Research Article

Persistent Human Papillomavirus Infection Is Associated with a Generalized Decrease in Immune Responsiveness in Older Women

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Abstract

The development of cervical cancer and its precursors are linked to persistent infection with oncogenic types of human papillomavirus (HPV). Host immune responses seem to be determinants of risk for this disease. However, little is known about the immunologic determinants of HPV persistence. Here, we examined the association between lymphoproliferative responses to antigens/mitogens and persistent HPV infection in women older than 45 years. Women included in this study were participants in a 10,000-woman population-based cohort study of cervical neoplasia in Costa Rica. Women older than 45 years and HPV DNA positive at a screening visit were selected as cases (n = 283). We selected a comparably sized control group of HPV DNA–negative women, matched to cases on age and time since enrollment (n = 261). At an additional clinical visit, women were cytologically and virologically rescreened, and cervical and blood specimens were collected. Proliferative responses to phytohemagglutinin (PHA), influenza virus (Flu), and HPV16 virus-like particle (VLP) were lower among women with persistent HPV infection [median counts per minute (cpm): 72,849 for PHA, 1,241 for Flu, and 727 for VLP] than for the control group (median cpm: 107,049 for PHA, 2,111 for Flu, and 2,068 for VLP). The decreases were most profound in women with long-term persistence and were only observed for the oldest age group (≥65 years). Our results indicate that an impairment in host immunologic responses is associated to persistent HPV infection. The fact that effects were evident for all studied stimuli is suggestive of a generalized effect. (Cancer Res 2006; 66(22): 11070-6)

Introduction

Infection with 1 of ~15 human papillomavirus (HPV) types is necessary for the development of cervical cancer (1). Although HPV infections are common and typically transient, it is the small subset of women with long-term, persistent infections with oncogenic HPVs that are at highest risk of progression to precancerous cervical lesions and cervical cancer (2, 3). Inadequate immunologic control of HPV infection resulting in viral persistence is likely an important determinant of risk of progression to cervical neoplastic disease. Previous studies have provided evidence supporting this view. Higher prevalence of HPV infection is observed in HIV-infected individuals (4, 5). Studies have also reported associations between deregulation of cytokine production and impairment of $CD4^+$ T cell–mediated immunity and cervical precancers (6–9). Finally, the consistent association observed between HLA alleles and cervical neoplasia argue for a role of the host immune response to HPV in cervical cancer pathogenesis (10).

Direct evidence linking host immunologic responses to risk of HPV persistence is sparse. The few studies that have been reported to date have been modest in size. Therefore, it is not surprising that results have been mixed. Although some studies have suggested that immune responses to HPV are associated with viral clearance (11, 12), others have not (13–15). Further evaluation of host immunologic factors associated with HPV persistence in well-characterized, larger studies is clearly needed.

Studies of HPV DNA prevalence with age have consistently shown that the highest rates of HPV infection are seen in the first few years following initiation of sexual activity. Prevalence of HPV infection typically drops in the late 20s and early 30s. In some studies, this reduced prevalence is sustained at higher ages (16–19). However, in others, a second peak in HPV prevalence has been reported at older ages (18, 20–24). This second peak in HPV prevalence is poorly understood and could be due to cohort effects, re-exposure through new sexual contacts, and/or physiologic changes at the cervix with aging that increase the efficiency of HPV detectability by current sampling methods (25, 26). It has also been suggested that this second peak might occur due to re-emergence of latent HPV infections in older women (27), following age-related declines in immunologic competence (28–40).

We were interested in evaluating whether host immunologic factors are associated with HPV persistence. Given that results from our 10,000-women population-based cohort in Guanacaste, Costa Rica indicated a second peak in HPV prevalence among women older than 55 years (41), and that immunologic competence has been reported to decrease with aging (28–40), we reasoned that targeting women in this age group for our investigation might prove fruitful.

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Materials and Methods

Study Design

Women included in the present study were participants in a 10,049-woman population-based cohort study of HPV and cervical neoplasia initiated in 1993 to 1994 in the province of Guanacaste, Costa Rica. Details of the cohort recruitment (42) and follow-up (43) have previously been described. In brief, women were identified by random sampling of selected censal segments of Guanacaste, Costa Rica. Over 93% of eligible women agreed to participate. At enrollment, women were offered a pelvic examination, and specimens for conventional and liquid-based cytology and HPV DNA testing were collected. A cervigram was also taken at this time. Women with evidence of cervical abnormalities were referred to colposcopy for evaluation and treatment and were excluded from the study. All others were eligible for the follow-up phase of the study (n = 9,095).

