Regression of Cervical Intraepithelial Neoplasia and Loss of Human Papillomavirus (HPV) Infection Is Associated with Cell-mediated Immune Responses to an HPV Type 16 E7 Peptide[1]

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Abstract

Most human papillomavirus (HPV)-associated cervical intraepithelial neoplasia (CIN) lesions in normal women regress spontaneously, but a small number persist and may progress to invasive cancer. To evaluate the role of immunity to HPV and the outcome of CIN and associated HPV infection, we examined cell-mediated immune (CMI) responses to HPV 16 E6 and E7 peptides. One hundred thirty-six women with biopsy-confirmed CIN I or CIN II were followed for 1 year at 3 month intervals. Study subjects were 58% Hispanic, 36% African American, and 6% of other ethnicity, and were attending a municipal hospital colposcopy clinic. At each visit, cervical cytology and cervicovaginal lavage for HPV detection and typing was done, and blood was obtained for immunological studies. Lymphoproliferative CMI responses to HPV 16 E6 and E7 peptides were tested. An end point biopsy was done after the 1-year follow-up. The association between CMI responses to specific peptides and the outcome of disease was evaluated. CMI responses to E7 peptide (37–54) correlated significantly with regression of disease and with resolution of viral infection within 12 months. The protective effects of CMI to this peptide were not HPV type-specific. CMI responses to several other peptides also showed an association with regression, although not significant at present sample size. E7 peptide 37–54 contains one or more human T-cell epitopes. Identification and mapping of "protective" epitopes in the HPV E6 and E7 proteins could lead to the development of immunological assays to determine the risk of CIN and the development of immunotherapeutic protocols for the management of premalignant and malignant HPV-associated neoplasia and, ultimately, for the prevention of cancer.

Introduction

Genital infection with specific HPV[4] types has been shown to be the major etiological factor for the development of abnormal cervical cytology and CIN as well as cervical cancer. HPV infection is highly prevalent in sexually active young females, with increased risk associated with younger age, specific racial/ethnic background, sexual promiscuity, alcohol consumption, anal sex, and other factors (1), including host HLA types (2, 3). Most HPV infections are transient and regress spontaneously over a period of several months, whereas a small number persist and may progress to invasive cervical carcinoma (4, 5) Invasive cervical carcinoma has been associated with persistent HPV infection with the same HPV type, whereas HPV type-specific persistence is relatively infrequent in control populations who are not at risk for carcinoma (6). Immunosuppressed subjects have an increased risk of anogenital HPV infection and associated dysplasia, suggesting that immune reactivity is associated with the elimination of the virus and the clearance of disease (7–9).

We have previously demonstrated human LP CMI responses to E6 and E7 peptides in women with CIN and have demonstrated that there are multiple reactive T-cell epitopes in the HPV 16 E6 and E7 proteins (10, 11). Women with LP responses to specific E6 and/or E7 peptides *in vitro* were likely to lose their abnormal cervical cytology and HPV infection within several weeks, which suggested that these peptides contained "protective" epitopes. Whether subjects with ongoing HPV 16 infection and associated disease are more likely to exhibit CMI reactivity to the E6 and E7 proteins than are normal women without disease has been controversial (12). Whereas some investigators have reported that CMI responses are associated with regression of disease, others have found that CMI responses were associated with persistence of disease and

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[4] The abbreviations used are: HPV, human papillomavirus; CMI, cell-mediated immune; LP, lymphoproliferative; CIN, cervical intraepithelial neoplasia; HR, high risk; CVL, cervicovaginal lavage; PBMC, peripheral blood mononuclear cell; OR, odds ratio; CI, confidence interval.

high-grade dysplasia but were found infrequently in normal women (13–15). Several investigators have reported on differences in cytokine production associated with the stage of HPV-associated cervical neoplasia (16, 17). It has been suggested that the immunosuppression seen with cancer may not be systemic, but might be caused by factors produced by neoplastic cells (18). CMI responses to HPV may thus be associated with local factors as well as with stage of disease and virologic factors.

The HPV 16 transforming proteins E6 and E7 are abundantly expressed in precancerous and malignant cervical lesions and are likely to play a role as tumor rejection antigens in humans, as has been shown in mice (19–21). Little is known, however, about the clinical relevance and type specificity of CMI responses to these proteins in women with ongoing genital HPV infection. We previously evaluated the effects of CMI on cervical cytologic findings over short time periods (3–6 months). We report here studies of women with biopsy-confirmed CIN grades I & II. Many of our study subjects have completed follow-up and have undergone end point cervical biopsy, so that we are able to evaluate disease and infection status at the end of one year. Our results support the role of CMI to specific HPV 16 E6 and E7 peptides in the natural history of genital HPV infection and associated disease.

