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# T-Cell Proliferative Response to Human Papillomavirus Type 16 Peptides: Relationship to Cervical Intraepithelial Neoplasia

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**The incidence of human papillomavirus (HPV)-related cervical intraepithelial neoplasia (CIN) and cervical cancer is increased with immunodeficiency, but the role of immune response, including cell-mediated immunity, in disease prevention is not well understood. In this study, T-cell proliferative responses to six synthetic peptides with predicted immunogenic determinants from the HPV-16 E4, E6, E7, and L1 open reading frames were analyzed in 22 sexually active women with new-onset CIN and 65 sexually active women without cervical disease, characterized by cytology, colposcopy, and HPV testing. T-cell proliferative responses were demonstrated to all six HPV-16 peptides. Although not statistically significant, rates of reactivity to E6 (24-45) were higher among sexually active women without disease (26%) than among women with current CIN (7%), as was the overall number of peptides stimulating a response. Women with CIN may not respond to selected HPV antigens as well as women without disease do.**

Detection of anogenital human papillomavirus (HPV) infection is very common among sexually active young women, but cervical intraepithelial neoplasia (CIN) is diagnosed in only a small proportion of these women. In conjunction with data showing increased rates of cervical disease among immunodeficient women, the epidemiologic data suggest that immune response may play a protective role against development of cervical disease once HPV infection is established (5, 24, 28, 29, 33).

On the basis of these considerations, we hypothesized that, if an immune response were associated with protection against disease, then women with CIN might lack responses to one or more HPV antigens that would be detectable in a subset of sexually active women with no cervical disease. Since the prevalence of antibody positivity to HPV proteins such as E7 increases as the severity of the cervical disease increases (4, 14, 15, 17, 18, 26, 31, 35, 38), the available data suggest that humoral response does not play such a protective role. We therefore sought to characterize cell-mediated immune (CMI) response in a population of women with and without cervical disease, as well as women with no history of sexual intercourse. We performed HPV testing to determine if the detection of HPV DNA correlated with T-cell proliferative (TCP) response and further extended these observations by including HPV testing at extracervical sites such as the anus and oral cavity because of data suggesting that infection at these sites may be common (5, 16, 24, 28).

A major obstacle in studying CMI to HPV is the lack of a suitable culture system to propagate the virus in vitro. As a result, several investigators have used synthetic peptides derived from HPV type 16 (HPV-16) sequences (1, 8, 9, 13, 19, 32, 34, 36) as well as recombinant viruses expressing HPV-16 genes (13, 39) as sources of antigens for CMI assays. In this study, we used one of the commonly studied measures of CMI response, TCP response, to investigate its association with respect to detection of HPV infection and to HPV-related cervical disease. HPV-16 peptide antigens from four different HPV-16 open reading frames were incubated with peripheral blood mononuclear cells (PBMC) in a standard 6- to 7-day dose-dependent assay.

TCP responses to all six putative T-cell dominant synthetic peptides derived from the HPV-16 E4, E6, E7, and L1 open reading frames were demonstrated. Although not statistically significant, compared with women with CIN, there was a trend toward higher response rates in women without disease for several peptides, as well as responsiveness to a larger number of peptides. The data suggest that systemic TCP responses to HPV-16 peptides are detectable in many sexually active women and that women with disease may not respond as well to selected HPV antigens.

## MATERIALS AND METHODS

**Subjects.** Twenty-six subjects from the University of California at San Francisco (UCSF) Dysplasia Clinic and 68 subjects from the San Francisco State University Health Center (SFSUHC) participated in this study. Subjects from the UCSF Dysplasia Clinic were patients who were referred with a history of a recently abnormal Papanicolaou smear but who did not have a history of prior CIN. The subjects from the SFSUHC were part of an ongoing prospective study of cervical HPV infection and associated cervical disease among adolescents. Each subject was examined by cytology and colposcopy. A cervical punch biopsy was performed if indicated. Twenty-two subjects (20 from the UCSF Dysplasia Clinic and 2 from SFSUHC) had biopsy-proven CIN, and 66 subjects (all from the SFSUHC) were free of cervical, vaginal, or vulvar disease. Six subjects referred to the UCSF Dysplasia Clinic were excluded from analysis when CIN could not be confirmed with biopsy at the time of recruitment. Fourteen women who by history were virgins were also studied.

