunisation failed to significantly alter the course of E7-associated tumour development in K14E7×A2.1 mice. These data are consistent with a model of central deletional CTL tolerance to E7-encoded epitopes recognised in the context of two distinct MHC class 1 restriction elements, and with the possibility of peripheral T-cell anergy maintained by expression of E7 in the skin.

INTRODUCTION

The E7 oncoprotein of human papillomavirus type 16 (HPV16) contributes to the transformation and maintenance of malignant phenotype in epithelial cells in vitro (von Knebel Doeberitz et al., 1994; M unger, 1995), and presumably also in E7 expressing premalignant and malignant lesions of the uterine cervix. HPV16 is associated with up to 90% of cervical squamous cell neoplasms (Bosch et al., 1995). Continued expression of E7 to maintain the transformed phenotype (von Knebel Doeberitz et al., 1994) thus provides a tumour-associated antigen to which immunomanipulative strategies can be directed (Tindle et al., 1995). A high percentage of cervical carcinoma patients make antibody to HPV16 E7 (Bleul et al., 1991), and E7 directed cytotoxic T-lymphocytes have been detected in some (Altmann et al., 1992; Ressing et al., 1996) as a result of endogenous expression of E7. Vaccination with HPV16 E7 induces cellular and humoral immunity in humans (Borysiewicz et al., 1996) and in animal models, with protection against challenge with E7-expressing tumour detected in the latter (Chen et al., 1993; Tindle et al., 1995).

The phenotypic sequelae of expression of an HPV E7 transgene in the epithelium of mice are diverse depending (at least in part) on the promoter used and the site of expression of the transgene within the epithelium. Mice expressing HPV16 E6 and E7 or E7 alone specifically in the basal or suprabasal epithelial cells driven from keratin 10 or keratin 14 promoters (Arbeit et al., 1994; Auewaralkul et al., 1994; Herber et al., 1996; Meler et al., 1997) display epithelial hyperplasia and dysplasia at multiple epidermal and squamous mucosal sites with a propensity to papillomatosis and squamous cell carcinoma, particularly at sites subject to mechanical or chemical irritation (Coussens et al., 1996). Such mice...
provide a model for E7-mediated epithelial cell transformation in humans (Frazer et al., 1995) and for testing of E7-directed immunopreventative and immunotherapeutic interventions (Tindle, 1996). In addition, these mice provide a model with which to probe fundamental immunological questions regarding presentation of neoantigen (E7) to the immune system by cells of epithelial origin.

We have previously shown that mice with constitutive expression of E7 restricted to epithelium in adult life, and without perinatal expression in the thymus (Griep et al., 1993), had no endogenous E7-directed CTL response, and generated specific cytotoxic T-lymphocyte (CTL) following E7 immunisation in a way indistinguishable from their non-E7 transgenic counterparts (Herd et al., 1997). In addition, these mice generated no antibodies to endogenous E7 unless skin wounding or local invasive tumours occurred, as a consequence of which E7 presentation to the immune system presumably occurred in professional antigen-presenting cells as well as in epithelium (Frazer et al., 1995).

In this paper, we investigate the primary E7-directed CTL responses induced by immunisation in mice derived from the K14.HPV16E7 transgenic line (Herber et al., 1996), in which expression of the E7 transgene is driven from the keratin 14 promoter and occurs in basal epithelial cells. Since previous work from our group has shown that the H-2b background of these mice does not provide a restriction element for any E7-derived CTL epitope (Herd et al., 1997), we crossed K14.HPV16E7 mice to the F1 generation with HLA A*0201 transgenic mice (Vitiello et al., 1991), thereby introducing CTL restriction elements for three defined E7 CTL epitopes (two HLA A*0201-restricted epitopes (Ressing et al., 1995) and one H-2Db-restricted epitope (Feltkamp et al., 1993)).

We demonstrate that E7 is expressed in the thymus before birth in K14E7×A21 mice. We show that, in immunised K14E7×A21 mice, CTL responses to two of the three CTL epitopes are down-regulated, and to one epitope, abolished, in these E7 transgenic mice compared to their non-E7 transgenic counterparts. In the case of at least one CTL epitope, down-regulation is due to a loss of CTL precursors across a wide range of CTL-MHC/peptide complex affinities. We demonstrate that the residual CTL response induced by specific immunisation in K14E7×A21 mice fails to protect them from development of E7-associated tumours. Ablation of the immune system by whole body irradiation abolished completely the residual CTL response in K14E7×A21 mice. The capacity to mount non-E7 transgenic levels of CTL activity could not be established in these ablated mice by adoptive reconstitution with thymus implants and immunocytes, both from syngeneic but non-E7 transgenic donors. The results are discussed within the context of central thymic tolerance induction and of maintenance of peripheral CTL nonreactivity to E7 in K14E7×A21 mice.

