Infiltration of Cervical Cancer Tissue with Human Papillomavirus-specific Cytotoxic T-Lymphocytes

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ABSTRACT

CTLS specific for high-risk human papillomaviruses (HPVs) have recently been found in the peripheral blood of cervical cancer patients. Although cell-mediated immunity is thought to be important in the control of HPV infection, the functional relevance and site of activation of HPV-specific CTLs are unclear. We identified HLA-A*0201-restricted HPV-16 E7 peptide-specific CTLs in the peripheral blood (four of five patients), draining lymph nodes (three of four patients) and tumors (one of three patients) of cervical cancer patients. In four of four cancer patients, the frequency of CTLs specific for a recombinant vaccinia virus expressing HPV-16 and -18 E6/E7 gene products was found to be higher in tumors and lymph nodes compared with that of peripheral blood. HPV-specific CTLs were not identified in any of seven healthy controls, but primary responses could be generated by peptide-pulsed dendritic cells (four of four controls). In a non-HLA-A*0201 subject with invasive carcinoma, other HLA alleles also presented HPV antigens. This is the first demonstration that virus-specific CTLs infiltrate the virus-associated tumor, where they may play an important role in restricting disease progression.

INTRODUCTION

Viruses, including HPV, EBV, hepatitis B virus, and human T-cell leukemia/lymphoma virus type I, are associated with approximately 20% of all human cancers worldwide. Epidemiologic studies have established a strong association between HPV and cervical cancer, independent of other risk factors (1); HPV DNA is found in 93% of all cervical cancers (2), when it is often integrated into the host genome (3). Cell-mediated immunity may be particularly important in HPV-associated malignancy, because there is an increased incidence of associated genital cancer in immunosuppressed patients (4). MHC class I-restricted CTLs are important in the destruction of virus-infected cells in acute and persistent infections (5), but HPV-specific CTLs are difficult to study, possibly because of the inability to propagate the virus in vitro, with a consequent reliance on a number of expression systems for HPV proteins. HPV-specific CTLs recognizing HPV E6 and E7 proteins have only recently been demonstrated in peripheral blood of cervical cancer patients in our laboratory (6) and by others (7), as well as in healthy donors (8–10). However, although E6 and E7 proteins of high-risk HPVs are constitutively expressed in cervical cancer cells (11), such HPV-specific CTLs have not been found in TILs.

Although there are over 20 oncogenic HPV genotypes, HPV-16 is the most prevalent and is present in up to 70% of cervical cancers regardless of geographical origin (2). HLA-A*0201-restricted peptide CTL epitopes derived from HPV-16 E6 and E7 have been identified by binding assays (12). These peptides have been used to induce CTL responses in vitro from healthy donors and in vivo in HLA-A*0201 transgenic mice that were also capable of lysing the cervical carcinoma cell line CaSkI (10). In this study, we used two of these HLA-A*0201-restricted HPV-16 E7 peptides to map the distribution of HPV-specific CTLs at different anatomical sites (peripheral blood, draining lymph nodes, and tumor tissue) in patients with cervical cancer. LDA of PBLs, LNLs, and TILs was used to identify CTLs lysing target cells infected with a recombinant vaccinia virus encoding modified forms of HPV-16 and -18 E6/E7 protein sequences (TA-HPV; Refs. 6 and 13). This is the first examination of HPV-specific CTL responses at sites of disease in cervical cancer patients.

PATIENTS AND METHODS

Patients. The study was approved by the South Glamorgan Local Research Ethics Committee, and cervical cancer patients were recruited before surgery. Normal controls were either laboratory staff or patients having a hysterectomy for nonmalignant reasons. Clinical details are shown in Table 1.

PBL Extraction and HLA Typing. PBLs were separated from heparinized blood samples by centrifugation on a Histopaque-1077 (Sigma Chemical Co., St. Louis, MO) density gradient. Screening of PBLs for HLA-A*0201 expression with the monoclonal antibody MA2.1 (provided by Prof. A. McMichael, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, United Kingdom) was performed before HLA class I typing at the National Blood Transfusion Service (Cardiff, United Kingdom) by microlymphocyte cytotoxicity.

HPV Typing. HPV type-specific (HPV-16, -18, and -31) PCR of cervical tissue was performed by Dr. L. Ho (University College London Medical School, London, United Kingdom; Ref. 14).