**HPV DNA detection.** Cervical swab specimens were taken from the patients from the UCSF Dysplasia Clinic, and cervical, vulvar, anal, and oral swab spec-

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TABLE 1. Synthetic peptides derived from HPV-16 open reading frames with predicted T-cell immunogenic determinants

| Peptide from HPV open reading frame | Sequence |
|---|---|
| E4 (4-29) | PAATKPLLKLLGSTWPTTPPRPIPK |
| E6 (24-45) | TELQTTIHDILECVYCKQQLL |
| E6 (109-122) | RCINCQKPLCPEEK |
| E7 (21-30) | DLYCYEQLND |
| E7 (44-57) | QAEPDRAHYNIVTF |
| E7 (62-79) | DSTLRLCVQSTHVDIRTL |
| L1 (382-396) | LNTNFKEYLRHGEEY |

imens were obtained from the subjects recruited from the SFSUHC. Specimens for HPV testing could not be obtained from the virginal women.

PCR analysis for the detection of HPV DNA was performed with L1 consensus primers and probes as previously described (37). Specimens positive with consensus probes were further typed with probes specific for HPV-6, 11, 16, 18, 31, 33, 35, 39, 45, 51, and 52 (types 39, 51, and 52 were detected with a mixture of probes). Specimens that were positive with the consensus probe but negative with these type-specific probes were considered to contain one or more other HPV type(s). As a positive control, PCR was performed on all specimens with primers specific for the human beta-globin gene. Samples negative for this human beta-globin gene were considered insufficient. Twenty-three subjects enrolled from the SFSUHC had HPV testing on specimens prior to study entry (22 subjects had cervical specimens, and 1 subject had anal and oral samples), and this information was included in data analysis.

**Selection of HPV peptide antigens for T-cell proliferation assays.** Synthetic peptides from the HPV-16 E4, E6, E7, and L1 open reading frames were chosen by the following criteria, in descending order of importance: (i) they specified possible T-cell determinants according to the Berzofsky algorithms (2); (ii) whenever possible, the sequences identified by these algorithms also included Rothbard motifs (30); and (iii) the sequences fitting the first two criteria specified the smallest degree of homology with the HPV-18 counterpart of the protein. Six peptides shown in Table 1 were chosen for study and were synthesized by Merrifield's solid-phase techniques on a Beckman Model 990C automated peptide synthesizer. The peptides were characterized by high-pressure liquid chromatography and amino acid analyses. The purity of the peptides was greater than 95% in all preparations.

**T-cell proliferation assays.** TCP assays were performed with modifications made to previously described methods (22). Approximately 60 ml of heparinized whole blood was drawn from each subject. The following procedures were used for all subjects including the sexually active subjects with CIN, the sexually active subjects without cervical disease, and the virginal women. PBMC were isolated with a Ficoll-Hypaque density gradient (Sigma, St. Louis, Mo.) from fresh whole blood anticoagulated with heparin. An average yield of PBMC from healthy subjects was approximately $10^6$ cells per ml of whole blood. The optimal number of PBMC for the TCP assays was determined to be $10^5$ cells. This number of cells was placed in each well of a 96-well flat-bottom microtiter plate with varying concentrations of synthetic peptide antigens in a total volume of 200 μl of RPMI 1640 with 15% pooled human serum (Norml Cera-Plus; NABI, Miami, Fla.), 1,000 U of penicillin G per ml, and 1,000 mg of streptomycin per ml. Each experiment was performed in triplicate. Initially, 10 different concentrations (0.01 to 100 μg/ml) of the same peptide were studied, with harvesting on days 3 through 7 (data not shown). The higher concentrations (12.5, 25, 50, and 100 μg/ml) and longer incubation periods (day 6 and day 7) were chosen for subsequent assays since more responses were demonstrated in these ranges. Tetanus toxoid (2.4 Lf per well in triplicate) was used as a source of recall antigen, and at least six wells of PBMC without antigen were used as negative controls. The microtiter plates were incubated at 37°C in a 5% $CO_2$ atmosphere.