RESULTS

Perinatal thymic expression of E7 in K14E7×A21 mice

We demonstrated E7 mRNA in the thymus of K14E7×A21 mice at least as early as 2–5 days before birth, and in neonates at 8 days (Fig. 1).

The CTL response to a H-2b-restricted CTL epitope is down-regulated in K14E7×A21 mice

Groups of K14E7×A21 and FVB×A21 mice were immunised with various doses of 8Q peptide, containing the H-2Db-restricted E7 CTL epitope RAHYNIVTF. Splenocytes from FVB×A21 mice specifically killed EL4.A2 cells pulsed with peptide 8Q, and EL4 cells expressing the E7 gene (C2 cells) irrespective of immunising dose used in the experiment (Figs. 2A–2C). In comparison, the percentage specific cytotoxicity against EL4.A2 cells pulsed with peptide 8Q, and EL4 cells expressing the E7 gene (C2 cells) irrespective of immunising dose used in the experiment (Figs. 2A±2C). In comparison, the percentage specific cytotoxicity against EL4.A2 cells pulsed with 8Q, and against C2 cells, was significantly lower in corresponding groups of K14E7×A21 mice immunised with 50 and 10 μg of 8Q peptide, and specific killing was not detected in K14E7×A21 mice immunised with 0.4 μg of 8Q (Figs. 2D±2F).

The CTL responses to two HLA A*0201-restricted CTL epitopes are down-regulated or abolished in K14E7×A21 mice

Splenocytes from K14E7×A21 and FVB×A21 mice immunised with 50 μg each of a mixture of peptides 597
and 598, were restimulated in vitro with 597 or 598. CTL activity was measured against EL4.A2 target cells pulsed with cognate or irrelevant (in this case 8Q) peptide. FVB×A21 splenocytes restimulated with 597 or 598 showed high specific killing of EL4.A2 targets pulsed with 597 or 598, respectively (Figs. 3D and 3B). K14E7×A21 splenocytes restimulated with 597 showed no killing of 597 pulsed EL4.A2 targets (Fig. 3C). K14E7×A21 splenocytes restimulated with 598 showed killing of 598 pulsed EL4.A2 targets (Fig. 3A). No killing by any group was observed on 597 or 598 pulsed EL4 cells (data not shown), indicating that CTL killing was mediated through the HLA A*0201 restriction pathway.

K14E7×A21 mice are competent to make CTL responses

Spleen cells from K14E7×A21 mice and FVB×A21 mice immunised with influenza matrix peptide displayed comparable levels of killing EL4.A2 cells pulsed with cognate peptide (percentage specific killing at 50:1 effector:target cell ratio, 55.72 +/- 2.88% and 56.63 +/- 1.97%, respectively). Similarly, spleen cells from K14E7×A21 mice and FVB×A21 mice immunised with ovalbumin both displayed killing of EG7.OVA cells which constitutively express ovalbumin (percentage specific killing at 50:1 effector:target cell ratio, 22.50 +/- 0.63% and 51.80 +/- 1.45%, respectively), but not EL4 cells. These observations rule out a constitutive defect in K14E7×A21 mice to make CTL responses restricted through the HLA A*0201 or the H-2d pathways, respectively, as the cause of the highly down-regulated E7-directed CTL responses displayed by K14E7×A21 mice.

The frequency of E7-specific CTL precursors is diminished in K14E7×A21 mice

We wished to determine whether the frequency of CTL precursors was reduced in E7 epitope-immunised E7 transgenic mice when compared with their identically immunised non-E7 transgenic counterparts. Figure 5 indicates that the frequency of E7-specific CTL precursor cells capable of expansion by specific in vitro restimulation, and lysis of cognate peptide-coated targets, was significantly lower in the spleens of 8Q-immunised K14E7×A21 mice than the frequency of such precursors in the spleens of 8Q-immunised FVB×A21 mice.

Peptide requirements for sensitising target cells for killing by K14E7×A21-derived CTL and FVB×A21-derived CTL

We wished to determine whether immunised E7-transgenic mice would generate E7-directed CTL with less affinity for cognate epitope than immunised non-E7
transgenic mice. Figures 4A and 4B show a more-or-less constant lower level of killing by effectors from K14E7×A21 mice compared with effectors from FVB×A21 mice irrespective of concentration of peptide epitope used to sensitize the targets. However, data presented in Fig. 5 indicate that (at least for 8Q-directed
CTL) the number of E7 epitope-specific precursor CTLs is 10- to 20-fold higher in FVB×A21 than in K14E7×A21 mice. Were the data in Fig. 4 normalised to equivalent numbers of E7-specific CTL in the FVB×A21 and K14E7×A21 effector populations, then the difference in cytotoxic efficacy between effectors from the two types of mice would disappear. Thus, the data in Fig. 4 suggest that the residual effective E7-directed precursor CTL population, which remains in K14E7×A21 mice, has a spread of affinities for cognate epitope + MHC essentially similar to that seen in the FVB×A21 mice where the E7-directed precursor CTL population is intact.

