Human Papillomavirus-specific Cytotoxic T Lymphocytes in Patients with Cervical Intraepithelial Neoplasia Grade III

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ABSTRACT

Human papillomaviruses (HPVs), especially types 16 and 18, are strongly associated with the development of cervical intraepithelial neoplasia (CIN) and invasive carcinoma. The HPV E6 and E7 proteins are expressed constitutively in the majority of CIN lesions and carcinomas. Therefore, they are targets for the immune response against HPV and candidates for active immunotherapy. We have previously detected HPV-specific CTLs from the peripheral blood mononuclear cells from a cervical cancer patient following immunization with a recombinant vaccinia using in vitro restimulation with adenovirus recombinants expressing HPV16 or HPV18 E6/E7 fusion proteins. In this study, we used a similar protocol to determine the prevalence of CTL responses against HPV16 and HPV18 E6/E7 in the peripheral blood mononuclear cells from 10 CIN III and 10 normal subjects. HPV-specific CTL responses were detected in 6 of 10 CIN III subjects. These CTL lines recognized HPV16 E6/E7 proteins presented by at least three MHC class I HLA alleles and by HPV-transformed CaSki cells. No HPV-specific CTLs were detected in normal subjects. This study demonstrates the presence of naturally occurring HPV-specific memory CTLs in a majority of CIN III patients and provides an approach for further study of their role in modulating cervical malignancy.

INTRODUCTION

Infection by HPV is a major risk factor for the development of preinvasive CIN and invasive carcinoma (1, 2). HPV DNA, predominantly subtype 16 and 18, is detected in 93% of cervical cancers (3) and 65% of CIN III lesions (1). This, together with the obligatory expression of HPV E6 and E7 proteins in transformed cells (4, 5), suggests that these viral proteins are candidates for prophylactic and therapeutic HPV vaccines.

However, HPV infection may be poorly immunogenic. Half of the patients with HPV16 positive cervical cancer did not have antibodies against HPV early proteins (6). Even when antibodies to early or late gene products were detected, they did not correlate with clinical outcome (7). However, HPV E6- and E7-specific CTLs are protective against transplanted tumor cells expressing HPV genes in animal models (8—11). This observation together with the high incidence of HPV-associated malignancy in immunocompromised patients suggests an important protective role for cell-mediated and CTL responses (12—14).

CD8+ CTLs clear viral infections and tumors in animal models (15, 16) and humans (17, 18). It has been difficult to generate HPV E6- and E7-specific CTLs in humans, and it has been suggested that they may be absent or present at low frequency (19). However, we found HPV-specific CTLs in peripheral blood from one of three patients after immunization with TA-HPV (20). A new approach to detecting CTL responses, using adenovirus recombinants expressing HPV16 or HPV18 E6 and E7 proteins for secondary in vitro restimulation, was used in that study (20). This approach was extended in the present study to determine whether HPV-specific CTLs were present in PBMCs of patients with noninvasive disease (CIN III) and normal volunteers with no cytological abnormality.

PATIENTS AND METHODS

Subjects

Ten subjects with CIN III diagnosed and confirmed by colposcopically directed biopsy, cytology, and histopathology (Departments of Gynecology and Pathology, University Hospital of Wales, Cardiff, Wales) and age- and sex-matched normal volunteers with no history of abnormal cervical cytology were studied (Table 1). All CIN III subjects had histological evidence of HPV infection. HPV typing was performed on paraffin-embedded tissue sections from CIN III lesions by PCR (21). Informed consent was obtained from all subjects in the study.

HLA Typing

HLA class I typing of unfraccionated PBMC- or EBV-transformed B-LCLs was performed by standard lymphotocytotoxicity (22) or by PCR using sequence-specific primers (23) in the Tissue Typing Laboratory of the National Blood Transfusion Service (Rhydylafar, Wales).

Viruses

Recombinant adenoviruses Rad 101 and Rad 102 containing HPV16 E6/E7 and HPV18 E6/E7 fusion proteins, respectively, were constructed by G. W. G. W. (20). Virus stocks were propagated in Graham-293 cells (MOI, 0.1:1). Cell-associated virus was harvested by centrifugation and released by freezing and thawing and fluorocarbon (Arkone P) extraction (24). Virus stocks with titers of 10^10 plaque-forming units/ml were aliquoted and stored at —70°C. TA-HPV (20, 25) was a gift from Cantab Pharmaceutical (Cambridge, United Kingdom). TA-HPV and parental Wyeth strain vaccinia were propagated and titrated in Vero cells; virus stocks with titers of 1.5—2.5 × 10^9 plaque-forming units/ml were stored at —70°C (25).