Cell Lines. The C1R.A2 cell line (provided by Prof. A. McMichael) expresses a transfected genomic clone of HLA-A*0201 (15). It was grown in RPMI 1640 with 10% FCS and 400 μg/ml G418 (Life Technologies, Inc., Gaithersburg, MD). EBV-transformed B-LCLs were established by conventional methods using the EBV-producing marmoset cell line B95.8 (ECACC 85011419). CaSkI is a HLA-A*0201 cervical carcinoma cell line expressing HPV-16 E6 and E7 proteins (Ref. 16; ECACC 87020501). The K562 human erythroleukemic cell line (ECACC 89121407) is a NK cell-sensitive target that was used to "cold-target compete" with NK activity in cytotoxicity assays (7). All these lines were grown in RPMI 1640 supplemented with 10% FCS (Life Technologies, Inc.). Human T-cell lines were cultured in RPMI 1640 supplemented with 10% pooled human AB serum (National Blood Transfusion Service, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, 1 mM sodium pyruvate, and 25 mM HEPES buffer (Life Technologies, Inc.), referred to as complete medium. This medium was further supplemented with 2 μg/ml fungizone (Squibb, Princeton, NJ) for culturing TILs/CILs.

Viruses and Synthetic Peptides. TA-HPV is a recombinant vaccinia virus expressing HPV-16 and -18 E6/E7 gene products (provided by Cantab Pharmaceuticals, Cambridge, United Kingdom; Refs. 6 and 13). Wyeth strain is the parental vaccine strain of vaccinia used in the construction of TA-HPV (13). HPV-16 E7_convert (YMMLDQPETT) and Plasmodium falciparum cp36 (YLKTIQNSL) peptides were synthesized by the Peptide and Protein Facility, University of Wales College of Medicine. HPV-16 E7 convert (TYLGVIQCVTP) and the influenza matrix M1 convert (GILGFVFTL) peptides were synthesized by Immune Systems (Bristol, United Kingdom). All peptides were >80% pure by LC/MS.
high-performance liquid chromatography analysis. In cytotoxicity assays for peptide-specific CTLs, the M158-50 peptide was used as the irrelevant peptide on control targets for HPV peptide-stimulated CTL lines, and the P. falciparum cp36 peptide was used as the irrelevant peptide on control targets for HPV peptide-stimulated CTL lines.

Extraction of LNLs and TILs. Lymph node and tumor samples were processed within 30 min of surgery. Lymph nodes were dissected free of surrounding tissue and teased open. LNLs were pelleted and washed several times with PBS before counting. Tumor samples were washed, and a small fragment was snap-frozen in liquid nitrogen for HPV PCR analysis. The remainder was minced into 1-mm² fragments, placed in fungizone-containing complete medium in 6-well plates, and incubated at 37°C. The medium was harvested at 6, 18, 24, and 36 h, and released cells were pelleted, washed, and purified by Histopaque-1077 density centrifugation before culture.

Flow Cytometric Analysis and Monoclonal Antibodies. Flow cytometric analysis of cell surface molecules was performed on a FACScan 440 flow cytometer (Becton Dickinson, Mountain View, CA) with anti-CD3(Leu-4) and anti-CD8(Leu-2a) monoclonal antibodies.

In Vitro Induction of Memory CTL Responses. A modified version of the method previously described by Ressing et al. (7) was used. Briefly, 4 x 10⁵ PBMCs or LNLs (and up to 2 x 10⁵ TILs) were resuspended in 1 ml of complete medium and incubated with 10 µg of peptide in 24-well plates. On day 1, wells were topped up with 1 ml of complete medium containing recombinant human IL-2 (final concentration, 10 IU/ml; Boehringer Mannheim, Indianapolis, IN). On day 7 and weekly thereafter, cells were restimulated as follows: (a) irradiated autologous PBMCs (3000 rads; 4 x 10⁶ cells/ml) were incubated for 2 h at 37°C in 1 ml of complete medium with 10 µg/ml peptide and 3 µg/ml β2-microglobulin (Sigma); (b) after removal of nonadherent cells by washing, 10⁶ responder T cells were added in 1 ml of complete medium; and (c) wells were topped up with 1 ml of complete medium containing IL-2 (final concentration, 10 units/ml) 2 days later. Cytotoxicity assays were usually performed on days 14 and 21.