Reconstituted (manipulated) K14E7×A21 mice acquire FVB×A21-derived CD4 and CD8 T cells

We wished to inquire whether the high levels of CTL activity seen in immunised FVB×A21 non-E7 transgenic mice would be observed were we to ablate the thymus and immune system of K14E7×A21 mice and replace it with thymus and immune system from FVB×A21 mice. We presumed that the K14E7×A21 T-cell system had been centrally tolerised with respect to E7 in the E7-expressing K14E7×A21 fetal thymus. The immune system of K14E7×A21 was ablated by thymectomy and whole body irradiation followed by injection of anti-Thy1.1-depleting antibody to remove radioresistant T-cells. The mice were reconstituted with FVB×A21 bone marrow (BM) cells depleted of CD4+ and CD8+ cells, and a thymus implant of FVB×A21 origin. When tested 1 week after irradiation and BM reconstitution, the mice exhibited profound peripheral leucocytopenia. At 6 weeks post-BM reconstitution (4 weeks post-thymus implant), the peripheral leucocytopenia was reversed but the mice had not regained circulating peripheral CD4 and CD8 cells (Figs. 6A and 6B). At 11 weeks post-BM-reconstitution, CD4 and some CD8 cells were detected in the periphery (Figs. 6C and 6D), and by 14 weeks CD4 and CD8 cells in the periphery had returned to levels approximately 50% of those of sham-manipulated control mice (Figs. 6E and 6F). Further examination of these manipulated mice revealed a vascularised cellular subcapsular renal thymus graft containing CD4/CD8 double positive cells and quantitatively normal spleen cellularity (not shown). The E7 gene could not be detected by PCR in DNA from peripheral blood.
mononuclear cells (PBMC) of manipulated mice, but was readily detectable in DNA from PBMC of sham-manipulated mice, indicating that the leucocyte compartment had been reconstituted by cells of FVB × A2.1 origin (Fig. 7).

A T-cell system of FVB × A2.1 origin fails to establish a "nontolerant" level of E7-directed CTL in the manipulated K14E7 × A2.1 mice

Splenocytes from sham-manipulated K14E7 × A2.1 mice immunised with peptides 597 and 598, and restimulated with 597 and 598 showed significantly down-regulated killing of EL4.A2 cells pulsed with cognate peptide, compared with similarly immunised sham-manipulated FVB × A2.1 mice (Figs. 8A and 8B). This residual response was lost completely in the manipulated K14E7 × A2.1 mouse examined 6 weeks after reconstitution (Fig. 8C), when no circulating CD4+ or CD8+ cells were detectable in the periphery (Figs. 6A and 6B). The CTL response did not reestablish in 597 and 598 immunised manipulated K14E7 × A2.1 mice 16 weeks after reconstitution with FVB × A2.1 bone marrow (12 weeks after FVB × A2.1 thymus), even though E7 gene negative CD4+ and CD8+ cells (i.e., of FVB × A2.1 origin) constituted the peripheral T-cell pool (Figs. 6E and 6F, and Fig. 7, Lanes 2 and 3).

A T-cell system of FVB × A2.1 origin reestablishes CTL responses to non-E7 antigen in manipulated K14 × A2.1 mice

To establish that functional reconstitution of CTL responses had occurred, spleen cells from manipulated K14E7 × A2.1 mice immunised with a mix of the three E7 peptide epitopes and influenza matrix epitope, were reacted with groups of EL4.A2 target cells pulsed with individual E7 peptides or influenza matrix peptide. Figure 8, II shows that the reconstituted mice displayed CTL responses to the influenza matrix CTL epitope, but not to any of the three E7 CTL epitopes. This indicates that the capacity to mount CTL responses to non-E7 antigen had been restored by FVB × A2.1 thymus and bone marrow grafting, and confirms that CTL responses could not be established against the three CTL epitopes of E7 origin.

Multiple immunisation of K14E7 × A2.1 mice with peptides encoding E7 CTL epitopes fails to prevent the development of E7-associated tumours or induce E7-directed CTL responses