Isolation of PBMCs

PBMCs were separated from heparinized peripheral venous blood by Ficoll-Histopaque (Sigma Chemical Co., St. Louis, MO) density gradient centrifugation. Cells were resuspended in RPMI 1640 containing 50% FCS (Life Technologies, Inc., Paisley, United Kingdom) and 10% DMSO (Sigma) and cryopreserved at 10^7 cells/ml.

Cell Lines

EBV-transformed B-LCLs were established by coculture of PBMCs with EBV-containing supernatant from the B95.8 marmoset cell line in the presence of 0.5 μg/ml cyclosporin A (Sandoz, Camberley, United Kingdom). B-LCLs were maintained in RPMI 1640 supplemented with 2 mM l-glutamine, 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.). CaSki, a cervical carcinoma cell line expressing HPV16 E6 and E7 proteins (European Collection of Animal Cell Cultures, Salisbury, United Kingdom), was maintained in RPMI 1640 supplemented with 10% FCS. MDA
In Vitro Stimulation of HPV-specific CTLs

Generation of Stimulator Cells. Fresh or cryopreserved autologous PBMCs were washed and resuspended at 5 × 10^5/ml in RPMI 1640 supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES, 5% heat-inactivated FBS (RPMI-5ABS) and cultured in the presence of 2.5 μg/ml PHA (Murex Biotechnology, Dartford, United Kingdom) in 24-well plates. Cells were incubated at 37°C in a 5% CO₂ humidified atmosphere for 3–5 days. The PHA lymphoblasts were harvested and washed four times by centrifugation and infected with Rad 101 or Rad 102 (MOI, 75:1) for 18–24 h. The infected blasts were γ-irradiated (3000 rad), washed, resuspended in RPMI with 10% ABS (RPMI-10ABS), and seeded into 24-well plates at 10^5 cells/well.

Generation of Effector Cells. Responder PBMCs were washed and resuspended in RPMI-10ABS at 2 × 10^6 cells/well and mixed with stimulator cells to establish bulk cultures. The cultures were supplemented with 5 ng/ml human rIL-7 (Genzyme, Cambridge, MA) and incubated at 37°C. After 7 days, these cultures were restimulated with autologous Rad-infected stimulator cells. Human rIL-2 (20 units/ml) was added to the cultures on day 10 and every 3–4 days thereafter. After 14 days of culture, a portion of cultures were assayed for CTL activity. The remaining cultures were restimulated and cultured for a further 7 days and assayed again on day 21.

Cytotoxicity Assays

Autologous and allogeneic B-LCLs were infected with TA-HPV or Wyeth strain vaccinia virus (MOI, 10:1) or mock-infected for 10–12 h of incubation, washed, and resuspended in 100 μCi of ^51Cr (Amersham, Little Chalfont, United Kingdom) for 90 min at 37°C. These labeled target cells were washed three times, and 2 × 10^5 cells/well were added to triplicate wells of 2-fold serially diluted effector cells. Control wells containing target cells alone to establish spontaneous release or target cells with 5% aqueous Triton X-100 (Biological Supplies, BDH, Poole, United Kingdom) to determine maximum release were included in all assays. After 4 h of incubation, supernatants were harvested and counted in a Beta-plate liquid scintillation counter (Wallac, Turku, Finland). Percentage of specific lysis was calculated as [(mean experimental release – mean spontaneous release) / (mean maximum release – mean spontaneous release)] × 100.

Cell Surface Phenotype Analysis

Anti-CD3 (OKT3), anti-CD4 (OKT4), anti-CD8 (OKT8; American Type Culture Collection), anti-CD16 (Leu-11b), and FITC-conjugated anti-TCR-αβ (Becton Dickinson, San Jose, CA) antibodies were used to characterize effector cell populations by direct and indirect immunofluorescence and analyzed on a FACSscan (Becton Dickinson).

Establishment of HPV-specific CTL Lines and Clones

To further characterize the HPV-specific CTLs generated in response to in vitro restimulation, clonal CTL lines were established from bulk cultures after three or four rounds of restimulation with HPV-infected autologous PHA lymphoblasts. The cells were diluted and added to 96-well plates at 10, 100, and 1000 cells/well with 5 × 10^5 irradiated allogeneic PBMCs as feeders and 0.5 μg/ml PHA in RPMI-10ABS supplemented with 25 units/ml rIL-2 and 10 ng/ml rIL-7. Cultures were fed with 25 units/ml rIL-2 every 2 days and restimulated every 10–14 days under the same conditions as above. Cultures that were, from input cell numbers, indicative of expansion from a single progenitor CTL, were assayed for CTL activity after 3 weeks, and positive wells were then placed in 24-well plates.

