Original Research Report

Inverse correlation of cellular immune responses specific to synthetic peptides from the E6 and E7 oncoproteins of HPV-16 with recurrence of cervical intraepithelial neoplasia in a cross-sectional study

Asis K. Sarkar[a], Guillermo Tortolero-Luna[b,c], Michele Follen[b,d],*, K. Jagannadha Sastry[a]

[a]*Department of Immunology, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030, USA*
[b]*Department of Gynecologic Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030, USA*
[c]*The University of Texas Health Science Center at Houston, the School of Public Health, TX 77030, USA*
[d]*The University of Texas Health Science Center at Houston, Department of Obstetrics, Gynecology, and Reproductive Sciences, TX 77030, USA*

Available online 26 September 2005

Abstract

Background. Epidemiological studies have clearly established that human papillomavirus (HPV) infection is the major risk factor for cervical cancer. Most cervical cancers and pre-cancers are HPV-positive. Not all pre-cancers progress to cancer; a significant number regress. The immunological basis for either spontaneous or treatment-mediated recovery from HPV-associated CIN is not clear. Currently, prophylactic vaccines are successfully inducing antibody responses in HPV negative patients. Therapeutic vaccines for HPV-positive patients with disease are needed. There is a need to understand the immunologic basis for the Cell-Mediated Immune (CMI) response and for histological regression to help the formulation of therapeutic vaccines.

Material and methods. Four groups of women were identified for this cross-sectional study of CMI. Group 1 consisted of six women without cytological or histological diagnosis of CIN and with an HPV negative test ($CIN^{(-)}/HPV^{(-)}$). Group 2 included 31 women with a new histological diagnosis of CIN and HPV positive test ($CIN^{(+)}/HPV^{(+)}$). Groups 3 and 4 were selected from women who had undergone ablative or excisional treatment for CIN at the colposcopy clinic at least 6 months before the study. The women in groups 3 and 4 were ($CIN^{(+)}/HPV^{(+)}$) before CIN treatment. Group 3 consisted of 22 women without evidence of recurrence of CIN ($Recur^{(-)}$), and group 4 included 10 with histological diagnosis of recurrent CIN ($Recur^{(+)}$). In particular, we investigated CMI responses to synthetic peptides from the E6 and E7 oncoproteins of HPV-16.

Results. Compared to patients with disease recurrence ($Recur^{(+)}$, $n = 10$), the majority of individuals who remained recurrence-free post-treatment ($Recur^{(-)}$, $n = 22$) exhibited significant proliferative responses to synthetic peptides from the E6 ($P = 0.001$) and the E7 ($P = <0.001$). In particular, significant responses were observed with the E6 peptide Q15L (aa 43–57, $P = 0.006$) and the E7 peptide Q19D (aa 44–62, $P = 0.002$) in $Recur^{(-)}$ patients but not $Recur^{(+)}$ individuals. Additionally, PBMC from women in the $Recur^{(-)}$ group, but not the $Recur^{(+)}$ group, produced predominantly TH1 cytokines upon stimulation with the peptides Q15L or Q19D.

Conclusions. These results indicate an association between significant cellular immune responses specific to synthetic peptides from the E6 and E7 oncoproteins of HPV-16 and recurrence-free survival in HPV patients treated for CIN. We predict that these peptides may be useful as indicators of protective immunity for recovery from CIN and also for potential inclusion in designing immunotherapeutic and immunoprophylactic reagents for HPV-associated CIN.

Keywords: HPV; CIN; Peptides; Cell-mediated immunity; Cytokines

Introduction

While it is well known that Human Papilloma Virus (HPV) causes most cervical pre-neoplastic and neoplastic lesions, there are few studies of the natural immune response to HPV. The cervical pre-neoplastic lesions regress in the majority of cases. The immunologic factors that lead to this spontaneous regression are not yet well understood.

Cervical cancer is the second most common malignancy in women worldwide; 80% of the burden is in the developing world where screening resources do not exist [1,2]. The association of HPV and cervical cancer is markedly stronger with HPV-16, the most prevalent HPV type, and the most frequently detected in high-grade squamous intraepithelial lesions and cancer [3–7]. Several prophylactic vaccine studies have successfully induced antibody responses to HPV. These prophylactic vaccines are aimed at HPV negative patients. It is estimated that if protection were conferred against HPV 16, 50% of cervical cancers would be prevented. Therapeutic vaccines would be used to treat HPV-positive patients with disease. Therapeutic vaccines are thus far, less successful at inducing uniform Cell-Mediated Immunity (CMI) and/or disease regression. A better understanding of the spontaneous regression of lesions could suggest new strategies for therapeutic vaccines.

The E6 and E7 genes of HPV-16 are frequently co-expressed and are the most abundant viral transcripts found in biopsies from HPV-16 positive cervical carcinomas [8,9]. There is strong evidence that co-expression of both E6 and E7 open reading frames, is necessary and sufficient for efficient malignant transformation of a variety of mammalian cells [10,11]. Furthermore, continued expression of the E6 and E7 regions of the viral genome appears to be required to maintain the malignant phenotype [12]. Also, Duensing et al. [13] showed that the E6 and E7 oncoproteins of HPV-16, but not the low-risk type HPV, HPV-6, cooperate with each other to induce mitotic defects and genomic instability.