In Vitro Induction of Primary CTL Responses. DCs were prepared by a modification of the method of Bakker et al. (17). Briefly, PBMCs were resuspended at 4 x 10⁶ cells/ml in RPMI 1640 with 5% FCS and distributed in 6-well plates at 3 ml/well. After a 2-h incubation at 37°C, nonadherent cells were removed, and 3 ml of medium containing 800 units/ml granulocyte macrophage colony-stimulating factor (Boehringer Mannheim) and 500 units/ml IL-4 (Genzyme Diagnostics, Cambridge, MA) were added to the wells. On day 6, the adherent cells were harvested by trypsinization and resuspended at 5 x 10⁶ cells/ml in serum-free medium containing 40 µg/ml peptide and 3 µg/ml β2-microglobulin. After incubation at 30°C for 4 h, the DCs were irradiated (3000 rads). Two x 10⁶ peptide-loaded DCs and 2 x 10⁶ responder cells/well were then cocultured in a 24-well plate in 2 ml of complete medium with 5 ng/ml IL-7 (Genzyme). The cells were restimulated on day 10 and weekly thereafter as follows: (a) after irradiation (3000 rads), 4 x 10⁶ autologous PBMCs were incubated for 2 h with 20 µg/ml peptide and 2 µg/ml β2-microglobulin in 1 ml of medium; (b) after washing, 10⁶ responder cells were added to each well in 1 ml of complete medium; and (c) the next day, 1 ml of complete medium was added, containing IL-2 and IL-7 at final concentrations of 10 units/ml and 5 ng/ml, respectively. Cytotoxicity assays were performed after a minimum of three rounds of restimulation.

Cytotoxicity Assays. Target cells (CIR.A2 cells, CaSki cells, or B-LCLs) were loaded with ³¹Cr (Amersham International, Little Chalfont, United Kingdom) for 1 h. Virus-infected target cells were infected overnight before labeling (multiplicity of infection, 10:1). Peptide-pulsed targets were incubated with peptide (10 µg/ml) for 45 min after ³¹Cr labeling. Various numbers of effector cells in 50 µl of medium were preincubated with 50 µl of unlabeled K562 cells (1 h at 37°C, in 20-fold excess of the "hot" target cells; Ref. 7). Two x 10⁵ ³¹Cr-labeled target cells were then added to triplicate wells of 96-well plates in a final volume of 200 µl. After 4 h at 37°C, 20 µl of supernatant were harvested onto glass-fiber mats (Wallac, Milton Keynes, United Kingdom) and measured on a β-plate counter (Wallac). Target and K562 cells incubated with 5% Triton X-100 (Merck, Poole, United Kingdom) or medium alone were used to determine maximum and spontaneous releases on each plate. Spontaneous release was usually less than 10% in most assays and never exceeded 20%. The percentage of specific lysis of each well was calculated by (experimental release — spontaneous release) X 100.

Limiting Dilution Cultures for TA-HPV-specific CTLs. Replicate microwells (100 µl; 60–600 replicates/cell input number) of freshly isolated PBLs, LNLs, and TILs were set up in 96-well round-bottomed plates, with dilutions ranging from 100–50,000 cells/well. Complete medium (100 µl) containing 5 x 10⁶ irradiated allogeneic PBMCs was added to each well, together with IL-2 and PHA (Sigma) in final concentrations of 25 units/ml and 1 µg/ml, respectively. On day 7, the wells were fed with fresh medium and IL-2, and on day 14, the cells were restimulated with 5 x 10⁴ irradiated allogeneic PBMCs, 25 units/ml IL-2, and 1 µg/ml PHA. The plates were assayed by split-well analysis on day 21. Two 30-µl aliquots were tested against CIR.A2 target cells infected with TA-HPV and the parental Wyeth strain. Wells with lysis greater than 10% above that of the control target were considered positive (18). Limiting dilution plots for virus-specific killing were produced by plotting the proportion of negative wells against the initial responder cell number/well on a semilogarithmic plot. From the single-hit Poisson model (a straight line relationship demonstrated by the C2 value for goodness of fit), the frequency of HPV-specific CTLs was estimated from the initial responder cell number, at which 37% of the wells were negative for cytotoxicity (19). Statistical analysis was performed using GLIM 3.77 (Royal Statistical Society, London, United Kingdom).
RESULTS