Follow-up visits were done at frequent intervals (every 6 or 12 months) for the subset of 2,121 women who at enrollment were defined as high risk of cervical disease by virtue of one of the following: evidence of low-grade or equivocal cytology, prevalent HPV infection (defined by the first generation Hybrid Capture 1 test available at that time), or high-risk sexual behavior (defined as five or more sexual partners in a lifetime). A group of 410 virginal women who were 26 years or younger at enrollment and a random sample of 535 women who did not fulfill the above criteria of high risk were also selected for these frequent follow-up visits. Follow-up for 5,134 of the remaining 6,029 (85.2%) women was done at their 5th or 6th anniversary of enrollment. Standardized pelvic examinations were done at the time of follow-up visits. Women who developed evidence of high-grade cervical intraepithelial neoplasia (CIN) or cancer during follow-up were not eligible for the follow-up phase of the study and were referred to colposcopy for evaluation and treatment.

For the present study, PCR-based HPV DNA testing results (see below) from the 5- to 7-year visit (referred to subsequently as the follow-up visit) available from 7,008 women were used to select as cases all women older than 45 years who were HPV DNA positive at that time point. As controls, we selected an equivalent number of women who were HPV DNA negative at their follow-up visit, frequency matched to cases on age and time since enrollment. Women selected for the study were asked to attend the study clinic (at their 7th to 9th anniversary of enrollment) for an additional study visit (referred to subsequently as the final visit to distinguish it from the enrollment and follow-up visits), at which time a questionnaire was given, cervical cells were collected for conventional and liquid-based cytology, additional cells were collected for PCR-based HPV DNA testing, and 40 mL of blood were collected in heparinized tubes from which peripheral blood mononuclear cells (PBMC) were isolated and cryopreserved.

Cases (n = 324) and controls (n = 310) were selected for this study. Of these, 298 cases (92.0%) and 293 controls (94.5%) participated. Among these 591 participants, blood was successfully collected, cryopreserved, and tested for all but seven participants (5 cases and 2 controls). An additional nine cases found to have cytologic evidence of high-grade cervical disease at the time of their final study visit were excluded, resulting in a final number of 284 cases and 291 controls included in this analysis. Among these individuals, the median time between enrollment and the follow-up visit was 61 months (61 months among both cases and controls; range: 57-98 months). The median time between the follow-up visit and their final visit for the present study was 41 months (42 months among cases and 40 months among controls; range: 12-54 months); 37.9% of participants were 46 to 54 years old, 36.7% were 55 to 64 years old, and the remaining 25.4% were ≥65 years old. By design, this distribution was similar for cases and controls.

The study was approved by ethical committees in Costa Rica and the National Cancer Institute (NCI). All participants provided informed consent.

Laboratory Analyses

Detection and genotyping of HPV. HPV DNA detection and typing was done using consensus-primer PCR for the L1 region of HPV using the MY09/MY11 primer system and the AmpliTaq Gold polymerase followed by dot blot hybridization of the amplification products, as previously reported (25). Additional PCR-based HPV DNA results available on the subset of women followed every 6 or 12 months was used in exploratory evaluations described in Results to further clarify virological patterns.

Cells. Heparinized peripheral whole-blood specimens obtained from participants at the final visit (years 7-9) were transported in coolers at ~20°C to the cryopreservation laboratory. PBMC isolation and cryopreservation were done within 24 hours after specimen collection. PBMCs were separated by Ficoll density gradient centrifugation using Ficoll-Paque Plus (Bio Whittaker, Walkersville, MD) and cryopreserved using a controlled rate freezer (Kryosave, Rockville, MD) in 22% FCS (Bio Whittaker), 7.5% DMSO (Sigma, St. Louis, MO)–supplemented RPMI 1640 (Bio Whittaker). Storage and transport to NCI (HPV Immunology Laboratory, Frederick, MD) were carried out in the vapor phase of liquid nitrogen.

At the testing laboratory, cells were thawed and resuspended in 50 mL of AIM-V media. To avoid cell aggregation, 150 units of RNase-free DNase I (Roche Diagnostics, Mannheim, Germany) were added, and the suspension was incubated at 37°C for 1 hour (44). After incubation, cells were centrifuged and resuspended at 2×10^6/mL. Viability was determined using trypan blue staining and was on average 94 ± 5%.