While some HPV-infected patients develop cervical neoplasia, others do not. Also, there is a high rate of spontaneous regression observed indicating the role of host immune responses. Based on reports in the literature describing a relation between increased prevalence of anti-HPV antibodies, particularly those directed against the E7 oncoprotein, and severity of cervical disease [14–19], it has been suggested that HPV-specific humoral response, directed against the E6 and/or E7 oncoproteins, may not play a protective role against HPV-associated cervical neoplasia [20]. On the other hand, it has been reported that individuals with defects in cell-mediated immunity (CMI) have an increased prevalence of HPV-associated cervical neoplasia, indicating that T cells participate in the control of HPV-associated neoplasia in humans [20–23]. Decreased IL-2 production and proliferative responses to mitogens such as PHA and concanavalin-A have been observed in patients with invasive cervical carcinoma [24]. A number of in vitro and in vivo strategies have been described to identify peptides from HPV-16 E6, E7, and L1 proteins that induce T cell activity in mice and humans [22,25–29]. Using a mouse model developed in our laboratory [30], we identified synthetic peptides corresponding with the E6 and E7 oncoproteins of HPV-16 that were effective in inducing HPV-specific CTL responses in vivo [31]. Nakagawa et al. [20,32] reported that systemic T cell proliferative responses and CTL responses to HPV-16 peptides and proteins were detectable in many virgin as well as sexually active women without cervical lesions but not in those with active disease. Similarly, Tsukui et al. [21] reported that helper T lymphocyte (TH) response, particularly IL-2 production, to HPV antigens was greater among cytologically normal women than in women with different degrees of progressive cervical neoplasia. Also, Clerici et al. [23] observed production of TH1 cytokines (IL-2 and IFN-γ), which potentially enhance CMI, to be defective in women with extensive HPV infection and progression to CIN to be associated with a shift from TH1 to TH2 cytokine production. Employing a long-term in vitro stimulation protocol for determining the TH activity, Kadish et al. [33] reported that lymphoproliferative responses to specific HPV peptides were associated with HPV clearance and regression of CIN. On the other hand, de Gruijl et al. [34] reported that T cell proliferative responses to HPV-16 E7 peptides correlated with persistence of HPV infection, but antigen-specific IL-2 production was associated with both virus clearance as well as progression of cervical lesions [35].

Clearly, a better understanding of CMI responses is needed to design future strategies for effective immunoprevention and immunotherapy of HPV-associated malignancies. A common clinical management strategy for CIN patients includes excisional or ablative treatment. However, a significant number of patients experience recurrence. At present, no clear understanding exists regarding the role of the immune system in disease recurrence or disease-free status in these patients. We conducted a cross-sectional study to investigate the role of cellular immune responses specific to synthetic peptides corresponding with the E6 and E7 oncoproteins of HPV-16 in four groups of patients attending a colposcopy clinic. In particular, in this cross-sectional study, we focus this report on the CMI responses of patients who had undergone ablative or excisional treatment for CIN 6 months prior to the study.

Materials and methods

Patients

Our study population was selected from patients seen at the colposcopy clinic of The University of Texas M.D. Anderson Cancer Center. The study was approved by the M.D. Anderson Cancer Center Internal Review Board.

Informed consent was obtained from the patients, and all procedures were performed according to an Institutional Review Board-approved protocol.

The women were 17 years of age or older and not pregnant with no medical history of immune disorders. Four groups of women were identified for this study. Group 1 consisted of six women without cytological or histological diagnosis of CIN and with an HPV negative test (CIN$^{(-)}$/HPV$^{(-)}$). Group 2 included 31 women with a histological diagnosis of CIN and HPV positive test (CIN$^{(+)}$/HPV$^{(+)}$). Groups 3 and 4 were selected from women who had undergone ablative or excisional treatment for CIN at the colposcopy clinic at least 6 months before the study. The women in groups 3 and 4 were (CIN$^{(+)}$/HPV$^{(+)}$) before CIN treatment. However, at the time of enrollment, which was a minimum of 6 months after CIN treatment, the women were only assessed for disease status. Group 3 consisted of 22 women without evidence of recurrence of CIN (Recur$^{(-)}$), and group 4 included 10 with histological diagnosis of recurrent CIN (Recur$^{(+)}$).

HPV testing

HPV positivity was determined using the Hybrid Capture I assay (Digene Technologies Inc., Gaithersburg, MD). In this protocol, the dot blot hybridization for HPV RNA is performed using exfoliated cervical epithelial cells obtained with cervical swabs. The assay method involves using a ^{32}P-labeled DNA probe-set, which identifies HPV by type: 6/11, 16/18, and 31/33/35. Cells were isolated and processed according to the manufacturer's instructions. At the time of the study, this test was used as part of the standard care program at the colposcopy clinic. These analyses were carried out in the Neuropathology laboratory at the M.D. Anderson Cancer Center.

HPV positivity was further confirmed by PCR using DNA extracted from paraffin-embedded biopsy material as previously described [36,37]. The consensus primers used for the PCR analysis were derived from the L1 open reading frame of the papillomaviruses (MY11, GCMCAGGGW-CATAAYAATGG and MY09, CGTCCMARRGGAWACT-GATC; where M = A + C, R = A + G, W = A + T, Y = C + T). The HPV-16 positivity was confirmed using a specific oligonucleotide probe (CATACACCTCCAGCACCTAA).