HPV-16 E7 Peptide-specific CTLs Are Present in Peripheral Blood of Patients with Invasive Cervical Cancer. We have previously shown that vaccination of patients with cervical cancer with a vaccinia recombinant expressing E6/E7 from both HPV-16 and -18 (TA-HPV) induced CTLs in PBLs (6). To determine whether HPV-specific memory CTLs were present without vaccination, two HLA-A*0201-restricted HPV-16 E7 peptides were used in an in vitro restimulation protocol (7) to screen peripheral blood of HLA-A*0201 cervical cancer patients and healthy controls (Table 1). In four of five cancer patients, peptide-specific CTLs to HPV-16 E711-20 were detected in cytotoxicity assays on day 14 (Fig. 1a). In one of these four positive patients, specific CTLs against HPV-16 E786-93 were also observed (Fig. 1b). CaSki, a HLA-A*0201 cervical carcinoma cell line expressing HPV-16 E6 and E7 proteins (16), and C1R.A2 cells infected with TA-HPV were used as targets to assess recognition of endogenously processed antigen. All four peptide-specific lines recognized TA-HPV-infected targets at lower levels of specific lysis than peptide-pulsed targets. The HPV-16 E786-93-specific CTL line from subject P2 (Fig. 1b) was the only peptide-specific line (one of four) to kill CaSki cells (Fig. 2). To determine whether these specific CTL responses were maintained in vivo, PBMCs from two patients (P1 and

![Graph](image-url)
CIR.A2 targets in a day 21 chromium release assay, in the presence of K562 cells. Other targets included CIR.A2 cells pulsed with an irrelevant HLA-A*0201-restricted peptide (□), no peptide (○), infected with TA-HPV (△) or the parental Wyeth strain vaccinia (λ), and CaSki cells (■). SD for all assays was <5% specific lysis.

P2) were reexamined 6 months after treatment, and similar HPV-specific CTLs were again obtained in both cases (data not shown).

After conventional HPV peptide secondary in vitro restimulation, no HPV responses were detected in PBLs from the HPV-negative CIN III patient (P(CIN)), or in those from seven normal controls. This suggested that these HPV-specific CTLs were memory CTLs rather than in vitro peptide-induced primary CTLs. This was supported by the induction of CTLs to the influenza A matrix peptide M158-66 in all subjects (Fig. 1c), but no CTLs to the P. falciparum cp36 peptide (20) were induced, to which neither patients nor controls were previously exposed (Fig. 1d).

**In Vitro Induction of HPV-16 Peptide-specific Responses in Normal Donors by DCs.** HPV-16 E7 peptide-specific responses were detected in cervical cancer patients but not in normal subjects, which suggested that the culture conditions used were only able to detect memory CTLs. This was further investigated using DCs derived from PBMCs cultured in granulocyte macrophage colony-stimulating factor and IL-4 as antigen-presenting cells (17). Under these conditions, HPV-16 E711-20 and E76-63 peptide-specific responses were generated from PBLs of all four control subjects tested (C1, C2, C4, and C5); these CTL lines also recognized CIR.A2 target cells infected with TA-HPV (Figs. 3, a and b). Again, the suggestion that pulsed DC restimulation induced primary in vitro responses was supported by the generation of P. falciparum cp36 peptide-specific CTL responses in all subjects (Fig. 3c), whereas no such CTLs were detected in the absence of DCs.

**HPV Peptide-specific CTLs Are Present in Draining Lymph Nodes of Patients with Invasive Cervical Cancer.** Having established a protocol for restimulating HPV-16 E7 peptide-specific CTLs from the PBLs of cervical cancer patients, we addressed the question of whether CTLs were present in tumor-draining lymph nodes. Peptide-specific CTLs to HPV-16 E711-20 could be demonstrated in LNLs from three of four cancer patients (P1, P3, and P6; Fig. 4a). Two of these three positive patients (P3 and P6) had lymph node metastases. All three positive lines killed TA-HPV-infected targets at levels of specific lysis 15–20% above those of parental Wyeth strain vaccinia-infected targets. No HPV-16 peptide-specific responses were seen in lymphocytes from pelvic lymph nodes in two patients (C6 and C7) having a hysterectomy for nonmalignant disease. CTLs recognizing the influenza M158-66 peptide were generated from LNLs of all patients (Fig. 4c).