CTLs directed to the E7 oncoprotein have been shown to specifically abrogate the growth of challenge doses of E7-expressing transplantable tumours (Feltkamp et al., 1993; Tindle et al., 1995). We wished to inquire whether the residual E7-directed CTL response elicited by immunisation would ameliorate the development and growth of induced E7-associated tumours in K14E7 × A2.1 mice. Florid E7-associated papillomas developed in K14E7 × A2.1 mice, beginning at ca. weeks 9 ± 10 after commencement of the tumour-inducing regimen. K14E7 × A2.1 mice typically had 20 ± 40 macroscopic papillomatous tumours and 0 ± few sebaceous tumours on the shaved dorsal surface exposed to tumour induction. (In contrast, non-E7 transgenic FVB × A2.1 mice developed 0 ± few tumours of any kind). We sought to (a) delay the appearance of tumours and/or abrogate the extent of tumour growth in K14E7 × A2.1 mice by multiple immunisation both before and during tumour induction, with peptides encoding E7 CTL epitopes, and (b) quantify CTL responses in these mice. Groups of K14E7 × A2.1 mice were immunised seven times with 8Q, or with 597/598, or with whole E7 fusion protein or with irrelevant peptide. Tumour induction commenced after the fourth immunisation. In all groups tumours first appeared at 9 to 10 weeks after commencement of tumour induction, and progressively increased in number until the experiment was terminated (to prevent distress to the mice) at 19 weeks. Intergroup comparisons of the number of tumours were made during weeks 16 ± 19, inclusive. The irrelevently immunised mice developed a mean of 22.7 tumours per mouse at 16 weeks, increasing to 29.7 at week 19 (Fig. 9C). The number of tumours per mouse at weeks 16 ± 19 did not differ significantly from the irrelevantly immunised group among mice immunised with 8Q ($\chi^2 = 7.8$, df = 7, $P < 0.1$), with 597/598 ($\chi^2 = 4.3$, df = 7, $P < 0.5$), or with whole E7 ($\chi^2 = 3.7$, df = 7, $P < 0.5$) (Figs. 9A, 9B, and 9D) (this conclusion was reached regardless of whether actual tumour numbers, or tumour numbers predicted from the linear regression line, were used for statistical analysis. Actual tumour numbers were used in the analysis given here). Similar observations were made in a second identical experiment, though in that case a somewhat lower (but not significantly different) number of tumours at 16 ± 19 weeks was recorded in 597/598 immunised mice (not shown).

CTL precursors were sought in the spleens of the 8Q-immunised mice at the end of the experiment (19
FIG. 8. (I) CTL responses of splenocytes from manipulated or sham-manipulated mice immunised with an equimolar mix of peptides 597 and 598 and restimulated with both 597 and 598 in vitro. (A) sham-manipulated K14E7×A21.1; (B) sham-manipulated FVB×A21.1; (C) manipulated K14E7×A21 6 weeks after bone marrow reconstitution; (D) manipulated K14E7×A21 16 weeks after bone marrow reconstitution. EL4.A2 cells pulsed with peptide 598 (open squares), peptide 597 (open triangle), irrelevant peptide 8Q (closed squares), and unpulsed EL4.A2 (closed triangle). Percentage specific killing by splenocytes from influenza matrix peptide immunised, sham-manipulated K14E7×A21.1 mice was 51.44 ± 1.76% at an effector:target cell ratio of 70:1.

Effect: Target Cell Ratio

- Peptide 597 (open squares)
- Peptide 598 (closed squares)
- Irrelevant peptide 8Q (open triangle)
- Unpulsed EL4.A2 (closed triangle)
weeks). Splenocytes restimulated in vitro with cognate peptide, from K14E7×A2.1 mice immunised with 8Q, failed to kill 8Q-loaded target cells. In contrast, splenocytes from FVB×A2.1 mice which had been identically immunised and subject to identical tumour induction, efficiently killed 8Q loaded targets (Fig. 10). In accordance with these in vivo and in vitro findings, it was found that in vivo depletion of CD8+ cells or CD4+ cells, or both CD4+ and CD8+ cells did not have a statistically significant effect on the outcome of tumour development in 8Q-, 597/598-, or E7-immunised K14E7xA2.1 mice (data not shown).

DISCUSSION

In this study we demonstrate that the specific immunisation-induced CTL response to two HPV16 E7 CTL epitopes is reduced, and to a further epitope, abolished completely in mice expressing the E7 transgene perinatally in the thymus and throughout life, in basal epithelial

FIG. 9. Linear regressions of mean cumulative papilloma counts per mouse following tumour induction in K14E7×A2.1 mice immunised with (A) peptide 8Q; (B) peptides 597 and 598; (C) irrelevant peptide; (D) E7/GST fusion protein. (Tumour induction in control FVB×A2.1 mice produced a mean of 0.40 papillomas per mouse at 19 weeks.)

FIG. 10. CTL responses of splenocytes harvested at week 19 from mice immunised on days 0, 14, 21, and 42, and thereafter at four weekly intervals until week 19, and in which tumour induction commenced at day 21. (A) K14E7×A2.1 mice immunised with peptide 8Q; (B) K14E7×A2.1 mice immunised with E7/GST; (C) FVB×A2.1 mice immunised with peptide 8Q. Closed squares, EL4.A2 cells pulsed with peptide 8Q; open squares, EL4.A2 cells pulsed with a mix of peptides 597 and 598; open diamond, C2 cells.
cells. The down-regulation was seen in CTL responses restricted by two restriction elements: H-2^b and HLA A^*0201. The observation that the K14E7×A21 mice made CTL responses to "irrelevant" (i.e., non-E7) H-2^b-restricted (ovalbumin) and HLA A^*0201-restricted (influenza) CTL epitopes of comparable magnitude to their non-E7 transgenic counterparts, argues against (a) a constitutive poorer ability to make CTL responses, and (b) lower expression of either of the two restriction elements, in the E7 transgenic mice as explanation(s) for the down-regulation of CTL responses to E7 epitopes observed in our study.