Materials and Methods

Study Design. Since 1995, women with histopathologically confirmed CIN I or CIN II have been recruited from the colposcopy clinics affiliated with the Albert Einstein College of Medicine in an observational, nontherapeutic follow-up study to identify factors associated with spontaneous resolution of HPV infection and regression of CIN. Subjects were recruited at the "baseline" visit, an average of 2 months after abnormal cervical cytology and diagnostic cervical biopsy. Subjects were then followed for 1 year at 3 month intervals with Pap smears and colposcopy. An end point (12 months), colposcopic cervical biopsy was performed. At each visit, CVL was collected for HPV DNA analysis. Heparinized blood was obtained by venipuncture for an assessment of CMI responses. Questionnaires were administered to collect information on demographic and behavioral factors. The primary outcome of the study was the presence (persistence) or absence (regression) of CIN lesions or type-specific HPV infection at 12 months.

In this ongoing study, 258 subjects have been recruited, and 162 have completed follow-up and had an end point biopsy. Of women enrolled, 20% have dropped out and others (17%) are still under active study. Biopsies were taken consistently from the most abnormal sites; if no colposcopic abnormality was found, end point biopsy was done at the site of the initial biopsy. The study pathologists (A. S. K. and M. A.) reviewed the initial and end point biopsy slides blinded. Of the 162 subjects who completed the study, 136 had confirmed CIN I or CIN II on initial biopsy and adequate findings for diagnosis on end point biopsy and were included in data analyses. The outcome of CIN was determined by comparing initial and end point biopsies. Regression occurred if end point biopsy showed normal histology and the Pap smear at 12 months was also negative (22). If the biopsy was negative but Pap smear showed changes consistent with CIN, the subject was considered to have persistent disease. Changes from CIN I to CIN II or from CIN II to CIN I were not considered progression or improvement. An end point diagnosis of CIN III or above was considered progression. Because the number of subjects with progression was small (3%), persistence and progression were analyzed together.

Lavage and blood samples were labeled with sample numbers only, which avoided possible bias in testing and interpreting serial samples from the same subject. Laboratory personnel did not have access to clinical information, nor did clinical personnel have access to laboratory results except Pap smears and biopsies during the follow-up period. HPV was detected in CVL samples by Southern blot and PCR, as reported previously (23, 24). This project was approved by the institutional review board, and informed consent was obtained from each patient.

Peptides. In our previous immunological studies, we have found that multiple E7 peptides contain reactive human epitopes (10). We include here E7 peptides 101, 1–18; peptide 103, 17–37; peptide 105, 37–54; and peptide 109, 72–97, which were the E7 peptides most often reactive in women in our patient population. Peptides overlapping the E6 protein, were also included, as reported previously (11). E6 peptides used were: peptide 369, 1–30; peptide 370, 16–30; peptide 371, 21–50; peptide 373, 41–70; peptide 377, 81–110; peptide 379, 101–130; peptide 381, 121–150; and peptide 383, 141–158. E6 and E7 peptides were tested separately, except for E7 peptides 101 and 103, which were tested together in most experiments and E6 peptides 373 and 377, which were used together in some experiments.

Lymphoproliferation Assay. LP assays were performed using 2-to-3-week "bulk" cultures with weekly restimulation with peptides as described previously (10, 11). Briefly, PBMCs were cultured with E6 and E7 peptides or with medium alone for 1 week. Fresh peptides, and irradiated autologous, cryopreserved antigen-presenting cells were added at 7 and 14 days. Recombinant interleukin 2 (Boehringer-Mannheim, Indianapolis, IN) was added with antigen-presenting cells (15 μg/ml) at 14 days. At 14 and 21 days, triplicate aliquots (100 μl each) were taken from each culture, assayed for blast transformation by thymidine uptake, harvested, and counted in a scintillation counter. A positive response was defined as stimulation index $\geq$3.0 (25). A sample was considered CMI positive if LP response to one or more peptides was demonstrated on either day 14 or day 21, or both, and negative if LP was absent to all peptides tested on both days. As control for T-cell reactivity, PBMCs were also cultured with the mitogens concanavalin A and phytohemagglutinin for 3–4 days and with *Candida* and tetanus antigens for 5–6 days.