Peptides

Peptide sequences corresponding to the E6 and E7 oncoproteins of HPV-16 were selected on the basis of the amphipathic structures and information related to known T cell epitopes described in the literature. Table 2 lists the peptides used in the present study. All peptides were made as reported earlier [31] using the Merrifield solid-phase method [38] either on a modified Vega 250 automatic peptide synthesizer (Vega Biochemicals, Tucson, AZ) or by the "bag" method as described by Houghten [39]. The purity of the peptides used was >95% as determined by amino acid analyses and mass spectrometry. In addition to the E6 and E7 peptides, we used a peptide from the c-mos protooncogene (aa 158–170, STRTPEDSNSLGT) as a negative control. Stock solutions of the peptides were prepared in PBS (pH 7.0) and filter sterilized.

T cell proliferation assay

Heparinized blood was collected from the study participants by venipuncture. The peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on a Ficoll-Hypaque density gradient (Histopaque-1073; Sigma Chemical Co., St. Louis, MO). The proliferative responses of PBMC from different individuals after stimulation with PHA as well as the c-mos peptide and individual E6 and E7 peptides (each used at 5 μg/ml final concentration) were determined using the [^{3}H]thymidine incorporation assay as previously described [40]. Briefly, each sample was seeded in triplicate in 96-well microtiter plates and incubated for 7 days at 37°C in a humidified 5% CO_2 atmosphere. During the final 16–18 h, 1 μCi of [^{3}H]thymidine (6.7 Ci/mmol; ICN Biomedicals, Inc., Costa Mesa, CA) was added. The cells were harvested onto filter strips to estimate [^{3}H]thymidine incorporation. The specific radioactivity of cells treated with various additions was calculated in each case by subtracting the counts per minute (cpm) values obtained with cells cultured in medium alone. Data from pilot experiments showed that, at 5 μg/ml, each peptide yields consistent levels of proliferation. The significance of T cell proliferative responses to the individual E6 and E7 peptides (in terms of stimulation index [SI]) was calculated as the fold increase of [^{3}H]thymidine incorporation by cells exposed to the peptide over that by the control to which no peptide was added. An SI value ≥ 3.0 was considered a positive response and was used for all statistical analyses to determine the significance of proliferative responses and the association with disease-free or disease-recurrence status. In all the experiments, data from triplicate samples were comparable with a standard error of <10%. None of the women in the four study groups tested showed proliferative responses specific to the control c-mos peptide (SI < 2.0).

Cytokine analysis

Cryopreserved PBMC were used for these assays. The PBMC (1×10^5) were incubated with various HPV peptides in RPMI-1640 medium (containing 10% fetal calf serum) in triplicate wells of 96-well round-bottom plates for 48 h at 37°C. Supernatants (100 μl) were removed from each well after centrifugation and stored frozen at -70°C in another 96-well plate. The plates were then thawed and the supernatants assayed for various cytokines (IFN-γ, IL-2, IL-4, IL-10, and IL-12) using the Cytoscreen immunoassay kits (Biosource International, Camarillo, CA) according to the manufacturer's instructions.

Statistical analyses

Differences in the SI values between the patient groups were assessed by Pearson χ^2 and Fisher's exact tests. For the purpose of the statistical analysis, significant proliferative response was defined as a SI $\geq$ 3.0. Statistical significance was set at $P < 0.05$.

For [^{3}H]-thymidine incorporation assays measuring proliferative responses, it is a standard practice to use a stimulation index (SI) value of $\geq$3.0 and above as a cutoff value to indicate significant positivity over background levels. Several studies from our own laboratory as well as others reported in the literature attest to this practice [41–45]. A SI cutoff value of $\geq$3.0, compared to $\geq$2.0 is used uniformly for all the analyses to be indicative of strong positivity for the proliferative responses. Therefore, although used in other published studies, an SI of $\geq$2.0 was not used for these analyses.

Results

The clinical characteristics, including HPV status, of the study subjects are listed in Table 1. A total of 69 women ranging in age from 17 to 54 years (median 31 years) were enrolled in the study. Of these 69 women, 52 were white, 8 each were African American and Hispanic, and one was Asian. The PBMC collected from blood samples obtained from all these women were analyzed for proliferative response to synthetic peptides corresponding with antigenic sequences of the E6 and E7 oncoproteins of HPV-16 (Table 2).

Analyses of proliferative responses specific to various E6 and E7 peptides in each of the four different groups of patients revealed that the majority of patients in group 3 (Recur$^{(-)}$) exhibited positive responses (SI $\geq$ 3.0) to all the

Table 2
Amino acid sequences of the E6 and E7 peptides from HPV-16

Peptide	Residues	Sequence
E6 peptides		
K9L	(aa 18–26)	KLPQLCTEL
E10I	(aa 25–34)	ELQTTIHDII
C10R	(aa 37–46)	CVYCKQQLLR
Q15L	(aa 43–57)	QLLRREVYDFAFRDL
V10C	(aa 49–58)	VYDFAFRDLC
P9L	(aa 66–74)	PYAVCDKCL
P10I	(aa 102–111)	PLCDLLIRCI
E7 peptides		
T10Q	(aa 7–15)	TLHEYMLELQ
M9T	(aa 12–20)	MLDLQPETT
D9L	(aa 14–22)	DLQPETTDL
Q19D	(aa 44–62)	QAEPDRAHYNIVTFCCKCD
R9F	(aa 49–57)	RAHYNIVTF
R9V	(aa 66–74)	RLCVQSTHV
L9V	(aa 82–90)	LLMGTLGIV
G10C	(aa 85–94)	GTLGIVCPIC

aa, amino acid.

seven E6 peptides and 7/8 E7 peptides tested (Fig. 1). On the other hand, only 5/31 untreated patients in the group 2 (CIN$^{(+)}$/HPV$^{(+)}$) and none in the groups 1 (CIN$^{(-)}$/HPV$^{(-)}$) and 4 (Recur$^{(+)}$) showed responses to any of the peptides tested.