The predominant tolerogenic mechanism for CTLs is generally thought to involve intrathyMIC negative selection of maturing T cells with reactivity to self-peptides presented in association with MHC-encoded molecules on the epithelial compartment of the thymic stroma (Hoffman et al., 1995). A prevalent view is that T cells with high affinity for self-peptide/MHC complex are deleted. We observed perinatal expression of E7 driven from the K14 promoter in K14E7×A21 mice. The K14 promoter directs transgene expression to thymic epithelium (Lauerer et al., 1996). Frequency analysis of E7-specific CTL precursors in K14E7×A21 mice and in their non-E7 FVB×A21 counterparts indicated a major diminution of CTL precursors in the E7 transgenic mice (Fig. 5). We suggest that this is likely due to deletion of maturing T-cells with receptors for cognate E7 CTL epitope in the E7-expressing developing thymus.

CTLs with reactivity to two of the E7 epitopes studied could still be detected in the E7 transgenic mice (Figs. 2 and 3). The existence of potentially autoreactive CTLs in the periphery is well documented in humans and experimental animals (Miller et al., 1997). Presumably, these cells escape thymus censorship by expressing antigen-specific TcRs of too low an affinity for the self-antigen, or there is insufficient expression of the antigen in the thymus, or the self-epitope may not exist in the thymus as a stable complex with MHC molecules (Miller et al., 1997). Our data suggest that (within the context of the peptide concentrations used) the residual populations of E7-reactive splenic T-cells in K14E7×A21 mice, which are directed to the HLA A^*0201 epitope 598 and the H-2^b epitope in peptide 8Q, have a similar spread of affinities for cognate epitope + MHC as E7-directed precursor CTLs in FVB×A21 mice where the E7-directed precursor CTL repertoire remains intact (Fig. 4).

The observation that T cells to some epitopes are totally depleted, and to other epitopes partially depleted leading to diminished specific T cell response, as reported in this paper and in other systems (Hoffman et al., 1992; von Herrath et al., 1994; Oehen et al., 1994), is consistent with this mechanistic process of a central deletional tolerance induction in the thymus, in which some precursors avoid deletion and escape to the periphery. Our observations are also consistent with proposed alternative processes in which (a) at least some high avidity self-reactive T cells may not be deleted, but positively selected and display effector functions of a regulatory type in the periphery (Coutinho, 1995; Modigliani et al., 1996), and (b) at least some lower affinity/avidity interactions may lead to clonal inactivation (unresponsiveness) of T cells exported to the periphery (Kawai and Ohashi 1995).

Multiple immunisation of K14E7×A21 mice with either E7 CTL peptide epitopes or whole E7 protein failed to significantly control the development of induced E7-associated skin tumours, and this correlated with a lack of E7-specific CTL responses in these mice. The apparent ineffectiveness of cellular immune response in control of tumour development in such mice was underscored by lack of effect of depletion of CD8^- or CD4^+ cells, or CD8^- and CD4^+ cells on tumour development. These observations are supported by experiments (G. Fernando et al., submitted for publication) which demonstrated that (K14E7×C57BL)F1 mice failed to control a challenge with syngeneic E7-expressing tumour following immunisation with peptide containing the H-2^b-restricted CTL epitope (RAHYNIVTF), whereas in identically immunised non-E7 transgenic (FVB×C57BL) F1 mice, the tumours did not establish. This in vivo finding correlated with the induction of RAHYNIVTF-specific CTLs in (FVB×C57BL)F1 mice but not in (K14E7×C57BL)F1 mice.