Statistical Analysis. The association of CMI responses with the outcome of CIN at 12 months was first examined univariately by the χ^2 test. A subject was considered CMI positive to a specific peptide if any of her samples during the 12-month study was positive and CMI negative if all of her samples were negative. Because younger age and higher education were associated with spontaneous regression of CIN, ORs for spontaneous regression of CIN, when women with a positive CMI response to a particular peptide were compared with women without a response, were adjusted for age (continuous) and education (less than high school *versus* high school or above) in logistic regression analyses. For the effects of CMI responses on the outcome of HPV infection, data on 114 women who were HPV positive at baseline and had at least one subsequent follow-up visit, regardless of whether they had an end point biopsy or not, were analyzed by time-dependent proportional-hazards regression analysis. Resolution of HPV infection occurred when all of the HPV types detected at baseline were no longer present, and time-to-event was estimated as the midpoint between visits. CMI response to a particular peptide at each

Table 1 Association between spontaneous regression of CIN in 12 months and CMI responses to HPV 16 E6 and E7 peptides

HPV 16 peptides	CMI responses to specific peptides[a]	No. with regression/total (%)[b]	Adjusted OR for regression (95% CI)	*P*
E7				
105				
	Negative	38/92 (41.3)	1	
	Positive	28/41 (68.3)	2.87 (1.29–6.40)	0.010
Any E7 peptide(s)				
	Negative	18/41 (43.9)	1	
	Positive	50/95 (52.6)	1.53 (0.71–3.30)	0.279
E6				
370				
	Negative	49/103 (47.6)	1	
	Positive	18/30 (60.0)	1.71 (0.72–4.07)	0.227
373/377				
	Negative	35/77 (45.5)	1	
	Positive	31/55 (56.4)	1.82 (0.87–3.82)	0.112
Any E6 peptide(s)				
	Negative	3/9 (33.3)	1	
	Positive	65/127 (51.2)	2.70 (0.62–11.79)	0.186

[a] ORs were adjusted for age and education.
[b] The numbers presented here do not include all of the subjects enrolled (136); not all of them could be tested with all of the individual peptides separately because, occasionally, insufficient PBMCs were obtained.

visit was entered as a time-dependent covariate in the regression model. The relative risks for HPV resolution, when women with a positive CMI response to a particular peptide were compared with women without a response, were adjusted for ethnicity (African Americans *versus* others) as well as current cigarette smoking status (yes *versus* no), HPV type (HR *versus* low-risk type), and the number of HPV types detected (≥2 *versus* 1) at baseline, because the reference categories of these variables were associated with resolution of HPV (1). HR types were HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and W13b, which were previously found in cervical cancer patients in a worldwide study (26). Comparisons between HR and "other" HPV types included any HPV type other than those listed here.

To further examine whether the protective effects of CMI responses were dependent on the HPV types with which a subject was infected, the logistic regression and time-dependent Cox regression analyses described above were repeated with stratification of the HPV types detected at baseline. HPV types at baseline were classified into one of three categories: (*a*) negative; (*b*) HPV 16 and related types (HPV 31, 33, 35, 52, 58); and (*c*) other types. In the stratified time-dependent Cox regression analyses, the variable HPV type (HR *versus* low-risk type) was not included in the model because of high correlation with the stratification variable.

Results

Subjects were recruited an average of 2 months (SD = 1) after the initial CIN diagnosis. Among the 136 subjects who completed a 12-month follow-up, there were 58% Hispanics, 36% African Americans, and 6% of other ethnicity; the mean age was 30.3 years (±9.8 years); of the women recruited, 21% were current smokers, and 79% were nonsmokers. At recruitment, the HPV prevalence was 65% for any HPV type, 37% for HR types, and 26% for HPV 16 or related types. The mean duration between initial and end point diagnoses was 15 months (SD = 3).

Of the women with CIN I ($n = 98$) and with CIN II ($n = 38$), 50% had spontaneous regression, 47% had persistent CIN, and 3% developed CIN III by 12 months. Regression and progression rates were equivalent in women with CIN I and CIN II. Regression occurred in 24% of women with type-specific persistent HPV infection at baseline and at 12 months ($n = 17$), in 33% of women who were HPV positive but with different types at baseline and 12 months ($n = 15$), and in 59% of the 85 women who were negative for HPV at both visits. Spontaneous regression was, thus, highest among women without persistent HPV infection ($P = 0.011$).

CMI Results. To date, 593 samples have been tested for LP responses to HPV 16 E6 and E7 peptides. All of the subjects tested to date have been immunocompetent as gauged by mitogen (phytohemagglutinin/concanavalin A) and standard recall antigen responses. CMI responses to E6 and E7 were highly correlated, with 68% of the samples either negative or positive to both E6 and E7 (repeated measurement analysis, $P < 0.001$).