The relationship between proliferative response to E6 and/or E7 peptides and post-treatment disease status in women in groups 3 and 4 are presented in Table 3. Whereas none of the patients in group 4 (Recur$^{(+)}$) showed response to any E6 or E7 peptide tested, in group 3 (Recur$^{(-)}$), 64% of patients had significant proliferative responses to the E6 peptides ($P = 0.001$), 82% to the E7 peptides ($P < 0.001$), and 86% to at least one of the E6 or E7 peptides ($P < 0.001$). There was no difference in proliferative response to a common mitogen like PHA ($P = 0.912$, data not shown)

Table 1
Characteristics of the study subjects

Characteristics	Total	Group 1	Group 2	Group 3	Group 4
No. of patients	69	6	31	22	10
Median age	31	31	31	32	27
Age range	17–54	17–43	21–50	18–54	20–39
Race					
White	52 (75.4%)	3 (50%)	26 (83.9%)	15 (68.2%)	8 (80%)
Hispanic	8 (12.6%)	3 (50%)	1 (3.2%)	4 (18.2%)	0 (0%)
African-American	8 (12.6%)	0 (0%)	3 (9.7%)	3 (13.6%)	2 (20%)
Asian	1 (1.4%)	0 (0%)	1 (3.2%)	0 (0%)	0 (0%)
HPV status					
Negative	6 (8.7%)	6 (100%)	0 (0%)	0 (0%)	0 (0%)
Positive	63 (91.3%)	0 (0%)	31 (100%)	22 (100%)	10 (100%)
HPV-16	57 (90.5%)	0 (0%)	31 (100%)	17 (77.3%)	9 (90%)
Other HPV types	6 (9.5%)	0 (0%)	0 (0%)	5 (22.7%)	1 (10%)
Initial diagnosis[a]					
Negative	6 (8.7%)	6 (100%)	0 (0%)	0 (0%)	0 (0%)
CIN1	4 (5.8%)	0 (0%)	0 (0%)	4 (18.2%)	0 (0%)
CIN 2 and 3	59 (85.5%)	0 (0%)	31 (100%)	18 (81.8%)	10 (100%)

[a] For group 3, the diagnosis at the time of recruitment into study was negative for CIN, while that for group 4 was positive for CIN 2/3.
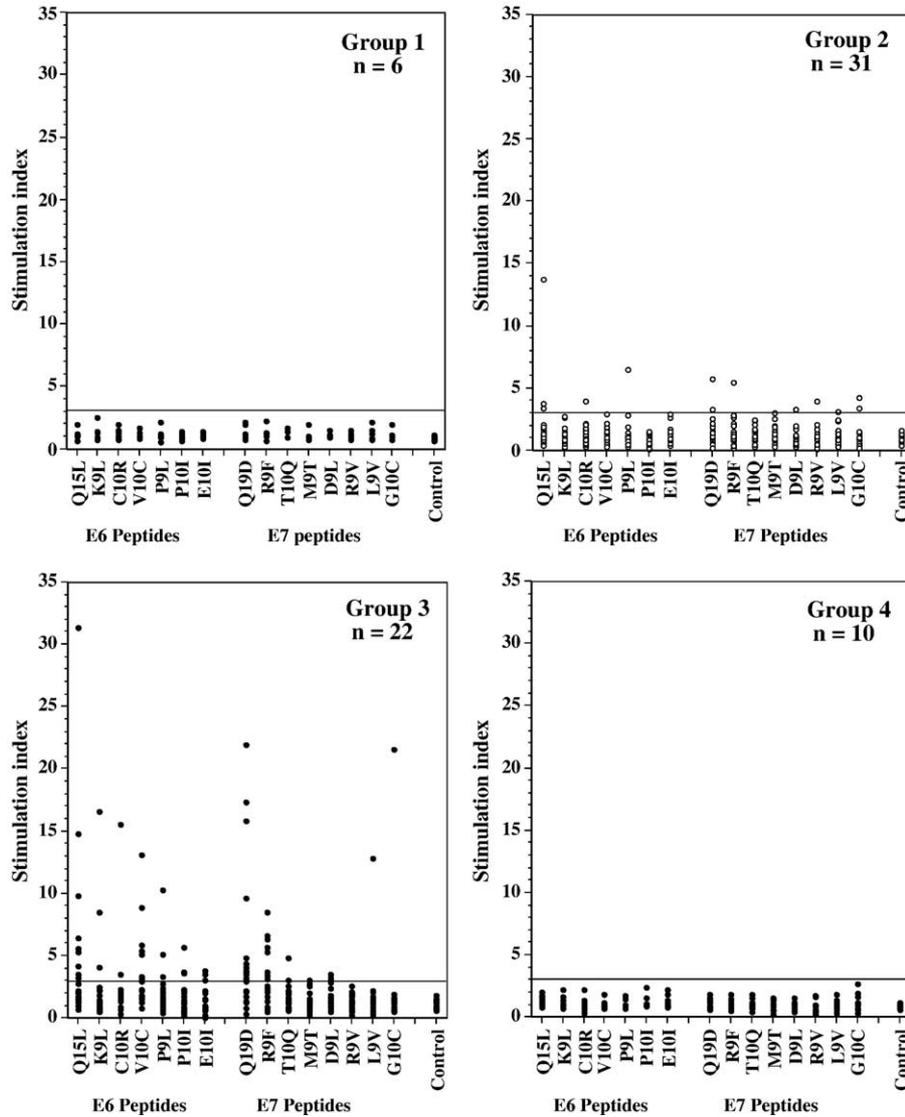
Fig. 1. Proliferative responses to different E6 and E7 synthetic peptides by PBMC from women in four different study groups. Women in group 1 were normal (CIN(−)/HPV(−), n = 6), in group 2 were freshly diagnosed to be HPV-associated CIN (CIN(+)/HPV(+), n = 31), group 3 were recurrence-free post-treatment (Recur(−), n = 22), and group 4 were with disease recurrence (Recur(+), n = 10). PBMC from women in the four different groups were tested for proliferative responses to peptides from the E6 or E7 oncoproteins of HPV-16. The stimulation index (SI) values calculated as fold increase in [³H] thymidine incorporation in peptide-treated samples over medium controls were shown for each patient for each of the peptides tested. See Materials and methods for details.