RESULTS

HPV-specific CTL Responses Were Detected in Peripheral Blood of CIN III Subjects. The in vitro restimulation protocol used to detect HPV-specific CTLs from cancer patients immunized with TA-HPV (20) was used to test 10 CIN III and 10 normal subjects (Table 1). By use of the appropriate recombinant adenoviruses (see "Patients and Methods") we restimulated cultures with HPV E6/E7 from either HPV16 or HPV18 before assay on HPV16/18 E6 and E7 recombinant (TA-HPV) or parental (Wyeth) vaccinia-infected autologous B-LCLs (Fig. 1). Four CIN III subjects (M. M., S. D., V. D., and V. M.) had specific CTLs against TA-HPV-infected autologous B-LCLs after restimulation with HPV16 E6/E7 (Fig. 1a). In each case, lysis of these targets by effector CTLs was more than 10% greater than that of control targets (autologous B-LCLs infected with Wyeth and uninfected B-LCLs) over at least two E:T ratios. The other subjects did not specifically lyse autologous TA-HPV-infected cells (Fig. 1a). In these, lytic activity was either uniformly low (S. B., B. J., and A. T.) or high but nonspecific (L. F., G. B., and H. F.).

Three CIN III subjects (A. T., V. D., and B. J.) had responses against TA-HPV after secondary in vitro restimulation with HPV18 E6/E7 (Fig. 1b). Similarly to HPV16 stimulation, other subjects had uniformly low lysis (M. M., S. B., and S. D.) or high but nonspecific lysis (V. M., G. B., L. F., and H. F.). In one subject (V. D.), both
HPV16- and HPV18-specific CTL responses were generated (Fig. 1, a and b).

One subject (S. B.) had low lytic activity in all experiments (Fig. 1, a and b); however, this was not due to decreased immunocompetence because specific allogeneic CTLs were generated in mixed lymphocyte culture (data not shown).

**HPV16- and HPV18-specific CTL Responses Were Not Detected in Normal Subjects.** PBMCs from 10 subjects with no history of abnormal cervical cytology were stimulated and maintained in culture under conditions identical to that used for PBMCs of CIN III subjects and assayed for HPV16-specific CTL activity on day 21. In contrast to the results obtained with HPV16 E6/E7 stimulation of PBMCs from CIN III subjects, no HPV-specific CTL activity was detected (Fig. 1c), and in three of these subjects (M. N., A. P., and S. P.), no HPV16-specific responses were observed on repeat examination (in contrast to V. M. and M. M.; see below). Similarly, when HPV18 restimulation was performed, no HPV-specific CTLs were detected in M. N., A. P., and S. P. (data not shown).

**Maintenance of HPV-specific CTL Responses.** CTL responses against either HPV16 or HPV18 E6 and E7 gene products were found in 6 of 10 CIN III subjects studied. Because 0 of 10 normal subjects had HPV-specific CTLs, it was possible that HPV E6 and HPV E7 that presented in the context of transformed cells, rather than normal, productively infected epithelium, were better able to generate and sustain this response in vivo. To address this possibility, two subjects (M. M. and V. M.) who had CTL responses against HPV16 were tested for up to 12 months, following surgical removal of the transformed cells. PBMCs were restimulated in vitro with HPV16 E6/E7 and tested for CTL lysis as before. Although significant lysis of TA-HPV-infected targets was evident at each time point, the magnitude of the specific CTL response decreased with time. This is clearly evident in the case of V. M. (Fig. 2a), despite the generation of alloreactive CTLs at the same time point (data not shown). In the case of M. M., this reduction was observed, but only in a context of increased nonspecific lysis at 15 months (Fig. 2b). These experiments also documented the reproducibility of the in vitro restimulation protocol. Furthermore, a series of CTL lines were subcultured from bulk cultures of these two patients at 10 cells/well and have been maintained for >4 months with sublines VM 1.2 and MM 5-D10 maintaining their in vitro specificity (Fig. 3).

**Cell Surface Phenotype and HLA Restriction of HPV-specific CTLs.** The phenotype of effector cell population was determined. Where HPV-specific CTL activity was observed, the predominant phenotype of the effector cells, after 21 days of in vitro culture was CD3⁺, CD8⁺, TCRαβ⁺ (49-70%); the ratio of CD4⁺:CD8⁺ cells was <1.0. However, the effector cell population of cultures not exhibiting HPV-specific CTL activity were predominantly CD3⁺, CD4⁺, TCRαβ⁻; the CD4⁺:CD8⁺ ratio was >1.0 (data not shown). Interestingly, one line stimulated, BJ HPV16 (Rad101), was 67% CD8⁺, yet no HPV16-specific CTL activity was observed.