Lymphoproliferation assays. PBMC specimens were randomly assigned to 98 testing batches. Laboratory personnel were blinded to case status. PBMCs were cultured in triplicate, at 2×10^5 per well, in 96-well round-bottomed plates (Costar, Corning, NY) in AIM-V media (Invitrogen, Frederick, MD) supplemented with penicillin-streptomycin (100 μg/mL to 100 units/mL; Invitrogen), glutamine (2 mmol/L), and HEPES buffer (10 mmol/L) at 37°C, 5% CO_2. Cells were cultured in the presence of one of the following conditions: AIM-V media as a negative control; HPV-16 L1 virus-like particle (VLP; noninfectious HPV-like particles, composed of the L1 major capsid protein, expressed in baculovirus-infected Sf9 insect cells, 10 μg/mL in AIM-V; Novavax, Malvern, PA), influenza A virus (Flu; infectious virus; H3N2, A/Hong Kong/8/68, 1:100; American Type Culture Collection, Manassas, VA) as recall antigens; or phytohemagglutinin (PHA; 1:100; Sigma), a mitogen. The purity of the HPV-16 L1 VLP was >96%, as determined with SDS-PAGE by the manufacturer. To control for the possible influence of contaminants from the cell system used to produce the HPV-16 L1 VLP, Sf9/baculovirus insect cell lysate (Bac, 0.1 μg/mL; Novavax) diluted in culture media was used as a negative control. No responses were detected in this condition (data not shown).

Cultures containing mitogens or antigens were pulsed with 1 μCi of [^{3}H]thymidine (Amersham Biosciences, Piscataway, NJ) for 18 hours after either 48 hours (3-day media and PHA) or 96 hours (5-day media, HPV-16 L1 VLP, Flu) of culture. Cultures were harvested and counted in an automated scintillation counter (Microbeta, Perkin-Elmer, Boston, MA). Results were expressed as mean counts per minute (cpm). For a small subset of specimens, the total number of PBMCs recovered after thawing was not sufficient to permit all laboratory tests (PHA, n = 0; Flu, n = 10; HPV-16 L1 VLP, n = 42). Consequently, results from these specimens were not available for analysis. There were no other reasons for excluding specimen results from the analysis.

To assess batch-to-batch variability, an aliquot from a normal donor was tested once in all but 3 of the 98 batches. The respective variation coefficients for net cpm were as follows: PHA, 17%; Flu, 30%; HPV-16 L1 VLP, 35%. As an additional quality control step, 50 blinded replicate aliquots from participant specimens were selected for testing. These 50 replicates were always included in a distinct batch from that in which the original aliquot was included, to assess interbatch reproducibility. Replicate results were classified into tertiles used for analysis and compared. Exact tertile agreement between the original and blinded replicate aliquots was observed for PHA for 49% of the specimens; 94% of specimens agreed within one tertile level. Exact and within-one-tertile-level agreements for Flu and HPV16 L1 VLP were 52% and 92% and 58% and 98%, respectively.

Flow cytometric analysis. Phenotype for major leukocyte subsets was determined using five-color flow cytometry on a FC-500 flow cytometer (Beckman-Coulter, Fullerton, CA) as previously described (45).

Statistical Analysis

For the analysis, the initial group of participants who were HPV DNA positive at the time of their follow-up visit were subdivided into two case

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groups: those whose HPV infection(s) were still present at the time of their final visit for the present study (HPV-persistent case group, n = 87) and those who cleared their HPV infection by the time of their final visit (HPV clearance case group, n = 196). These two case groups were compared against the control group, defined as women who were free of HPV infection at both time points (HPV-negative control group, n = 261). The HPV-negative group was selected as the control/comparison group because it is representative of women older than 45 years from our population-based study. Given how common HPV infection is among sexually active individuals, the vast majority of these women are expected to have been exposed to HPV and to have had the opportunity to develop persistent HPV infection. A group of 28 women who were HPV negative at the time of their follow-up visit were found to have acquired HPV infection at the time of their final visit. This group of women was not included in the primary analyses, although they were considered in subanalyses of interest. In addition, women with unknown HPV infection status at either time point (follow-up visit or final visit) were excluded from analysis (n = 3).