between groups 3 and 4, suggesting that there is no impairment in the innate immune status of these patients. These results strongly suggest a relationship of proliferative responses specific to synthetic peptides from the E6 and E7 oncoproteins of HPV-16 and disease-free condition after CIN treatment.

We identified higher levels of proliferative responses to two peptides each from the E6 (Q15L and V10C) and E7 (Q19D and R9F) with PBMC samples from patients in group 3 (Recur(−)). Representative proliferative responses, in terms of SI values to 7 synthetic peptides from the E6 oncoprotein and 8 from E7 oncoprotein of HPV-16 are shown in Fig. 2 for 2 patients each in groups 3 and 4. Comparative analyses of proliferative responses to theses

four peptides showed statistically significant differences between women in groups 3 and 4 (Table 4). Whereas no proliferative responses to these peptides were observed in group 4 patients, in group 3 patients, a total of 11 women exhibited responses to peptide Q15L (P = 0.006), 10 to peptide V10C (P = 0.006), 13 to peptide Q19D (P = 0.002), and 10 to peptide R9F (P = 0.013). As seen in Table 2, 9 of the 10 amino acids in the E6 peptide V10C overlap with those of Q15L peptide. Similarly, the 9 amino acids of the E7 peptide R9F overlap with amino acids of the Q19D peptide. Proliferative responses specific to these four peptides together could account for all the responses (19/22 women) observed in group 3 patients (Recur(−)). These results suggest that, with respect to HPV-specific protective

Table 3
Association between proliferative response to all synthetic peptides of HPV-16 oncoproteins E6 and/or E7 and recurrence status following CIN treatment

	Proliferative response	Group 3 (recurrence-free) ($n = 22$)	Group 4 (recurrence) ($n = 10$)	Significance
E6 peptides	Yes[a]	14 (64%)	0	
	No[b]	8 (36%)	10 (100%)	$P = 0.001$
E7 peptides	Yes[a]	18 (82%)	0	
	No[b]	4 (18%)	10 (100%)	$P < 0.001$
Any E6 or E7 peptide	Yes[a]	19 (86%)	0	
	No[b]	3 (14%)	10 (100%)	$P < 0.001$

[a] SI ≥ 3.0.
[b] SI < 3.0.

Table 4
Association between proliferative response to specific synthetic peptides of HPV-16 oncoproteins E6 and/or E7 and recurrence status following CIN treatment

	Proliferative response	Group 3 (recurrence-free) ($n = 22$)	Group 4 (recurrence) ($n = 10$)	Significance
E6 peptides				
Q15L	Yes[a]	11 (50%)	0	
	No[b]	11 (50%)	10 (100%)	$P = 0.006$
V10C	Yes[a]	11 (50%)	0	
	No[b]	11 (50%)	10 (100%)	$P = 0.006$
E7 peptides				
Q19D	Yes[a]	13 (59%)	0	
	No[b]	9 (41%)	10 (100%)	$P = 0.002$
R9F	Yes[a]	10 (46%)	0	
	No[b]	12 (54%)	10 (100%)	$P = 0.013$

[a] SI ≥ 3.0.
[b] SI < 3.0.

cellular immune responses, the amino acid sequences for the Q15L and Q19D peptides within the HPV-16 oncoproteins E6 and E7, respectively, may be immunodominant regions.