Cell proliferation was measured by pulse-labeling cells for DNA synthesis for 6 h with 1 μCi of [$^3$H]thymidine per well. Cellular DNA was collected onto a glass fiber filter with a multiple automated sample harvester, and counts per minute were determined in a liquid scintillation counter.

**Statistical analysis.** Subjects were grouped with respect to history of sexual activity and the presence or absence of HPV infection at the time of study entry or documented HPV infection in the past. The time of study entry was defined as the date at which blood was drawn to perform T-cell proliferation assay for a given subject. Sexually active women were also classified with regards to the presence or absence of cervical disease. Since one of the hallmarks of the immune system is the ability to mount memory responses, the subjects were grouped on the basis of both past and present PCR results for HPV typing. A subject was therefore entered into the HPV-16-positive group if she had HPV-16 in any prior or current specimen.

Raw data from TCP experiments expressed in counts per minute were double-entered into data files for checking data entry errors. The count per minute values of the negative control wells (PBMC without antigen) were compared within and across subjects for each harvest day, and outliers were eliminated. The outliers were defined as values greater than the 75th percentile plus three times the interquartile range (the difference between the 75th and 25th percentiles of the data), or less than the 25th percentile minus three times the interquartile range (21). Three subjects had all three negative control wells from harvest day 7 excluded from analysis, and three additional subjects had all values from both harvest days excluded from analysis. For two of these subjects, data from subsequent assays performed at a follow-up visit were included in the analyses. The third subject had no subsequent assays performed and was excluded from all further analyses.

Throughout this analysis, the data were analyzed by nonparametric statistical methods with SAS software (SAS Institute, Cary, N.C.) in order to minimize the effects of outliers. These methods are preferred to their parametric analogs when samples are small and when responses are skewed, as in this data set. For each subject, the median counts per minute from replicate wells, for a given harvest day and concentration, was used to calculate her stimulation index (SI) by the following formula: SI = (median counts per minute of antigen-stimulated wells)/(median counts per minute of negative control [antigen-free] wells).

For comparisons of proliferative responses among groups of subjects, SIs instead of counts per minute were used to eliminate the effect of interassay variability. There was no obvious dose response over harvest days and antigen concentrations; therefore, the maximum SI response to peptides for each subject was analyzed, regardless of dose or length of incubation. Since the definition of biologically relevant positive proliferative responses is not known, data were analyzed by using SI cutoffs for positivity of 3.0, 4.0, and 5.0.

Kruskal-Wallis *P* values were determined for age and tetanus toxoid SI to compare three groups of subjects: sexually active with CIN, sexually active without disease, and virgins. This test was also used to compare these groups with respect to distributions of number of peptides stimulating a positive TCP response. All other comparisons were made with the two-sided Fisher's exact test.

## RESULTS

Twenty-two sexually active women with new-onset CIN (six subjects with CIN I, eight subjects with CIN II, and eight subjects with CIN III), 65 sexually active women without cervical disease, and 14 virginal women were studied. This sample excludes one woman from the sexually active-no disease group from whom all count per minute values from the negative control wells were outliers and six women without biopsy confirmation of CIN at study entry. The age, race, and response to tetanus toxoid were compared among subjects grouped by history of sexual activity and the presence of cervical disease (Table 2). The sexually active-CIN group were older (median age, 26.0 years) than the sexually active-no disease group (median, 22.0 years) and the virginal women (median, 21.0 years; $P < 0.001$, Kruskal-Wallis test). The tetanus toxoid SI value did not differ significantly across groups ($P = 0.36$, Kruskal-Wallis test). The median was lowest in the sexually active-CIN group (SI = 65; cpm = 5,163) compared with that of the sexually active-no disease group (SI = 136; cpm = 15,607) and that of the virginal group (SI = 125; cpm = 12,452).