K14E7×A21 mice in which the lymphopoietic system and mature T-cells had been ablated, and which had been reconstituted with a FVB×A21 lymphopoietic system, whose T-cells had matured through a FVB×A21 (i.e., non-E7 transgenic) thymic graft, (manipulated mice), failed to acquire the E7-directed CTL responses typical of FVBxA21 mice, following immunisation with E7 peptide epitopes. (That these mice were functionally reconstituted for CTL responses was confirmed by the capacity to mount influenza matrix peptide (i.e., non-E7)-directed CTL responses.) Nor were there overt signs of skin-directed autoimmunity in (unimmunised) manipulated mice. This begs the question whether the donor (FVB×A21) T-cells were anergised peripherally by E7 expressed in host (K14E7×A21) epithelium. Evidence from other systems indicates that tolerance to self-antigens is maintained by peripheral as well as central (thymic) mechanisms (reviewed in Miller et al., 1997). Processed self peptides (in this case E7 peptides) presented at the keratinocyte surface in the context of MHC class 1 in the absence of appropriate second signals (B71/CD28,CTLA) may be tolerogenic rather than immunogenic (Bal et al., 1990; Miller et al., 1997). Evidence that E7 expression in epithelium stimulates neither the afferent nor the efferent arms of the immune system, for a positive response, is accumulating. Thus, E7-containing full-thickness skin grafts from E7 transgenic mice to syngeneic non-E7 transgenic recipients were not re-
jected, even following prior E7 immunisation of the recipients to generate demonstrable E7-specific CTL responses (Dunn et al., 1997). The questions of whether E7 expression in epithelium can induce specific peripheral immune unresponsiveness, and whether the persistence of E7 expression in skin throughout life is necessary to maintain the down-regulation of E7-directed CTL responses which we observe in K14E7×A21 mice, are currently being addressed in our laboratory. These questions have implications for the development and persistence of both endogenous- and vaccine-induced E7-directed immune responses in epithelially derived HPV16-associated cervical cancer in humans, since a majority of these cancers express E7 constitutively.

In H-2b mice expressing HPV16 E6 and E7 off the keratin 14 promoter in skin and neonatal thymus, Melero and co-workers observed no down-regulation of the CTL response induced by immunisation with E7 peptide epitope RAHYNIVTF, and concluded that the mice remained immunologically ignorant of this CTL epitope (Melero et al., 1997). This result stands in contrast to ours, and the reason(s) for the difference is not immediately apparent. The expansion of CD8+ precursors in mice is enhanced by Freund's adjuvant, as used by the Melero group, with the possibility of "bystander killing" (Doherty, 1995). An alternative explanation is that, unlike the K14E7×A21 mice used in our experiments, the E7 gene in their mice was expressed as a fusion with the E6 gene, with possible implications for antigen processing and presentation of E7 CTL epitopes in the thymus. Another possibility that a more complete restimulation occurred in their system by using cells expressing whole E7 for in vitro restimulation of splenocytes from immunised mice is unlikely to explain the difference in results, since CTL tolerance has been observed in spleens of E7 transgenic mice restimulated with E7-expressing syngeneic cells (I. Jochmus, personal communication).

MATERIALS AND METHODS

Mice

K14.HPV16E7 mice are FVB (H-2b) mice made germ-line transgenic for a DNA fragment containing HPV16 sequence from nt 79–883 and engineered to transcribe E7-specific mRNA driven from the human keratin (K14) promoter which is restricted in its activity to the stratum basale of epithelium (Herber et al., 1996).

Transgenic A21Kb mice express a chimeric HLA class 1 molecule composed of the α-1 and -2 domains of HLA A*0201, and the α-3 transmembrane and cytoplasmic domains of H-2Kb (Vitiello et al., 1991), on a predominantly C57Bl (H-2b) background.

In the experiments reported here, male K14.HPV16E7 mice were crossed with female A21Kb mice. All parents were homozygous for their respective transgenes. The F1 offspring were designated K14E7×A21. They displayed a characteristic phenotype of stunted growth, wrinkled skin as newborns and striated fur as adults, and pycnotic eyes with cataracts characteristic of K14 promoter-driven epithelial E7 expression. The presence of the E7 gene was confirmed by PCR. HLA A*0201 was expressed in the epithelium as determined by immunocytochemistry using Mab MA21 (ATCC, MD). F1 control mice (designated FVB×A21) were obtained by crossing male FVB mice with female A21Kb mice. Founder mice were derived by caesarean section, and all mice were housed under specific pathogen-free conditions. Genetic authenticity was tested at intervals. Mice were used at 7±15 weeks of age, but within a given experiment were littermates or closely age and sex matched.

Expression of the E7 gene in perinatal thymus

Messenger RNA was extracted from thymuses removed from fetal mice at 2±5 days before birth, or newborn mice at 8 days after birth using the poly(A) tract system (Promega Corp.) and was analysed by RT±PCR. E7 transgene-specific cDNA was synthesised using reverse transcriptase (SuperScript II, Gibco) and subsequently amplified by Taq polymerase using E7 oligonucleotide primers, representing nt 562–582 (5’-ATGCATGGGATACACCTACA-3’) and 858–887 (5’-TGGTATAGCTCGGGATTATTGGT-3’) of the HPV16 sequence, in the presence of RNA-ases H and T1 (Boehringer and Gibco, respectively). Bands corresponding to full-length E7 were detected on a 1% agarose gel. Amplified E7 DNA from plasmid pJ410E7 was used as positive control.