We also tested, on a subset of patients in groups 3 and 4 of the study population, whether production of various cytokines in response to these peptides is differential. Cryo-preserved PBMC from 8 women in group 3 (Recur$^{(-)}$), and 6 from group 4 (Recur$^{(+)}$) were stimulated in vitro with peptides Q15L and Q19D. The amounts of the various cytokines in the culture supernatants, after adjusting to unstimulated cultures were shown in Fig. 3. PBMC from 7 of 8 (87.5%), and 5 of 8 (62.5%) women in group 3 (Recur$^{(-)}$) showed production of IFN-γ, and IL-2, respec-

tively, in response to both Q15L and Q19D (Table 5). Additionally, IL-12 production was observed in response to Q15L in PBMC from 3 of 8 women in this group, whereas Q19D-mediated production of IL-12 was evident in 6 of 8 women. On the other hand, none of the PBMC from women in this group secreted IL-4 in response to stimulation with peptides Q15L or Q19D, and only 3 women showed IL-10 production in response to either of the peptides. In contrast to women in group 3 (Recur$^{(-)}$), women in group 4 (Recur$^{(+)}$)
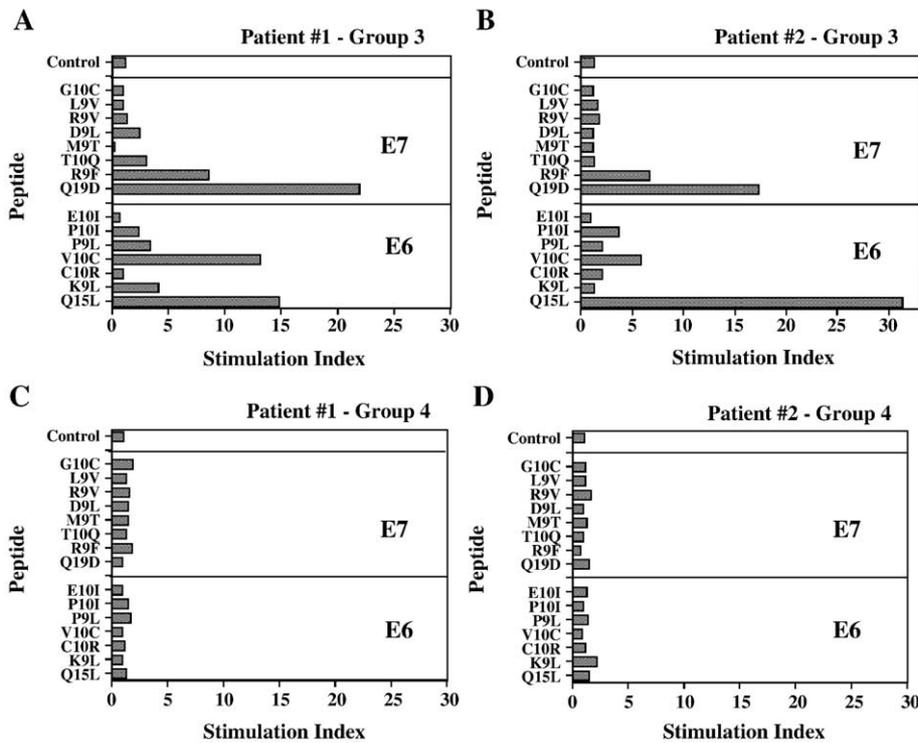
Fig. 2. Representative data showing proliferative responses of PBMC from patients in groups 3 (Recur$^{(-)}$) and 4 (Recur$^{(+)}$) to synthetic peptides from the E6 and E7 oncoproteins of HPV-16. PBMC from women in these groups were tested for proliferative responses to a control peptide (control), and 7 and 8 peptides each from the E6 and E7 oncoproteins, respectively, of HPV-16. Representative data showing SI values from two patients each from Recur$^{(-)}$ group 3 (panels A and B) and Recur$^{(+)}$ group 4 (panels C and D) are shown. See Materials and methods for details.
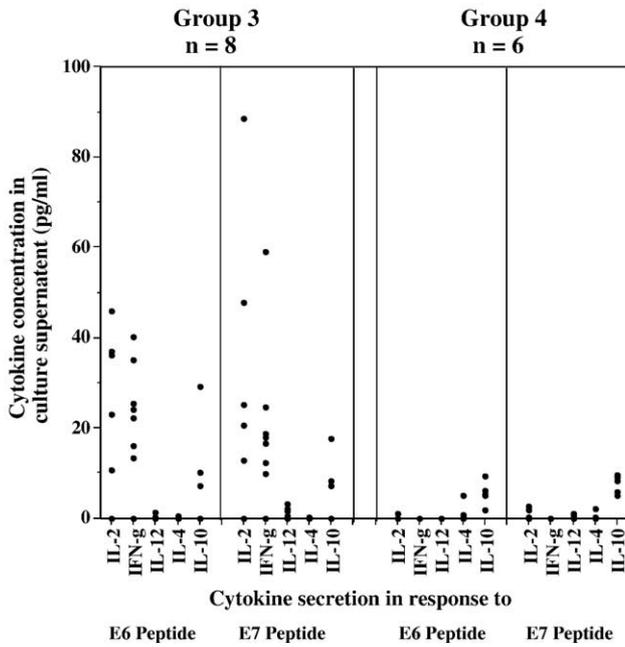
Fig. 3. Production of TH1 cytokines by PBMC from women in group 3 (Recur[(−)]) in response to stimulation with selected E6 and E7 peptides. PBMC from women in groups 3 (Recur[(−)]) and 4 (Recur[(+)]) were cultured in the presence of the E6 peptide Q15L and E7 peptide Q19D for 2 days and the supernatant fluid in each case was analyzed for the presence of various cytokines (IL-2, IFN-γ, IL-12, IL-4, and IL-10) by ELISA. The amount of each cytokine produced, after adjusting to medium controls was shown for each of the patient tested. See Materials and methods for details.

predominantly showed IL-10 production (5 of 6 with Q15L, and 6 of 6 with the Q19D). In 1 of the 6 women in this group, IL-4 production was observed when the PBMC were stimulated with either of the two peptides tested (Table 5). Overall, these results showed that patients in group 3 (Recur[(−)]) predominantly exhibited TH1 cytokine production (IL-2, IFN-γ, and IL-12), whereas women in group 4 (Recur[(+)]), despite not exhibiting specific proliferative responses directed against the HPV peptides, showed production of IL-10.