Positive LP responses to E7 peptide 105 (37–54) were significantly related to regression of CIN (Table 1). Of nonresponders 41% (38 of 92) underwent regression, compared with 68.3% (28 of 41) of responders. Responses to other E7 peptides tested (101 & 103 and 109) were not protective (data not shown). There was an association between CMI to E6 peptides 370 (adjusted OR, 1.71; 95% CI, 0.72–4.07; $P = 0.227$) and 373 & 377 (adjusted OR, 1.82; CI, 0.97–3.82; $P = 0.112$) and regression of CIN, although not significant at current sample size. CMI responses to several other E6 peptides (369, 371, 379, 381, and 383) showed no association with regression.

CMI responses to E7 peptide 105 were also significantly associated with the loss of the HPV type(s) detected at baseline (Table 2). Women who had positive CMI responses to two or more E7 peptides showed an increased likelihood of HPV resolution than did patients responding to only one peptide (adjusted RR, 2.72; 95% CI, 1.04–7.16; $P = 0.042$). CMI responses to specific E6 peptides were not significantly associated with loss of HPV infection.

Whereas most responders to peptide 105 were infected with HPV 16 or related types at baseline (31, 33, 35, 52, or 58), suggesting some degree of type specificity, the association between positive LP response to peptide 105 and improvement in disease status was seen among subjects infected with HPV 16 or related types as well as among those infected with other (not

Table 2 Resolution of HPV infection at baseline and CMI responses to HPV 16 E6 and E7 peptides: time-dependent Cox regression analysis

HPV 16 peptides	Positive CMI responses to specific peptides	Adjusted RR (95% CI) for HPV resolution	*P*
E7			
	105	2.36 (1.07–5.19)	0.033
	Any E7 peptide(s)	1.17 (0.67–2.04)	0.587
E6			
	370	1.06 (0.31–3.56)	0.929
	373/377	1.27 (0.61–2.63)	0.527
	Any E6 peptide(s)	0.88 (0.52–1.49)	0.639

[a] RR, relative risks were adjusted for ethnicity as well as cigarette smoking status, HPV types, and number of HPV types infected at baseline.

Table 3 Resolution of infection with HPV 16 and related types at baseline and CMI response to HPV 16 E7 peptide 105: time-dependent Cox regression analysis stratified by HPV types at baseline[a]

HPV types at baseline[b]	Adjusted RR[c] (95% CI)	*P*
HPV 16 or related (31/33/35/52/58) (*n*, 37)	2.81 (0.70–11.3)	0.145
Others (*n*, 53)	3.15 (1.22–8.11)	0.017

[a] Only women who were HPV DNA positive at baseline are included here.
[b] Some patients with untypable HPV DNA in entry CVL are included here, and, therefore, the numbers presented do not include all of the 114 subjects whose samples were HPV positive at baseline.
[c] RR, relative risks were adjusted for ethnicity as well as cigarette smoking status and the number of HPV types detected at baseline.

16-related) HPV types. Both subjects infected with HPV 16 and related types and those infected with other HPV types responding to peptide 105 were likely to clear HPV infection (Table 3). Regression of CIN was associated with HPV type present at baseline, although not significant at the current sample size. Adjusted OR for regression of CIN in 12 months for subjects HPV negative at baseline was 1.54 (95% CI, 0.34–6.97; $P = 0.571$); for subjects with HPV-16-related types was 4.34 (95% CI, 0.70–26.84; $P = 0.115$); for those with other HPV types was 3.37 (95% CI, 0.82–13.80; $P = 0.091$).

Discussion

In the present study, we have followed patients with biopsy-proven CIN grades I and II at 3-month intervals for 1 year. At each visit, PBMCs were tested for CMI responses to E6 and E7 peptides. During the 12-month follow-up, most patients were shown to be reactive to one or more E6 or E7 peptides. The LP assay done here tests the patients' ability to respond to E6 and/or E7 peptides *in vitro*, not actual *in vivo* immunological responses. Not all of the LP responses observed, however, were associated with regression of disease. We have identified one E7 peptide for which CMI responses were associated with both spontaneous regression of disease and resolution of viral infection (peptide 105, 37–54).

We have previously shown that a human T-cell epitope is present in COOH-terminal HPV 16 E7 peptide 109 (72–97; Ref. 10). In a short term (3-month) study of CMI responses to HPV 16 E7 peptides, we also showed that women who demonstrated LP CMI responses to E7 peptide 109 and to NH_2-terminal E6 peptide 369 (1–30) were likely to lose HPV infection and abnormal cytology, which suggests that these peptides contain protective epitopes (11). These two peptides did not appear to be protective in the present study. In that study (Ref. 11), only COOH-terminal E7 peptides 108 and 109 were studied. Peptide 105 was not included. The racial/ethnic backgrounds of subjects in our previous immunology studies (10, 11) have been the same as in the present study, so that the differences in results are not caused by differences in the populations studied.