**HPV Peptide-specific CTLs Infiltrate Tumor Sites.** Having demonstrated HPV-16 E7 peptide-specific CTLs in both PBMCs and LNLs in patients with cervical cancer, we examined the tumor for HPV-specific CTL activity in TILs. HPV-16 E711-20 peptide-specific CTLs were detected in TILs in one of three cancer patients (P4). The HPV peptide-specific line from P4 did not recognize CaSki cells but killed TA-HPV-infected targets at 13–15% lysis above those of Wyeth-infected targets (Fig. 5a). Due to the limited amount of tumor tissue available, phenotypic analysis of fresh TILs was only performed in one patient with invasive cancer. This showed the cells to be 53% CD3 positive and 21% CD8+ (data not shown); functional studies could not be performed in this patient due to insufficient numbers of TILs. No HPV peptide-specific CTL activity was detected in lymphocytes from a cervical biopsy specimen of a patient with CIN III (P(CIN)) or from normal cervical tissue (C7). Again, M158-66 peptide-specific responses were induced from TILs/CILs of all subjects (Fig. 5c).

**HPV-specific CTLs Are Enriched in TILs and LNLs Compared to PBLs in Patients with Cervical Cancer.** If HPV antigen presentation occurs not just in the tumor but also in draining lymph nodes, HPV-specific CTLs not requiring antigen-specific secondary in vitro restimulation may be present at both sites. Furthermore, such CTLs might aggregate at these sites preferentially and be present at higher...
MHC class I restriction. Therefore, the ability of HLA alleles other than HLA-A*0201 to present HPV antigens was investigated in LNLs and TILs of PS (HLA-A*2401, -A*2501, -B*0801, and -B*4401). The autologous B-LCL and two allogeneic B-LCLs matched at two HLA alleles, either HLA-A*2401 and -B*4401 or HLA-A*2501 and -B*0801, were infected with either TA-HPV or the parental Wyeth strain vaccinia, and used as targets in a day 21 cytotoxicity assay with LNLs or TILs. In both cases, the percentage of positive wells, in which lysis was more than 10% above the lysis of control wells (18), was highest for the autologous B-LCL and for the allogeneic B-LCL matched at HLA-A*2501 and HLA-B*0801, indicating that HLA alleles other than HLA-A*0201 can present HPV-16/18 E6/E7-derived antigens (Table 3).

**DISCUSSION**

We and others have previously identified HPV-specific CTLs in peripheral blood of patients with cervical cancer and CIN (6, 7, 22). The present studies were performed to determine whether such CTLs localized to anatomical sites of disease and whether their in vivo generation was dependent on the presence of tumor tissue. Initially we studied PBLs, LNLs, and TILs/CILs from HLA-A*0201 patients frequencies relative to PBLs. We addressed this by culturing fresh PBLs, LNLs, and TILs in limiting dilution. No specific antigen was used, but IL-2 and PHA were added to allow polyclonal expansion of all normal resting lymphocytes (21). The cytotoxicity of individual culture wells was subsequently tested by split-well analysis on C1R.A2 target cells infected with TA-HPV or the parental Wyeth strain. Under these conditions, HPV-specific CTLs were found in PBLs, LNLs, and TILs of three cancer patients and in the LNLs and TILs of one cancer patient. No CTLs were detected in the HPV-negative patient with CIN III (P_CIN) or in a normal control (C7; Table 2). Single-hit kinetics enabling further comparative analysis by maximum likelihood analysis (19) were confirmed for HPV-specific CTLs in LNLs from three of the patients and for TILs from two patients. In these subjects, HPV-specific CTL frequencies were highest in TILs and lowest in PBLs; CTL frequencies were also higher in the two patients with lymph node metastases (P3 and P6). The highest HPV-specific frequencies in TILs were found in P3, where there is significant enrichment of HPV-specific CTLs in TILs over LNLs and in LNLs over PBLs (Table 2).