Twenty of 22 subjects with CIN were from the UCSF Dysplasia Clinic. Cervical swab specimens were collected from 18 of these 20 subjects and were analyzed for the presence of HPV-DNA by PCR. Ten subjects were HPV-16 positive (coinfection with HPV-6, one subject; coinfection with HPV-31, one subject), two subjects were positive for HPV types other than 16 (HPV-35, one subject; other HPV type[s], one subject), four subjects were HPV negative, and two subjects had insufficient samples. No specimens from these subjects were available prior to study entry. The two subjects from SFSUHC with CIN were HPV-16 positive (cervix) prior to study entry.

Swab specimens from four sites (cervix, vulva, anus, and mouth) were collected for PCR analysis from 64 of 65 sexually active SFSUHC subjects without cervical disease at study entry. Six subjects were HPV-16 positive (cervix and vulva, two subjects; vulva, two subjects; anus, one subject; mouth, one subject). Twenty-eight subjects were positive for HPV types other than 16 (HPV-6, 31, 33, 35, 45, 39/51/52, and other), and 30 subjects had no HPV detected. At the time of study entry, 11 subjects had more than one HPV type detected and 14 subjects had HPV detected at more than one site. Twenty-one

TABLE 2. Demographic characteristics and TCP response to tetanus toxoid

| Characteristic | Value for group | | |
|---|---|---|---|
| | Sexually active | | Virgin ($n$ = 14) |
| | CIN ($n$ = 22) | No disease ($n$ = 65) | |
| Age (yr) (median [range]) | 26.0 (19–48) | 22.0 (19–25) | 21.0 (19–26) |
| Race (%) | | | |
| White | 77.3 | 49.3 | 78.6 |
| Hispanic | 13.6 | 12.3 | 0 |
| Afro-American | 9.1 | 7.7 | 0 |
| Asian | 0 | 16.9 | 21.4 |
| Other[a] | 0 | 13.8 | 0 |
| Tetanus response (median [range])[b] | | | |
| SI | 65 (1–954) | 136 (2–2,140) | 125 (1–433) |
| cpm | 5,163 (101–144,125) | 15,607 (75–123,113) | 12,452 (65–54,112) |

[a] Mostly of mixed race.
[b] Maximum SI over two harvest days (day 6 and day 7) was used.

(32%) of the 65 subjects were tested prior to study entry. Seven subjects were HPV-16 positive (cervix, six subjects; anus, one subject), and two subjects were positive for HPV-18 in the cervix. The subjects in the virginal group declined to have specimens taken for PCR analysis.

At the time of study entry, a higher proportion of subjects in the sexually active-CIN group was positive for HPV-16 (10 of 16 [63%]) compared with the sexually active-no disease group (6 of 64 [9%]) at the time of study entry ($P < 0.0001$). The same was true for high-risk (HPV-16, 18, 31, 33, 35, 39, 45, 51, and 52) HPV types (11 of 16 [69%] in the sexually active-CIN group versus 15 of 64 [23%] in the sexually active-no disease group; $P = 0.002$). However, there was little difference in the infection rates with all HPV types (12 of 16 [75%] in the sexually active-CIN group versus 34 of 64 [53%] in the sexually active-no disease group; $P = 0.16$).

Six HPV-16 peptides predicted to specify T-cell epitopes according to Berzofsky algorithms and Rothbard motifs were chosen for studies of TCP response (Table 1). The rates of positive TCP responses to these peptides, based on an SI cutoff of 5.0, are shown in Table 3. Responses to each of the six peptides were detected in some subjects. The maximum response to each peptide ranged from SI of 16.4 for E6 (109-122) to that of 83.4 for E4 (4-29) with corresponding maximum median counts per minute of 1,397 and 2,334, respectively. The highest response rate was seen with E6 (24-45) (26% in sexually active-no disease group, 14% in virginal group, and 7% in sexually active-CIN group). The lowest response rate was seen for E7 (21-30), with only 8% of subjects in sexually active-no disease group showing positive response and no responses seen in the other groups. Similar results were obtained with cutoffs of 3.0 and 4.0 (data not shown).