The diagnosis of CIN I is often difficult, and low-grade lesions are often over-diagnosed. We used definitive diagnostic criteria (27) and may have characterized some biopsies with minimal and questionable findings as negative. Patients without definitive histological findings of CIN I were excluded. Therefore, the regression rate for subjects with CIN I in our study may be lower than in some other published studies (28). When we used our diagnostic criteria, the regression rates for CIN I and II were similar, which is different from data published in other studies. It has been suggested that biopsies of colposcopically identified abnormal sites might affect the immune response, either because of antigen exposure or because of danger signals caused by associated inflammation. It is possible that the biopsies may have stimulated CMI responses; however, all of the patients in the study had biopsies both on entry and at end point.

In this study, LP responses to E7 peptide 105 (amino acids 37–54) were associated with both resolution of HPV infection and loss of associated CIN. This region of the E7 protein has been reported by several groups to contain both class I CTL epitopes and class II Th epitopes in murine models (29, 30). CMI responses to epitopes present in this region have also been described in humans (27, 31). Recently, a major immunogenic region of the HPV 16 E7 protein has been identified in the central portion of the E7 protein (amino acids 41–72). Using LP and HLA class II binding assays in human subjects, three different Th epitopes were identified in this area for different DR types (50–62, DR15; 43–77, DR; and 35–50, DQ2). Using IFNγ ELISPOT analysis, CMI responses were detected in several donors, whereas several patients did not demonstrate IFNγ production (32). Although many subjects had protective IFNγ responses, other responders did not.

Our data suggest that LP responses to one or more E6 peptides may also be associated with regression of disease during a 1-year follow-up (peptides 370, 373, or 377), although not significant at current sample size (Table 1). Two E6 sequences were recently described in mice immunized with overlapping E6 peptides that resulted in proliferative responses of lymph node cells in the context of multiple MHC class II haplotypes, indicating a "promiscuous" E6 T-epitope (33). These epitopes were identical to sequences present in our peptides 373 (60–68) and 377 (98–107). The E6 and E7 peptides with protective activity identified in the human studies presented here are thus similar to those that have been described in murine experimental systems.

The association of CMI to specific peptides and regression/resolution was not related to type-specific infection. Both subjects infected with HPV 16 and those with other HPV types were likely to clear HPV infection (Table 3). The explanation for these results is complex. The peptides used in this study were all HPV 16 peptides. Most of our patients did not have current HPV 16 infection. Women currently infected with other HPV types were likely to have been infected with HPV 16 in the past, because HPV 16 is the most common HPV type infecting women in our study population. The CMI responses to HPV 16 peptides tested here may represent a true CMI response to HPV 16 because of either present or past infection with HPV 16, or it may represent cross-reactivity between other HPV types and HPV 16. The CMI responses observed in our *in vitro*

model system may thus represent either primary *in vitro* responses or secondary memory (*in vivo*) responses, which cannot be distinguished in our assay system.

Our LP assay required repetitive antigen stimulation as has been shown for peptides containing subdominant epitopes or when responder cells are immature, as has been shown for cytotoxic T-cell responses in mice (30). In humans, it has been shown that naive precursor Th cells of specific DR types can be activated in unprimed individuals to exhibit LP activity against naturally processed epitopes using repeated *in vitro* stimulation, similar to our CMI protocol (34). It is unclear whether our activated T cells would be able to process and respond to the whole E7 protein, or just to the specific peptide to which they were sensitized, as would be expected for *in vitro*-primed T cells. Memory T cells, activated *in vivo*, would be expected to recognize other E7 and E6 epitopes as well as the immunizing peptide. The long time required for determining CMI in our studies is likely attributable to the small numbers of circulating PBMCs reactive with HPV antigens expressed in microscopic CIN lesions.

Although it has been widely accepted over the past several years that CTLs are the major effectors in determining tumor immunity in murine and human systems, CD4 T-helper cells are also likely to play a significant role (35). The LP responses described here appear to be CD-4-mediated; however, based on present data, we cannot exclude the possibility that CD8 or CTL activity is the major immunoprotective mechanism in humans with HPV-associated cervical neoplasia. Our results demonstrate that *in vitro* proliferative CMI responses to E7 peptide 37–54 peptide in women with CIN are associated with regression of disease and loss of HPV infection *in vivo* and suggest that inclusion of this portion of the E7 protein in HPV vaccines or immunotherapeutic regimens might be useful.

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