The MHC class I restriction of CTL response that was suggested by the predominant CD8⁺ phenotype was confirmed by the fact that fully allogeneic TA-HPV-infected target cells were not killed efficiently (Fig. 4). To determine more precisely the MHC restriction of these HPV-specific CTLs, sublines from M. M. were tested against a panel of HLA-matched and partially matched B-LCLs infected with TA-HPV (Table 2). Under these conditions, HLA-A2-, A24-, and B7-restricted CTLs were detected in the same subject. Furthermore, the HPV-transformed cell line CaSki was included to determine whether CTLs recognizing HPV16 E6/E7 after infection of B-LCLs with TA-HPV could also recognize endogenous HPV16 E6/E7 antigens by a naturally transformed cell line. CaSki cells were lysed by HLA-A2- and HLA-B7-restricted sublines 5-D10 and 10-B5. In contrast, MDA231, an HLA-A2 expressing breast carcinoma epithelial cell line that was not transformed with HPV, was not lysed.

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**Fig. 1.** CTL responses against HPV E6 and E7 in short-term bulk cultures. PBMCs from the subjects indicated were stimulated weekly for three weeks with recombinant adenovirus encoding either HPV16 or HPV18 E6/E7 proteins (see "Patients and Methods"). The cultures were assayed against autologous B-LCL targets, which were uninfected (a), infected with parental Wyeth strain vaccinia (b), or infected with TA-HPV (c). Each assay was performed at four different E:T ratios or at a minimum of E:T ratio of 2. CTL responses were measured following stimulation by HPV16 E6/E7 stimulation in CIN III subjects (a), HPV18 E6/E7 stimulation in CIN III subjects (b), or HPV16 E6/E7 stimulation in normal subjects (c). Columns, mean percentage specific lysis at an E:T ratio of 60:1; bars, SE.
HPV16- AND HPV18-SPECIFIC CTLs IN CIN III PATIENTS

class I restricted. The HPV-specific CTLs obtained were predominantly CD8+, although the presence of CD4+ HPV-specific CTLs could not be formally excluded because CD4 depletion experiments were not done; these CTLs did not efficiently lyse HLA-mismatched targets, and different sublines developed from M. M. recognized HPV antigens in the context of HLA-A2, A24, and B7, specifically. These CTLs also recognized HPV antigens expressed by the HPV-transformed line CaSki in the context of A2 and B7 but not epithelial tumor cells not expressing HPV. Therefore, HPV-specific CTLs recognizing E6 and E7 are present in peripheral blood of approximately 60% of CIN III patients and can be restricted by multiple MHC class I alleles in an individual.

Until recently, there have been few reports of HPV-specific CTLs in human subjects. Most HPV-specific CTLs have been documented in mice, in which HPV is not a natural pathogen (8, 10, 26). This has lead to the suggestion that HPV has coadapted to the human host by evading the immune system (19). However, two studies have recently demonstrated low levels of HPV-specific CTL lysis in occasional CIN III and cervical cancer patients. In one of these studies, HLA-A2
binding peptides from HPV16 E7 were used to stimulate HPV-specific CTLs in 2 of 11 cancer patients and 2 of 11 CIN III patients (27). The second study used the HPV-transformed cell line CaSki to restimulate HPV-specific HLA-A2-specific CTLs in 1 of 11 CIN III patients (28). It was suggested that the low response in both studies (9% and 19%) was due to a low frequency of HPV-specific CTLs in the circulation. The higher response observed in this study (60%) suggests that differences in the methods used for CTL detection may be an equally important factor. Both Ressing et al. (27) and Evans et al. (28) specifically examined HLA-A2 responses. This may lower the rate of detection of HPV-specific CTLs because those CTLs restricted by other HLA alleles will be missed. In addition, HLA-A2-restricted CTLs may not dominate the response against a complex virus (29). Furthermore, using only peptide epitopes, researchers may miss CTL responses to other epitopes not restricted by HLA-A2. Therefore, from the outset, we used full-length proteins rather than single predicted CTL epitopes, allowing an equal opportunity of detecting all CTLs against E6 and E7. This approach has been used in the context of melanoma-specific CTLs and has demonstrated additional MHC-restricted responses to those predicted against individual peptides (30). We suggest that the additive effect of full-length proteins and therefore multiple MHC class I alleles for restimulation contributed to the higher detection rate in this study.