Several differences in TCP responses to different peptides among the groups were noted, but none reached statistical significance, in part because of the small number of subjects studied. Among women with HPV-16-associated CIN, responses were seen to four of six peptides, in contrast to only two of six peptides in women with HPV-16-negative CIN. Among sexually active women without disease, response rates between HPV-16 DNA-positive and -negative women were similar, with rates slightly higher among the latter for five of the six peptides.

Among all women with HPV-16 infection, the most marked difference in response was a 36% response rate to E6 (24-45) in women without disease compared with none among women with CIN. Furthermore, response to this peptide was higher among all sexually active women without disease (26%) compared with sexually active women with CIN (7%). TCP response rates were also slightly higher for three of the other six peptides [E4 (4-29), E6 (109-124), and E7 (21-30)] among the sexually active women without disease and were similar for the other two peptides [E7 (44-57) and L1 (382-396)]. Data were also analyzed by comparing subjects who were positive for high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, and 52) with those in whom no high-risk HPV types were detected, but the results did not differ significantly.

The distribution of number of peptides to which the subjects responded was also analyzed (Table 4). Most subjects responded to none of the peptides or one peptide. Only 1 of 13 sexually active women with CIN (8%) and 1 of the 14 virgins

TABLE 3. TCP responses of SI ≥ 5.0 to HPV-16 synthetic peptides

| Peptide | No. of subjects with SI ≥ 5.0/no. of subjects tested (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Sexually active | | | | | | Virgin |
| | CIN | | | No disease | | | |
| | All[a] | HPV-16$^+$ | HPV-16$^-$ | All[a] | HPV-16$^+$ | HPV-16$^-$ | |
| E4 (4-29) | 3/21 (14) | 2/11 (18) | 0/6 (0) | 12/60 (20) | 2/12 (17) | 10/47 (21) | 2/14 (14) |
| E6 (24-45) | 1/15 (7) | 0/8 (0) | 1/4 (25) | 15/58 (26) | 4/11 (36) | 11/46 (24) | 2/14 (14) |
| E6 (109-122) | 1/20 (5) | 1/11 (9) | 0/5 (0) | 8/65 (12) | 1/13 (8) | 7/51 (14) | 1/14 (7) |
| E7 (21-30) | 0/22 (0) | 0/12 (0) | 0/6 (0) | 5/65 (8) | 0/13 (0) | 4/51 (8) | 0/14 (0) |
| E7 (44-57) | 3/22 (14) | 3/12 (25) | 0/6 (0) | 9/65 (14) | 1/13 (8) | 8/51 (16) | 1/14 (7) |
| L1 (382-396) | 3/16 (19) | 1/7 (14) | 1/5 (20) | 10/65 (15) | 1/13 (8) | 9/51 (18) | 2/14 (14) |

[a] Subjects who had no or insufficient samples for PCR determination of HPV type(s) were included only in "all" columns.

TABLE 4. Distribution of number of peptides with proliferative responses[a] of SI ≥ 5.0