**HLA Class I Alleles Other than HLA-A*0201 Present HPV-16 E6/E7-derived Epitopes.** The LDA approach used in this study allowed polyclonal expansion of all HPV-specific CTLs regardless of MHC class I restriction. Therefore, the ability of HLA alleles other than HLA-A*0201 to present HPV antigens was investigated in LNLs and TILs of PS (HLA-A*2401, -A*2501, -B*0801, and -B*4401). The autologous B-LCL and two allogeneic B-LCLs matched at two HLA alleles, either HLA-A*2401 and -B*4401 or HLA-A*2501 and -B*0801, were infected with either TA-HPV or the parental Wyeth strain vaccinia, and used as targets in a day 21 cytotoxicity assay with LNLs or TILs. In both cases, the percentage of positive wells, in which lysis was more than 10% above the lysis of control wells (18), was highest for the autologous B-LCL and for the allogeneic B-LCL matched at HLA-A*2501 and HLA-B*0801, indicating that HLA alleles other than HLA-A*0201 can present HPV-16/18 E6/E7-derived antigens (Table 3).
undergoing hysterectomy, using peptides derived from HPV-16 E7 as a specific marker of the HPV CTL response in vivo. HPV peptide-specific CTLs were found at all these sites and, under these in vitro conditions, in cancer patients but not in normal subjects. Furthermore, this peptide-specific response was probably secondary, because influenza M135-36, but not P. falciparum cp36 CTLs were detected in the same samples. In contrast, peptide-pulsed DCs generated primary in vitro CTLs to HPV peptides, as well as to P. falciparum cp36, in normal volunteers.

To determine whether there was aggregation of CTLs at sites of disease, we used LDA to establish the frequency of HPV-specific CTLs after polyclonal activation of T cells. These studies confirmed the presence of these CTLs in PBLs, LNLs, and TILs as well as suggesting that they were retained in higher relative numbers at the sites of disease. The use of polyclonal activation has also enabled detection of HPV-specific CTL responses restricted by MHC class I for which peptides have not been identified.

HPV is only one of several viruses associated with human malignancies. EBV, a ubiquitous γ-herpes virus, is associated with Burkitt’s lymphoma, Hodgkin’s disease, nasopharyngeal carcinoma, and B-cell lymphomas in immunosuppressed patients. During normal EBV persistence/latency, MHC class I-restricted CTLs play a major role in maintaining the virus/host equilibrium (23). In fact, the development of EBV+ B-cell lymphomas in immunosuppressed subjects is associated with loss of EBV-specific CTLs, and their direct replacement by adoptive transfer is therapeutically effective (24). These transferred CTLs not only restored cellular responses against EBV but persisted in vivo for up to 18 months (25). Whether this therapeutic approach is applicable to other EBV-associated tumors such as Hodgkin’s disease is an important question. EBV is detected in approximately 40% of Hodgkin’s disease, and EBV-specific CTL responses in TILs (from clinically affected lymph nodes) and PBLs have been investigated (26). In contrast to our findings with HPV, MHC class I-restricted killing of autologous targets by TILs in Hodgkin’s disease was found only in EBV+ tumors, whereas TILs from EBV− tumors were either noncytotoxic or exhibited LAK activity. Comparison of TILs and PBLs in one patient showed EBV-specific cytotoxicity only in the PBL population. These differences highlight the important role of factors that may suppress CTLs locally (e.g., tumor-derived cytokines) and the fact that such effects may vary both with tumor type and the nature of the virus-induced transformation. The latter effect may determine whether virus gene products expressed in the tumor cells are efficiently processed, such as the failure of EBNA-1 to be efficiently processed (27), or interfere with MHC class I presentation in the transformed cell (28, 29).

Peptide-specific CTLs detected in this study were probably in vivo reactivated secondary responses, implying that in vivo priming has occurred in patients with a HPV-associated tumor. Generating naive responses in vitro usually requires longer term culture (30) or specialized antigen-presenting cells (10, 17, 31). In this study, both HPV- and P. falciparum-specific responses were only elicited in normal subjects when peptide-pulsed DCs were used, making it unlikely that the HPV-specific CTLs were memory responses with a very low precursor frequency. HPV-16 infection is widely prevalent even in women with normal cervical cytology (2, 3), and it has been suggested that infection with high-risk HPV types can occur in the perinatal period (32). This suggests that localized HPV infection of normal genital or mucosal epithelial cells fails to elicit a CTL response against HPV E6 and E7 proteins. This may be because epithelial cells fail to generate CTL responses effectively, because they do not present antigenic peptides with appropriate costimulatory molecules (e.g., B7; Ref. 33). However, the observation that HPV-specific CTL responses are detected in cancer patients indicates that this may be a CTL response akin to those generated against other tumor antigens. In these circumstances, priming of CTLs can use bone marrow-derived antigen-presenting cells in lymphoid tissue (34), which would bypass any defect in presentation by the epithelium, or alternatively suggests that the transformed cervical epithelium is more efficient at processing and presenting E6- and E7-derived peptides.