We were unable to assess the cross-reactivity of T cell responses to E6 and E7 peptides of different HPV types. The peptides used correspond to sequences in the E6 and E7 proteins of HPV-16, and the majority of patients analyzed were positive for HPV-16. Since the patients with HPV types other than HPV-16 are very few, the study did not provide the required numbers for proper statistical analyses and any comments regarding cross-reactivity.

We were also unable to analyze the cytokine response. No statistical correlations between the cytokines in response to stimulation by the selected E6 and E7 peptides, and between the positive proliferative responses for the different peptides are presented. The number of samples analyzed for cytokine production, with respect to the sample size of the study, is too small to allow statistical analysis with adequate power. Thus, no comparisons were made for cytokine responses with proliferative responses.

Discussion

Despite the exciting successes of prophylactic vaccines, there is still a need for therapeutic vaccines. The rationale for therapeutic vaccines that stimulate CMI is the evidence that: (1) patients with CMI deficiencies have an increased incidence of HPV infections, (2) HPV tumors show macrophages and T lymphocytes in areas of regression, (3) HPV-specific circulating T cells have been detected in healthy patients, and (4) anti-HPV IgG1 and IgG2 can be detected in patients with regression [46–55].

Our results suggest a relationship between the presence of cellular immune responses directed against certain E6 and E7 peptides and recurrence-free survival after excisional or ablative treatment for HPV-associated CIN. Surprisingly, none of the women that exhibited disease recurrence after at least 6 months post-treatment (group 4) showed proliferative responses to any of the HPV peptides tested. We did not measure antibodies. These results are consistent with those of others who have reported a correlation between HPV-specific cellular immune responses and CIN stage or cervical pathology [20,21,32]. However, unlike the reports in the literature, which analyzed patients at different stages of disease, our study focused on the relevance of HPV-specific immunity as a protective factor against recurrence of HPV-associated CIN. Our results suggest that these responses may help or determine the risk of recurrence for HPV-associated CIN. Since only a small number of peptides were used for the analyses, the data obtained could potentially underestimate the association and the significance of these findings. These results strongly support data from epidemiological studies [56,57] which have found an increased risk of HPV-associated disease among women with immunosuppressive conditions, in particular, among women with CMI deficiency but with normal humoral immune function, such as in certain groups of transplant recipients and patients with HIV [21,56,57]. Further evidence for the importance of CMI in HPV-associated cervical neoplasia comes from other related observations in

Table 5
Cytokine production of PBMC from patients in groups 3 and 4 in response to stimulation with synthetic peptides from the E6 and E7 oncoproteins of HPV-16[a]

Cytokine[b]	Group 3 (n = 8)		Group 4 (n = 6)	
	Q15L[c]	Q19D[d]	Q15L	Q19D
IFN-γ	7/8	7/8	0/6	0/6
IL-2	5/8	5/8	0/6	0/6
IL-12	3/8	6/8	0/6	1/6
IL-4	0/8	0/8	1/6	1/6
IL-10	3/8	3/8	5/6	6/6

[a] Number of patients positive/number tested.
[b] Positivity for cytokine production is based on values above the sensitivity of the test kit used for each cytokine in terms of pg/ml: IL-2 = 8.7, IFN-γ = 4.0, IL-12 = 1.0, IL-4 = 2.0, and IL-10 = 5.0.
[c] Q15L peptide from the E6 oncoprotein of HPV-16.
[d] Q19D peptide from the E7 oncoprotein of HPV-16.

the literature that include: (i) infiltration of helper T cells and CTLs in spontaneously regressing warts [58], (ii) depletion of antigen-presenting cells in the cervix of women with cervical neoplasia [59], and (iii) in some instances, an association of certain HLA haplotypes with cervical cancer risk [60,61]. Since patients in our study were not HLA typed, it is not possible to comment on any relation of particular HLA haplotype(s) with either the proliferation results or disease-free and/or disease recurrence condition.