| No. of peptides | No. of subjects who responded to a specified no. of peptide(s)/no. of subjects tested (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Sexually active | | | | | | Virgin |
| | CIN | | | No disease | | | |
| | All[b] | HPV-16$^+$ | HPV-16$^-$ | All[b] | HPV-16$^+$ | HPV-16$^-$ | |
| 0 | 8/13 (61) | 4/6 (66) | 2/4 (50) | 26/57 (45) | 5/11 (46) | 21/45 (46) | 8/14 (57) |
| 1 | 4/13 (31) | 1/6 (17) | 2/4 (50) | 14/57 (25) | 4/11 (36) | 9/45 (20) | 5/14 (36) |
| 2 | 1/13 (8) | 1/6 (17) | 0/4 (0) | 10/57 (18) | 1/11 (9) | 9/45 (20) | 0/14 (0) |
| 3 | 0/13 (0) | 0/6 (0) | 0/4 (0) | 4/57 (7) | 1/11 (9) | 3/45 (7) | 1/14 (7) |
| ≥4 | 0/13 (0) | 0/6 (0) | 0/4 (0) | 3/57 (5) | 0/11 (0) | 3/45 (7) | 0/14 (0) |

[a] Eighty-four subjects, who were tested with all six peptides, were entered into this analysis.
[b] Subjects who had no or insufficient samples for PCR determination of HPV type(s) were included only in "all" columns.

(7%) studied responded to two or more peptides. In contrast, 17 of 57 (30%) sexually active women without disease responded to two or more peptides. These data were not statistically different given the small sample size ($P = 0.22$, Kruskal-Wallis test).

## DISCUSSION

To date, little is known about the nature of the CMI response to HPV, the role of CMI in the pathogenesis of cervical disease, or the clinically relevant HPV epitopes. Similarly, little information is available about peripheral TCP responses to anogenital HPV infection. Several methods to measure TCP responses in vitro in PBMC have previously been described (3, 6, 7, 10–12, 20, 23, 27). Attempts have been made to amplify measurable responses by adding exogenous recombinant interleukin 2 (37, 38) and by establishing short-term cultures (27). Other investigators have also used limiting dilution methods (7, 12) to yield quantitative data. In our study, we used 6- and 7-day bulk culture of PBMC instead of a short-term culture, T-cell lines, or T-cell clones to obtain a better representation of sensitized PBMC and to obviate unpredictable skewing of data resulting from addition of cytokines and prolonged culture in vitro.

The interpretation of assays such as these necessarily depends on a satisfactory definition of a negative or positive response. Various cutoff points for positivity commonly used in the literature were explored (SI ≥ 3.0, 4.0, and 5.0). The differences observed among the groups did not change with these cutoff points, and the results of this study are therefore presented with the most conservative cutoff, i.e., an SI of 5.0. Furthermore, since SI ≥ 5.0 is the most stringent of these thresholds, it is less likely to misclassify subjects as responders to TCP when they are actually nonresponders.

Our data confirm that a peripheral TCP response to one or more HPV-16 peptide antigens can be detected in approximately one-half of all women tested. Overall, however, the absolute SIs were relatively low compared with recall antigens such as tetanus toxoid. Given the bulk culture method that we used for the TCP assays, this may indicate that there are only a small number of HPV-16-specific T cells circulating in the periphery.

In one previous study using T-cell lines or T-cell clones, E7 (5-18), E7 (17-38), and E7 (69-86) were shown to be immunogenic in seropositive individuals without obvious HPV-related diseases (1). In another study, positivity with borderline statistical significance was described against peptides E7 (17-37), E7 (37-54), and E7 (62-80) by a 3-week bulk culture method (19). The investigators reported that 22.2% (2 of 9) of subjects with HPV-16 DNA-positive cervical dysplasia and 7.7% (1 of 13) of controls (laboratory workers without history of abnormal Papanicolaou smear) responded to both E7 (17-37) and E7 (37-54). Two of the peptides included in our study, E7 (21-30) and E7 (44-57), overlap with these peptides, and our subjects responded similarly to E7 (44-57), with 25% of subjects with CIN and HPV-16 and 14% of sexually active subjects without cervical disease demonstrating a positive response. However, in our study no response was detected with E7 (21-30) in subjects with CIN and HPV-16 while 8% of sexually active subjects without disease responded. While fewer proliferative responses to E7 (17-37), E7 (37-54), E7 (62-80), and E7 (72-97) were demonstrated in control subjects compared with subjects with CIN in the previous study (19), our data with a different set of peptides showed overall more responses in subjects without CIN than with CIN. This discrepancy may be due to differences in the way that the subjects were characterized and that the assays were performed in the two studies.