The role of HPV-specific CTLs in vivo is unknown, but their detection and relative accumulation at sites of disease suggest an ability to localize as an antitumor response. We used two human HLA-A*0201-restricted HPV-16 E7 peptides (12) to restimulate memory CTLs. C1R.A2 cells either pulsed with peptide or infected with a recombinant vaccinia virus expressing E6 and E7 gene products of HPV-16 and -18 (TA-HPV) were killed, the latter at lower levels of specific lysis. However, most peptide-specific lines did not recognize the HLA-A*0201+ HPV-16 E6/E7-expressing CaSki cervical carcinoma line. This suggests that the high-affinity HPV peptides are not processed as efficiently or in sufficient quantity from naturally transformed cervical carcinoma cells. Unfortunately, in this study, no autologous tumor cell lines were available as targets, which would have enabled us to assess whether the natural in vivo target cell could process and present these peptide epitopes. This is of particular importance if peptide vaccination is to be used for immunotherapy.

Peptide vaccination can induce CTLs that reject HPV-16-induced tumors in experimental models of disease (35). In man, vaccination with peptide-pulsed autologous DCs may induce specific CTLs. DCs express high levels of HLA class I and II and accessory/costimulatory molecules (CD54, CD58, CD40, CD80, and CD86), secrete cytokines, and migrate into lymphoid organs where optimal priming of T cells can occur (36). Protective and therapeutic antitumor immunity has been generated in animals by this method (37, 38), and this effect is mediated by CTLs (39, 40). In this study, we have shown that this approach can induce, even as a primary response, peptide-specific

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**Table 1: HPV-specific CTLs in cervical cancer**

<table>
<thead>
<tr>
<th>Subject (stage)</th>
<th>PBL</th>
<th>LNL</th>
<th>TIL/CTL</th>
</tr>
</thead>
<tbody>
<tr>
<td>P3 (IB)</td>
<td>0.8 (0.6-1.3)</td>
<td>2.5 (2.2-3.8)</td>
<td>17.7 (13.6-23)</td>
</tr>
<tr>
<td>P4 (IB)</td>
<td>0.5 (0.3-1.2)</td>
<td>0.9 (0.8-1.2)</td>
<td>2.8 (2.3-6.8)</td>
</tr>
<tr>
<td>P5 (IB)</td>
<td>0.9 (0.8-1.2)</td>
<td>0.9 (0.4-2.7)</td>
<td>4 (3.1-5)</td>
</tr>
<tr>
<td>P6 (IB)</td>
<td>&lt;0.01</td>
<td>2.33 (1.9-2.9)</td>
<td>9.7 (6.8-13.5)</td>
</tr>
<tr>
<td>P5m1 (CIN III)</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>P7</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Results fulfilled single-hit kinetics by the method of x² goodness of fit with P < 0.05.

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**Table 2: HPV-specific CTLs are concentrated at sites of disease**

PBLs, LNLs, and TILs/CILs were cultured in limiting dilution in the presence of PHA (1 μg/ml) and IL-2 (25 IU/ml). On day 21, cytotoxicity assays were performed by split-well analysis using C1R.A2 cells (or the autologous B-LCL, for P5) infected with TA-HPV or parental Wyeth strain vaccinia. A positive result was taken as a percentage of specific lysis >10% above that of the control wells. The HPV-specific CTL frequency shown (per 10⁶ lymphocytes) was estimated from the initial responder cell number at which 37% of the wells were negative. Values in brackets represent 95% confidence limits.

**Table 3: HPV antigens are presented by HLA alleles other than HLA-A*0201**

LNLs and TILs from patient 5 (HLA A24 A25 B8 B44) were cultured with allogeneic irradiated PBMCs, low-dose IL-2, and PHA and assayed for cytotoxicity on day 21. Targets used for split-well analysis were autologous B-LCL, two allogeneic B-LCLs, each matched for two HLA alleles (shown in bold), and one allogeneic B-LCL, mismatched for all HLA class I alleles. Target cells were infected with TA-HPV or Wyeth strain vaccinia, and a positive result was regarded as a well showing a percentage of specific lysis >10% above that of control wells. The percentage of positive wells at an input number of 100 cells/well is shown for LNLs and TILs.