Overall persistence of HPV infection was defined as the persistence of one or more individual HPV types at the follow-up visit and the time of the final visit for the present study (i.e., type-specific persistence). Among women with persistent infection, 86.2% (n = 75) had a single persistent HPV type, 10.3% (n = 9) had two persistent HPV types, and the remaining 3.4% (n = 3) had three or four persistent HPV types. In addition to the overall analysis, analyses were done stratified by age (<55, 55-64, and 65-74 years) and considering as persistent infection the subset of women with evidence of very long-term HPV persistence (defined as type-specific HPV detected at enrollment into our cohort study, at the follow-up visit, and at the time of the final visit for the present study; n = 32; median duration of persistence = 108 months). Analyses were also done considering clearance and persistence of oncogenic HPV types (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68), nononcogenic HPV types (HPV types 6, 11, 26, 32, 34, 40, 42, 53, 54, 55, 57, 61, 62, 64, 66, 67, 69, 70, 71, 72, 73, 74, 81, 82, 83, 84, 85, and 89), and HPV-16 only.

Lymphocyte proliferation responses were expressed as net cpm, defined as cpm for antigen/mitogen of interest minus background cpm observable in the media alone. These proliferation results were evaluated as continuous and as categorical variables (using tertiles and quartiles based on the distribution among the entire study population). A variable that combined across the PHA, Flu, and HPV-16 L1 VLP results was also created to jointly evaluate immune responsiveness to these three stimuli. This joint variable was defined by grouping together women with consistent proliferation responses across antigen/mitogen type. Women with a consistently poor response, as defined by net cpm in the lowest tertile for $\geq$2 of the 3 assays, were compared with women having a consistently high response (net cpm in the highest tertile for all three assays). Women with responses in the middle tertile or with inconsistent responses across assays were grouped into an intermediate category. A similar joint variable combining across the PHA and VLP assays was also used.

When testing for differences between means and medians by persistence, the ANOVA F test and Kruskal-Wallis tests were used, respectively. When evaluating categorical variables, odds ratios (OR) and 95% confidence intervals (95% CI) were estimated using logistic regression. Models were adjusted for age and time from enrollment to the follow-up visit (both matching factors in our study). Additional adjustment for potential confounding by use of oral contraceptives, lifetime number of sexual partners, number of pregnancies, menopausal status, time between the follow-up visit to the final visit for the present study, and leukocyte phenotype markers (CD3, CD4, CD8, CD45, CD19, CD16/CD56, and CD14) did not materially affect estimates. For parsimony, these variables were not included in the final models presented. Heterogeneity of effects across age strata was evaluated by including in the logistic model the main effect variables along with their cross-product.

Results

Persistence of HPV infection is associated with a global decrease in lymphocyte proliferative potential. Mean and median proliferative responses to PHA, Flu, and HPV-16 L1 VLP were compared across study groups (Table 1). The weakest proliferative responses were consistently observed among women with persistent HPV infections, regardless of stimulus used (PHA, Flu, and HPV-16 L1 VLP). In analyses that restricted the HPV clearance and persistence case groups to those infected with oncogenic HPV types, nononcogenic HPV types, or HPV-16, the tendency for mean proliferative responses to be weakest among those with persistent infection was consistently observed, with the strongest effect seen for oncogenic HPV types. Because decreased immune proliferation among women with persistent infection was observed in all HPV groups evaluated, subsequent comparisons focused on the overall HPV group.

We also evaluated whether the proliferation levels observed among the group with persistent HPV infection differed for those with a single versus a multiple infection. No significant difference was observed when mean or median proliferation in response to PHA (mean/median cpm = 86,993/75,931 for single infection; 88,165/70,682 for multiple infection; P = 0.94/0.83), Flu (mean/median cpm = 3,681/1,346 for single infection; 4,386/959 for multiple infection; P = 0.64/0.80), or HPV-16 L1 VLP (mean/median cpm = 2,494/774 for single infection, 2,988/689 for multiple infection; P = 0.61/0.86) was compared.