Fewer sexually active women with CIN responded to the antigenic peptides tested than sexually active women without cervical disease. The most marked difference was with peptide E6 (24-45). Although these data support our hypothesis that immune response may be associated with protection against disease, the differences were not statistically significant given the small sample size. In addition, it is possible that the attenuated proliferative responses detected in women with CIN may reflect a suppressive effect of cervical disease on the immune response.

Among sexually active women with no cervical disease, rates of TCP response were slightly higher among the HPV-16-negative group than those with HPV-16 infection, possibly reflecting cross-reactivity to other HPV types. However, since HPV infection may be transient in nature (25), it is also possible that some of the sexually active HPV-16-negative subjects may have had HPV-16 infection in the past that was eradicated by the time of study entry. Evidence supporting specificity of the response for HPV-16 antigens includes the observation that the HPV-16-positive subjects in the sexually active-CIN group responded more frequently to several HPV-16 peptides, E4 (4-29), E6 (109-124), and E7 (44-57), than the HPV-16-negative women with CIN.

For comparison, we studied 14 virgins, since it was presumed that these women had not had any exposure to genital HPV types. Our data indicate that a small number of virgins show a TCP response, and this may be explained in one of three ways: (i) these women acquired HPV infection through exposure other than sexual intercourse, (ii) their sexual history was inaccurate, or (iii) there was cross-reactivity to antigens of nongenital HPV types.

The results of this study must be interpreted with caution, as we were unable to determine the HPV or cervical disease status of the women in the virginal group. Misclassification may also have occurred within our study groups since we did not perform HPV testing of extracervical sites in the women with CIN, and we did not perform HPV testing prior to study entry for many of the study subjects. Most of the subjects (21 of 23) who were tested prior to study entry belonged to the sexually active-no disease group, and the subjects in this group were tested more frequently and at multiple sites. This makes it more likely stochastically to detect HPV infection including HPV-16 in the sexually active-no disease group. However, in our study, HPV-16 infection was more common in sexually active women with CIN than in sexually active women without disease. Therefore, while a bias in classifying subjects due to more frequent testing in the sexually active-no disease group may have been introduced, it likely did not exert any significant effect. Furthermore, spontaneously resolved prior HPV infections or, for that matter, spontaneously regressed CIN could not be excluded in any of the study subjects. Lastly, the study groups differed with respect to age, as the sexually active women with CIN were older than the sexually active women without disease and the virgins. While this may be a reflection of differences in the recruitment strategies between these groups, this finding was expected, since the development of CIN as a consequence of HPV infection may require a relatively long period of time.

To determine if the differences in response rates reflected nonspecific differences in reactivity among the groups, we measured TCP response to a common recall antigen, tetanus toxoid. A trend toward decreased response to tetanus toxoid was seen in the sexually active-CIN group compared with sexually active women with no disease.

In summary, we have demonstrated the presence of measurable TCP cell response to HPV-16 in PBMC to all six HPV-16 peptides tested (Table 3). TCP responses in our study did not correlate significantly with the virologic and disease status of the subjects. However, our data show TCP responses in a large proportion of sexually active women without cervical disease, and a trend toward increased reactivity in this group compared with subjects with CIN. Among the peptides tested, the clearest difference was seen with E6 (24-45). Furthermore, while only a small proportion of the virginal women and women with CIN reacted to two or more peptides, almost one-third of sexually active women without disease reacted to multiple peptides. Our results also show that responses may be seen to a wide variety of HPV antigens, including both early and late region proteins, and future studies should include assessment of the antigenicity of these proteins at different stages of infection and disease. While these results confirm that CMI to HPV-16 antigens can be measured in PBMC, the biological relevance of these findings is not yet clear. Prospective studies will be needed to determine if changes in measurable TCP correlate over time with the changes in the clinical status of individual subjects.

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