Our results also suggest that the regions composed of amino acids 43–58 in the E6 oncoprotein (covering peptides Q15L and V10C), and amino acids 44–62 in the E7 oncoprotein (covering peptides Q19D and R9F) of HPV-16 are immunodominant and may be important for protection against recurrence of HPV-associated CIN. These are the same peptides that we previously identified in our murine studies to be capable of inducing HPV-specific CTL responses [31]. Similarly, Feltkamp et al. [22] reported that an E7 peptide similar to the R9F peptide employed in our studies, when used as a vaccine in a mouse model to prime CTL responses protected mice against tumors induced by HPV-16-transformed cells. It is interesting to note that while helper T cell epitopes are typically made up of 16–25 amino acids, in our study, efficient proliferative responses were observed with the E7 peptide R9F consisting of 9 amino acids and the E6 peptide V10C with 10 amino acids (Table 4). In this regard, Nakagawa et al. [62] reported that CD4-poistive and CD8-positive CTL contribute towards HPV-16 E6 and E7 responses in women free of lesions associated with HPV-16. Both the T cell proliferative responses and IL-2 production in response to peptides overlapping with the amino acid sequence of the Q19D peptide used in our studies were also reported in other human studies [20,21]. Importantly, in these studies, the presence of HPV-peptide-specific cellular immune responses correlated with either healthy status or less-severe stages of cervical pathology [20,21,32]. Similarly, the Q15L peptide from E6 was within a sequence of peptides used by Kadish et al. [33] that showed positive association of lyphoproliferative responses with clearance of HPV infection and regression of CIN.

It is important to point out the significant aspect of the assay protocol we used in determining the peptide-specific proliferative responses, which is different from the procedure reported by others [33,34]. Basically, our protocol involved culturing the PBMC for 1 week whereas the procedures of Kadish et al. [33] and de Gruijil et al. [34] involved a 2–3 week culturing period. In particular, the protocol described by Kadish et al. [33], which shows a correlation between HPV-peptide-specific cellular immune responses and disease-status, involved stimulation of patient PBMC with HPV peptides for 21 days with repeated additions of peptide-pulsed irradiated autologous PBMC and IL-2 before a strong proliferative response was achieved. It was hypothesized that such a lengthy stimulation protocol was needed because the patients have very few precursor T cells responsive to the peptides studied [33].

Also, in these studies, the patient selection depended on the clinical status rather than any specific treatment outcome. On the other hand, the association we observed between HPV-peptide-specific cellular immune responses and disease-free condition after surgical intervention for HPV-CIN involved a 7-day stimulation protocol with a single addition of the test peptide at the beginning of the assay. Based on these differences in assay protocol and study population, it is reasonable to speculate that there may be more precursor T cells responsive to the peptides selected for our study in the PBMC obtained from disease-free patients, which may account for the response of these individuals to the treatment. It is important to confirm this hypothesis in a prospective study of a bigger cohort, which will help us fully realize the clinical significance of cellular immune responses directed against the HPV peptides tested in the present investigation.

We also obtained preliminary evidence for TH1 cytokine production in response to E6 and E7 peptides by PBMC from women remaining disease-free after a minimum of 6 months following CIN treatment. On the other hand, we observed production of IL-10 in women with disease recurrence within 6 months of treatment. Although these cytokine studies were not extensive and did not include a large number of patients [21,23,61–68], the results obtained are in agreement with those of Tsukui et al. [21] who reported that IL-2 production by PBMC in response to HPV-derived peptides correlated with less severe cervical pathology. Moreover, Clerici et al. [23] observed that production of TH1 of cytokines (IL-2 and IFN-γ) was defective in women with extensive HPV infection and that progression to CIN was associated with a shift from TH1 to TH2 cytokine production. However, Santin et al. [63] reported that IL-10 in combination with IL-2 increased the intracellular expression of TH1 cytokines and cytolytic activity of HPV-specific CTL. In general, a possible protective role for TH1 cytokine production, which is indicative of strong CMI responses, has been described in certain chronic parasitic diseases, leprosy, and HIV [62–65]. Thus, there is compelling evidence in the literature related to various malignancies, including HPV-associated cervical neoplasia, for a protective role of specific CMI responses. In this regard, studies by Nakagawa et al. [19,31] showed that T cell proliferation and CTL responses to HPV-16 peptides and proteins are present in many virgin as well as sexually active women without cervical lesions but not in women with active disease.

It would be of interest to know whether the peptides are homologous to E6/E7 from other high-risk types or not. In other words, can these responses be generalized to other high-risk types, or are they potentially a marker for exclusively HPV 16? In terms of sequence homology, there is not a high degree of homology with other high-risk HPV types like HPV-18. However, since very few patients with HPV other than HPV-16 were analyzed, the sample size for the other HPV types is too small and accordingly it is

difficult to make any comments related to the generality of the responses observed in the present study.

These reports, together with our data showing an association between proliferative responses directed against peptides from the E6 and E7 oncoproteins of HPV-16 and recurrence-free survival after surgical intervention, strongly suggest that cellular immune responses specific to the E6 and E7 peptides may have a role in the protective immunity against HPV-associated CIN. However, it is important to confirm these results in a prospective study involving a larger patient population. It is also important to determine the pattern of cellular immune responses over time, from initial diagnosis of CIN to post-treatment for CIN, and its relationship with HPV-related factors (persistence of infection and viral load). Such a study would also help determine the value of these peptides as prognostic indicators of recurrence-free status after treatment for CIN and/or the potential of these peptides as immunoprophylactic or immunotherapeutic reagents against HPV-associated cervical neoplasia. Indeed, recent studies [67,68] described generation of HPV-specific CD4$^+$ and CD8$^+$ T cell responses by using autologous dendritic cells pulsed with E7 protein or peptides as a potential immunotherapeutic strategy for HPV-associated cervical cancer. Other strategies are also being employed in commercially and university-based vaccine trials [52–55]. Hopefully, these findings will help the effort to create a therapeutic vaccine.

Acknowledgments

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