We next examined risk associated with decreasing proliferative responses to PHA or recall antigens. For this analysis, the group of women who cleared their infection and those with persistent infection were each compared against the HPV-negative control group. Analyses based on tertile cuts are summarized in Table 2. Similar patterns were observed when quartile cuts were evaluated (data not shown). No significant association of lymphocyte proliferative responses to PHA or recall antigens was detected when the group that cleared HPV infection was compared with the HPV-negative control group, with the exception of a trend of increasing risk observed with decreasing responsiveness to HPV-16 L1 VLP (Table 2; OR, 1.4; 95% CI, 0.90-2.2 and OR, 1.8; 95% CI, 1.0-3.0 for the middle and low tertiles, respectively, compared with the highest tertile; P_{trend} = 0.03). When women with persistent HPV infection were compared against the HPV-negative control group, increases in risk of persistence were observed with decreasing proliferative responses for both PHA (Table 2; OR, 1.6; 95% CI, 0.8-3.1 and OR, 2.5; 95% CI, 1.3-4.9 for the middle and low tertiles, respectively, compared with the highest tertile; P_{trend} = 0.007) and HPV-16 L1 VLP (Table 2; OR, 1.3; 95% CI, 0.7-2.6 and OR, 2.3; 95% CI, 1.1-4.6 for the middle and low tertiles, respectively, compared with the highest tertile; P_{trend} = 0.02). No association was observed when Flu was used as the antigenic stimulus (P_{trend} = 0.80). When the group with persistent HPV infection was compared with the group that cleared infection, similar but weaker patterns were observed, but these effects were no longer significant (P_{trend} for PHA = 0.332, P_{trend} for Flu = 0.925, and P_{trend} for HPV-16 L1 VLP = 0.534).

Effects were even more pronounced in analyses that compared women with evidence of long-term persistence (as defined in Materials and Methods) against HPV-negative controls (Table 2). In this analysis, women in the lowest tertile of proliferative response (relative to those in the highest tertile) to PHA, Flu, and HPV-16 L1 VLP had OR estimates of 6.4 (95% CI, 2.1-19; P_{trend} = 0.002), 3.1 (95% CI, 1.0-9.8; P_{trend} = 0.05), and 16 (95% CI = 3.5-72; P_{trend} < 0.001), respectively.

Proliferative responses to PHA, Flu, and HPV-16 L1 VLP were correlated with each other, with Pearson's correlation coefficients

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Table 1. Lymphoproliferative responses in net cpm by study group

Antigen/mitogen	PHA	Flu	VLP	VLP	VLP	VLP
Restrictions*	None	None	None	Oncogenic HPV types	Nononcogenic HPV types †	HPV16
HPV-negative control group						
n	261	258	247			
Median	107,049	2,111	2,068			
Mean	114,015	4,994	4,225			
SD	73,474	7,966	5,477			
HPV clearance group						
n	196	191	180	65	128	12
Median	96,601	1,897	1,368	1,608	1,368	918
Mean	112,258	4,853	4,025	4,285	4,466	4,664
SD	75,910	7,652	6,448	6,263	7,130	7,713
P ‡	0.6	0.72	0.15	0.70§	0.30§	0.41§
HPV persistence group						
n	87	84	77	28	55	5
Median	72,849	1,241	727	408	1,094	798
Mean	87,408	3,967	2,693	2,674	2,701	1,012
SD	70,664	6,712	4,100	4,411	3,852	1,118
P ‡	<0.01	0.27	<0.01	0.01§	0.03§	0.13§

NOTE: Cell culture was done as described in Materials and Methods. Median cpm for media = 565 (3 days) and 648 (5 days).
*Restriction applied to the HPV clearance and HPV persistence groups.
†Because it is uncertain if types 66, 73, and 82 should be considered carcinogenic, these types were included in the nononcogenic group.
‡From Kruskal-Wallis test for difference between medians. HPV persistence and clearance groups were each compared against the HPV-negative control group.
§*P* is based on comparison against the HPV-negative control group (n = 247).

of 0.48 (for PHA/Flu), 0.56 (for PHA/HPV-16 L1 VLP), and 0.71 (for Flu/HPV-16 L1 VLP). This suggests that each of the three proliferative assays might be capturing the same general effect of reduced proliferative capacity among women persistently infected with HPV. Therefore, a combined index of proliferative responsiveness was developed (as defined in Materials and Methods) and risk of HPV persistence associated with this proliferative index evaluated. When this was done, women who were classified into

Table 2. Risk associated with decreasing lymphoproliferative responses to PHA, Flu, and VLP