Detection of E7 gene in PBMCs

PBMCs were extracted from the retro-orbital blood by hypotonic lysis of erythrocytes. Cellular DNA was extracted from 10^7 PBMC using a QIAamp tissue kit (QIAGEN Pty. Ltd). Approximately 550 ng was amplified for 35 cycles by PCR using E7 primers (above) to generate full-length product which was detected as a band on a 1% agarose gel. Lane loading was standardised by parallel amplification of ornithine carboxylase (OTC).

Peptides and epitopes

Peptides representing fragments of the HPV16 E7 protein were synthesised with free ends using 9-fluorenyl-methoxycarbonyl (F-moc) chemistry and analysed by HPLC by Chiron Corp. Peptide 8Q (44QAEPDRAHYNIVTF57) was synthesised using a QIAamp tissue kit (QIAGEN Pty. Ltd). Approximately 550 ng was amplified for 35 cycles by PCR using E7 primers (above) to generate full-length product which was detected as a band on a 1% agarose gel. Lane loading was standardised by parallel amplification of ornithine carboxylase (OTC).
Cell lines

The cell line designated C2 was derived from EL4 cells (a H-2b thymoma) transfected to stably express the full-length HPV16 E7 gene (Tindle et al., 1995). The cell line EL4.A2 was derived by transfecting EL4 with a plasmid containing the A2.1Kb gene and a hygromycin selection marker (Vitiello et al., 1991). Following antibiotic selection, and passage at low cell density, HLA A*0201 cell surface expression was confirmed in EL4.A2 cells by flow cytometry (FACS IV, Becton–Dickinson & Co.) using Mab MA2.1 and F(ab’)2-anti mouse IgG/FITC (Caltag) secondary reagent. All cells in the EL4.A2 cell line displayed a log increase in fluorescence intensity, indicating a high level of HLA A*0201 α-1 and α-2 chain expression. EL4.A2 cells are susceptible to specific CTL lysis through both the H-2b and the HLA A*0201 pathways. Ovalbumin-expressing EG7.OVA cells were derived from EL4 cells by transfection with a full-length ovalbumin gene (Moore et al., 1988). Cell lines were maintained in Dulbecco’s modified eagles minimal essential medium (DMEM, Gibco) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 20 mM HEPES, 5 × 10⁻⁵ M β-mercaptoethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% foetal bovine serum (FBS).

Induction and measurements of CTL responses

In standard experiments, groups of three mice were immunised once with 50 μg of 80 peptide, or 50 μg each of a mixture of 597 and 598 peptides, plus 10 μg of Quil A adjuvant (Fossum et al., 1990), in 100 μl PBS, subcutaneously at the base of the tail. Mice receiving the 597/598 mixture also received 2.5 μg of tetanus toxoid (TT) (CSL Ltd.) as a source of a T-helper epitope(s). Ten days later, spleens were removed and splenocytes were restimulated in vitro for 6 days in 24-well tissue culture plates (3 × 10⁶ to 5 × 10⁶ cells per well) in the presence of 1 μg per ml of cognate peptide. CTL assays were conducted as previously described (Herd et al., 1997). In summary, target cells (10⁶ per well) sensitised at 37°C for 1 h with 1 μg/ml cognate or irrelevant peptide, or expressing the E7 gene, and labelled with 100 μCi ⁵¹chromium (Cr), were incubated with effector cells at various effector:target ratios in triplicate in 96-well microtitre plates. Negative controls included wells containing targets but no effectors (background). In positive controls for CTL responses to non-E7 antigen, mice were immunised with 50 μg of ovalbumin or influenza matrix peptide, plus Quil A. Spleen cells from these mice were restimulated in vitro with irradiated EG7.OVA cells or cognate peptide, respectively, and CTL responses were measured against EG7.OVA or influenza matrix peptide-pulsed EL4.A2 cells. Supernatants were harvested from CTL assays at 4 h, and ⁵¹Cr release was quantified by gamma counting. Results are expressed as percentage cytotoxicity (⁵¹Cr release in experimental wells minus background/detergent-mediated total release minus background) × 100%. In experiments to determine the affinity of CTL in bulk cultures of effectors from immunised E7 transgenic and non-E7 transgenic mice, the target cells were coated with varying concentrations of cognate peptide from 10 to 0.0001 μg/ml. The target cells were then reacted with effector cells in standard CTL assays as above.

Radiation chimeras

Radiation chimeras were made following the technique of Bertolino et al. (1995). In summary, K14E7×A21 mice were thymectomised at 4±5 weeks of age, lethally irradiated with 1000 rad at 7±8 weeks, and reconstituted on the same day with 5 × 10⁶ to 5 × 10⁷ bone marrow cells depleted of CD4⁺, CD8⁺, and Thy1⁺ cells (see below) from FVBxA21 donors. One day after reconstitution, the mice were injected intraperitoneally with 100 μl of complement fixing anti-Thy1 ascitic fluid to remove radioresistant cells. After 2 weeks, they were grafted under the kidney capsule with a thymus lobe from a newborn FVB×A21 mouse. Mice were rested for a number of weeks before analysis of PBMC for absence of the E7 gene and for presence of surface expression of CD4 and CD8 by flow cytometry, using monoclonal antibodies GK1.5 and 2.43, respectively (hybridomas from ATCC). These mice are referred to as “manipulated mice.” Control “sham-manipulated” mice underwent surgical procedures, without the cell/organ transfers.