There may also be a difference in the patient groups examined by these three studies. We examined patients as they were referred for colposcopy, in most cases prior to first treatment. Interestingly, two of our best responders (M. M. and V. M.) had recurrent CIN III lesions, perhaps contributing to a continuing induction or persistence of CTLs in vivo while also raising the question of why recurrence of lesions in the face of detectable CTL response in PBMCs occurred? It was
noticeable that HPV-specific CTL responses (but not alloreactive CTL responses) from V. M. disappeared approximately 12 months after hysterectomy (Fig. 2a), and this suggests a possible link between the continued presence of transformed cells and detection of CTLs.

All of these studies failed to detect HPV-specific CTLs in PBMCs of normal subjects. In view of widespread exposure to these viruses (1, 31), this may be a surprising finding, when in other persistent virus infections there is a high frequency of virus-specific CTLs in acutely or persistently infected subjects (32—34). This may reflect the unique tropism of HPV for the epithelium and its failure to present appropriate epitopes below the detection limits of current assays. How or persistently infected subjects (32—34). This may reflect the unique tropism of HPV for the epithelium and its failure to present appropri ate epitopes below the detection limits of current assays. However, an alternative explanation may be that the transformed cells may both enhance antigen presentation and allow antigen presentation to be mediated by professional bone marrow-derived antigen-presenting cells (37).

The demonstration of HPV-specific CTLs among CIN III patients has important implications for immunotherapy. This study suggests that multiple HLA alleles can present HPV antigens to CTLs, and work in progress to identify which peptide epitopes are bound. Although single peptide epitopes have been shown to protect against tumors after immunization in animal models (10), they have also been shown to have adverse effects (38). The use of a few peptide epitopes is even less likely to be effective in the outpatient human population. To combat these problems and the possibility of viral escape (39), potential HPV vaccines will need to incorporate multiple peptide epitopes (40). Such an approach may minimize the hazards of using transforming genes, such as HPV E6 and E7, in vaccines. An alternative to vaccination would be direct immunotherapy based on adoptive transfer of CTLs. This study demonstrates that it is possible to culture HPV-specific CTLs from patients and maintain their specificity in long-term culture.

The role of CTLs in the natural history of cervical neoplasia has yet to be defined. It is clear from this study that some of the subjects (M. M., V. D., and B. J.) producing HPV-specific CTL responses have evidence of previous infection with HPV, but how these responses are generated in vivo is not known. One subject (B. J.) had HPV-specific CTLs after restimulation with HPV18 E6/E7 but not HPV16 E6/E7, despite having a biopsy sample that tested positive for HPV16 DNA. This subject, however, had recurrent disease, and it is possible that infection with multiple HPV types could have occurred. Determining the correlation between HPV infection, cervical disease, immune responses, and clinical outcome will require long-term prospective studies. This would be of particular interest in the CIN III group, in which 25—30% of patients have natural regression of disease and 40% have progression to cancer.

It would be of immense interest to determine the local immunoregulatory events occurring in CIN III lesions and whether HPV-specific CTLs could be found at the site of a CIN III lesion. Recent studies from our laboratory (41) have demonstrated that HPV-specific CTLs can be detected at the site of tumor and in draining lymph nodes in cervical cancer patients. This suggests that CTLs may be able to home in on HPV-transformed cells in the cervix; however, local immunoregulatory events may suppress cytotoxic activity in vivo. Any successful immunotherapy based on HPV-specific CTLs will need to circumvent this.

Regardless of the role that CTLs play in the natural history of disease, there is no doubt that the reliable detection of HPV-specific CTLs in vitro has implications for direct immunotherapy. We have completed one clinical trial in cancer patients in which no CTL responses could be detected prior to immunization (20). However, these patients had invasive carcinoma and could have been immunosuppressed by the disease or treatment (42). In the immunocompetent CIN III population, we were able to detect naturally occurring CTLs in most patients. One would predict that immunization of this population with the TA-HPV vaccine would result in the induction and boosting of HPV-specific CTLs to a far greater extent than in cancer patients, and this is currently under investigation.

ACKNOWLEDGMENTS

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REFERENCES


Table 2. HLA restriction pattern of HPV-specific lines from M. M. bulk CTLs

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<th>B-LCL target</th>
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<th>5-D10</th>
<th>10-B4</th>
<th>108-S</th>
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<td>M. M.&quot;</td>
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" Autologous B-LCL.
" HPV-transformed carcinoma cell line.
" Breast carcinoma cell line.


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