Antigen/mitogen	HPV negative control group*, *n*	HPV clearance group*		HPV persistence group*		HPV long-term persistence group*	
		n	OR (95% CI)	*n*	OR (95% CI)	*n*	OR (95% CI)
PHA							
High tertile	98	69	1.0	19	1.0	6	1.0
Middle tertile	89	63	1.1 (0.7-1.7)	28	1.6 (0.8-3.1)	9	2.3 (0.7-7.4)
Low tertile	74	64	1.6 (1.0-2.6)	40	2.5 (1.3-4.9)	17	6.4 (2.1-19)
P_{trend}			0.08		0.007		0.002
Flu							
High tertile	90	64	1.0	26	1.0	6	1.0
Middle tertile	92	65	1.0 (0.7-1.7)	27	0.8 (0.4-1.6)	11	1.8 (0.6-5.5)
Low tertile	76	62	1.3 (0.8-2.1)	31	1.1 (0.6-2.1)	12	3.1 (1.0-9.8)
P_{trend}			0.30		0.8		0.05
VLP							
High tertile	103	62	1.0	21	1.0	3	1.0
Middle tertile	87	67	1.4 (0.9-2.2)	26	1.3 (0.7-2.6)	12	6.9 (1.7-29)
Low tertile	57	51	1.8 (1.0-3.0)	30	2.3 (1.1-4.6)	11	16 (3.5-72)
P_{trend}			0.03		0.02		<0.001

*As defined in Materials and Methods. HPV persistence and clearance groups were each compared against the HPV-negative control group.

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Table 3. Risk of HPV persistence associated with decreasing lymphoproliferative responses to PHA, Flu, and VLP stratified by age

Antigen/mitogen	Ages 46-54		Ages 55-64		Ages 65-74	
	n (cases/controls)*	OR (95% CI)	*n* (cases/controls)*	OR (95% CI)	*n* (cases/controls)*	OR (95% CI)
PHA						
High tertile	5/38	1.0	10/35	1.0	4/25	1.0
Middle tertile	7/34	1.0 (0.3-3.8)	12/34	1.1 (0.4-2.9)	9/21	3.4 (0.9-14)
Low tertile	9/30	1.1 (0.3-4.3)	11/30	1.1 (0.4-3.2)	20/14	13 (3.2-49)
P_{trend}		0.93		0.82		<0.001
Flu						
High tertile	7/39	1.0	14/29	1.0	5/22	1.0
Middle tertile	5/30	0.69 (0.2-2.6)	7/37	0.35 (0.1-1.0)	15/25	2.6 (0.8-8.8)
Low tertile	9/32	0.7 (0.2-2.5)	10/32	0.55 (0.2-1.5)	12/12	4.8 (1.3-18)
P_{trend}		0.6		0.22		0.02
VLP						
High tertile	4/38	1.0	13/37	1.0	4/28	1.0
Middle tertile	6/32	1.1 (0.3-5.0)	7/32	0.56 (0.2-1.6)	13/23	5.0 (1.3-18)
Low tertile	7/30	0.8 (0.2-3.9)	10/20	1.3 (0.5-3.6)	13/7	19 (4.0-86)
P_{trend}		0.8		0.8		<0.001

NOTE: $P_{heterogeneity}$ = 0.04 for PHA, 0.12 for Flu, and 0.02 for VLP.

*Cases comprised of women with type-specific HPV persistence as defined in Materials and Methods. Controls comprised of women who were HPV negative as defined in Materials and Methods.

the lowest tertile of proliferative response on ≥2 assays (n = 95) were found to have a 1.5-fold increased risk of HPV persistence relative to women classified into the highest tertile of proliferative response for all three assays (n = 54; 95% CI, 0.6-3.6). Compared with controls, women who were classified into the lowest tertile of proliferative response in both the PHA and VLP assays (n = 63) were at 1.8-fold increased risk of HPV persistence relative to those classified into the highest tertile of proliferative response for both assays (n = 75; 95% CI, 0.7-4.4).

Association between lymphocyte proliferation and HPV persistence is restricted to women older than 65 years. To further evaluate the effect of age on the association between lymphoproliferative responsiveness and HPV persistence, analyses were done within three approximately equally sized age strata (ages 45-54, 55-64, and 65-74 years). Results are summarized in Table 3 and indicate that the association between reduced proliferative responsiveness and HPV persistence is restricted to the oldest age group ($P_{heterogeneity}$ between strata = 0.04 for PHA, 0.12 for Flu, and 0.02 for VLP). Within this age group, and compared with women in the highest tertile of proliferative responses to PHA, Flu, and HPV-16 L1 VLP, women classified in the lowest tertile of proliferative responsiveness had OR estimates of 13 (95% CI, 3.2-49; P_{trend} < 0.001), 4.8 (95% CI, 1.3-18; P_{trend} = 0.02), and 19 (95% CI, 4.0-86; P_{trend} < 0.001), respectively.