Bone marrow cells for transplant

Long bones were flushed with DMEM + 2.5% FBS, the cells were washed twice, and were incubated at 5 × 10⁷ cells per ml for 30 min at 4°C with a mixture of anti-CD4, anti-CD8, and anti-Thy 1.2 antibodies. Cells were then incubated at 5 × 10⁷ cells per ml in DMEM + 10% FBS plus rabbit complement (C-Six Diagnostics Inc.) for a further 45 minutes at 37°C. Following three washes, the cells were injected intravenously in a volume of 100 μl.

Flow cytometry

Approximately 10⁶ cells were reacted with primary antibody or control antibody for 45 min on ice. After three washes, cells were stained with F(ab’)2 anti-mouse IgG/FITC (Caltag) or anti-rat IgG/FITC (Caltag) as appropriate for a further 45 min on ice in the dark. Following further washes, analysis was performed on a FACS IV (Becton-Dickinson), gated accordingly.

CTL Precursor (CTLp) frequency

CTLp frequencies were determined as described previously (Tripp et al., 1995). One hundred microlitres of dilutions of responder cells in medium were added to round-bottom wells of a microtitre plate. Twenty repli-
cates were plated for each dilution and cocultured with $10^6$ 2500-rad gamma-irradiated syngeneic spleen cells in 100 µl plus cognate peptide at 1 µg/ml. Human r-IL-2 (GIBCO/BRL) was added to 25 units/ml. Cultures were incubated for 7 days at 37°C in a humidified atmosphere. At the end of the culture period, individual cultures were divided in half, and incubated for 4 h with $^{31}$Cr-labelled EL4.A2 cells either pulsed with cognate peptide, or without cognate peptide. Estimates of CTLp frequency were obtained by interpolation from Poisson distribution frequency as the linearly regressed slope of the line when the numbers of negative cultures were plotted against number of responder cells per well, with 95% confidence intervals of the frequency estimate.

Tumour induction and promotion

Parental strain K14.HPV16E7 mice homozygous for the E7 transgene spontaneously develop two types of skin tumour late in life, namely highly keratotic lesions representing low grade squamous carcinomas (papillomata), and cystic sebaceous epitheliomas (Herber et al., 1996). In contrast, K14E7×A2.1 F1 mice used in the present study, which are hemizygous for E7, do not spontaneously develop skin tumours. This may represent a gene dosage effect since, in multiple lines of K14.HPV16 E7 mice, penetrance of phenotype correlated with levels of E7 gene expression (Herber et al., 1996). Thus, tumours were induced on the shaved dorsal surface of K14E7×A2.1 mice by application of a single dose of 0.03 mM dimethylbenzanthracene (Sigma Chemical Co.) in 100 µl of acetone. Seven days later applications of the tumour promoter 15 mM phorbol 12-myristate 13-acetate (Sigma Chemical Co) in 100 µl acetone were made one to two times a week. This regimen induced florid papillomomas expressing HLA A*0201 and E7 in K14E7×A2.1 mice, but virtually no papillomas in FVB mice, but these were too few in number to be useful in analysis.

Effect of immunisation on tumour growth

Groups of six K14E7×A2.1 mice in two separate experiments were immunised with 50 µg BQ + 10 µg Quil A, with 50 µg each of a 597/598 mixture + 2.5 µg TT + 10 µg Quil A, with 50 µg irrelevant peptide + Quil A, or with 50 µg E7GST fusion protein (Fernando et al., 1995) + 10 µg Quil A, on days 0, 14, 21, and 42 and thereafter at four weekly intervals until week 19. Tumour induction and promotion commenced at day 21. Mice were maintained in a shaved state. Papillomas appeared at weeks 9 and 10. The number of papillomas per mouse and per group was quantified weekly, to 19 weeks when mice were euthanised to prevent suffering.

CD4,CD8 T-cell subset depletion

Mice immunised on days 0, 21, and 42, and subjected to the tumour induction regimen commencing on day 21 (as above) were injected intraperitoneally with 0.1 ml ascitic fluid diluted 1:1 in phosphate-buffered saline of Mab GK15 (anti-CD4 ), or 2.43 (anti-CD8), or a 50:50 mix of GK15 and 2.43, at 3-day intervals over a 15-day period, commencing on day 42. Effective subset depletion immediately after the antibody administration regimen and at intervals to the end of the experiment (week 12) was confirmed by FACS analysis on PBMC.

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