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**Table 4: No. of positive wells**

<table>
<thead>
<tr>
<th>Target B-LCL</th>
<th>LNL</th>
<th>TIL</th>
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</thead>
<tbody>
<tr>
<td>HLA A24 A25 B8 B44</td>
<td>13/180 (7.2%)</td>
<td>45/420 (10.7%)</td>
</tr>
<tr>
<td>HLA A24 B7 B44</td>
<td>1/180 (0.5%)</td>
<td>8/540 (1.5%)</td>
</tr>
<tr>
<td>HLA A25 B8 Cw7</td>
<td>12/180 (6.7%)</td>
<td>37/420 (8.8%)</td>
</tr>
<tr>
<td>HLA A2 A11 B7 B51</td>
<td>0/180 (0%)</td>
<td>0/180 (0%)</td>
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</tbody>
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CTLs that recognize processed E6 and E7 peptides (after TA-HPV infection). Potential binding epitopes from HPV-16 E6 and E7 proteins have been predicted for five human HLA alleles (HLA-A*0101, A*0201, A*0301, A*1101, and A*2401; Ref. 12), although only HLA-A*0201-restricted CTL responses have been investigated in detail (7, 22). However, we do not know which epitope may be dominant in an individual. We have found HPV-specific CTL restriction by alleles other than HLA-A*0201 in this study, and analysis of the fine specificity of CTLs induced by adenoviruses encoding HPV-16/18 E6/E7 from patients6 (6) suggests that multiple peptides would be required to minimize viral escape and provide adequate coverage of the outbred population. CTLs recognizing subdominant epitopes may in fact be effective in tumor rejection (35), whereas a recent report that peptide vaccination may result in accelerated tumor outgrowth due to induction of T-cell tolerance (41), and the potential induction of escape mutants/peptide antagonism, stresses the need to proceed with caution.

Lymphocyte infiltration is seen in cervical cancer (42) and other solid tumors (43), yet there is little evidence that it controls tumor growth and spread. Directly isolated TILs are poorly cytotoxic unless stimulated with IL-2 (44). Higher concentrations of IL-2 (>6000 units/ml) favor nonspecific activation of NK and LAK cells (45). Previous studies of CD8+ TILs from cervical cancers found predominantly non-MHC-restricted NK- or LAK-activated killing (46, 47). Some clones killed autologous tumor cells specifically, but their antigen specificity or MHC restriction was never determined (46). We studied the E6/E7 specificity of polyclonally activated TILs/CILs using low concentrations of IL-2 (25 units/ml) to promote outgrowth of T cells. The precise in vivo relationship between tumor cells and the HPV-specific CTLs detected in TILs in this study cannot be established; in particular, expansion of circulating PBLs cannot be excluded. However, quantitative analysis by LDA suggests that the increased number of HPV E6/E7-specific CTLs relative to their frequency in PBMCs may be due to a selective accumulation of these effector cells. The presence of these CTLs in TILs and LNLs does not necessarily reflect an effective antitumor response. Local factors in the tumor may prevent destruction of tumor cells, e.g., MHC class I down-regulation (29), induction of T-cell tolerance by tumor cells that fail to deliver costimulatory signals (48), or secretion of soluble inhibitory factors such as transforming growth factor β by tumor cells (49). However, the ability of CTLs to localize to sites of disease does suggest the possible use of adoptive immunotherapy with CD8+ CTLs for cervical cancer patients with nonresectable disease. We have maintained HPV-specific CTL clones established from bulk cultures of these cancer patients, and they retain their specificity in vitro for up to 8 months.5

Some HPV types are closely related with respect to E6 and E7 primary structure, suggesting that cross-reactive CTLs could be generated. Interestingly, PCR typing revealed the tumor present in P6 to be HPV-31 positive. HPV-16 is the most closely related high-risk genotype to HPV-31; the HPV-16 E71-20 amino acid sequence be HPV-31 positive. HPV-16 is the most closely related high-risk

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Infiltration of Cervical Cancer Tissue with Human Papillomavirus-specific Cytotoxic T-Lymphocytes

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