When the effect of age on proliferative potential was evaluated separately among women with persistent HPV and those in the HPV-negative control group, no evidence of immune down-regulation was observed among controls (in fact, some indication of increases in immune response with age were observed), whereas reductions in immune responsiveness to PHA, Flu, and HPV-16 L1 VLP were observed among women with persistent HPV infection (Fig. 1).

Women who "acquire" an HPV infection despite lack of sexual activity have lower proliferative responses. A group of 28 women who were HPV negative at the time of the follow-up visit acquired HPV infection by the time of their final visit. Interestingly, of these 28 women, 7 reported no sexual activity in the time period of interest. To ensure that the HPV infection detected at the final visit was not previously evident, complete virological histories since enrollment were reviewed. Two women were excluded after finding evidence of HPV-positive results in previous visits.

We compared the five remaining women (median age = 62; range = 46-71) with evidence of new infection despite lack of sexual activity against the 21 women who acquired infection and were sexually active during the period of interest (median age = 54; range = 49-68). When these two groups were compared (Fig. 2), the median proliferative response to PHA, Flu, and HPV-16 L1 VLP was lower among women who acquired infection despite a lack of sexual activity compared with women who were sexually active. The differences observed did not reach statistical significance, possibly due to the small number of women evaluated.

Discussion

In this study, we investigated whether persistent infection with HPV in women older than 45 years of age was associated with changes in immune competence by assessing immune responsiveness *in vitro* to mitogens and antigens. We found that there was a negative association between the magnitude of the immune response, as measured by the ability of PBMCs to proliferate in response to mitogenic/antigenic stimulation in culture, and HPV persistence. This effect was observed in response not only to HPV L1 VLP but also to a polyclonal stimulus (PHA) and less consistently in response to Flu. The magnitudes of the proliferative responses observed were correlated between stimuli, suggesting

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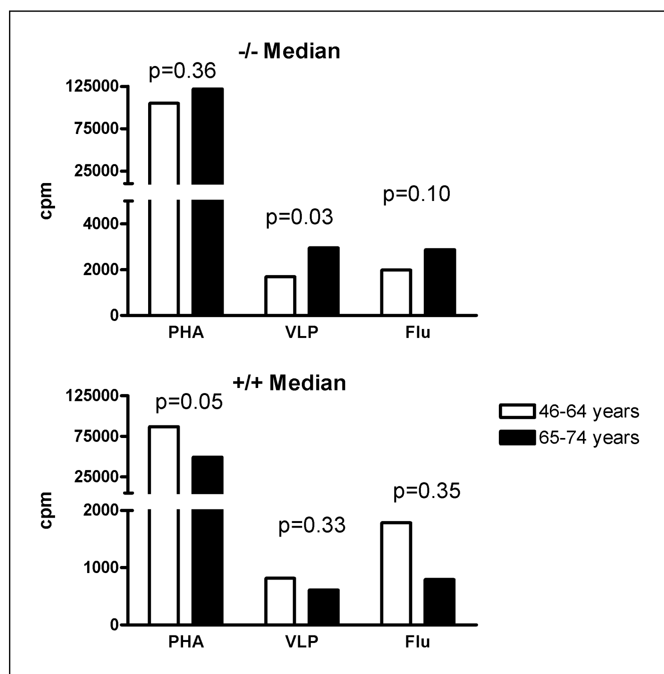

Figure 1. Median lymphoproliferative responses to PHA, Flu, and VLP in the HPV-negative control group (−/−) and in the HPV persistence group (+/+). PBMCs were cultured in media, recall antigens (HPV-16 L1 VLP, 10 μg/mL or influenza virus/Flu, 1:100), or a mitogen (PHA, 1:100) and incubated for 3 days (media, PHA) or 5 days (media, VLP, Flu). T-cell proliferation was assayed by [^{3}H]thymidine incorporation. Median net cpm calculated by subtracting cpm from media cultures. Median cpm for media (46-64 years): 3 days, 680 cpm (−/−) and 435 cpm (+/+); 5 days, 776 cpm (−/−) and 543 cpm (+/+). Median cpm for media (65-74 years): 3 days, 692 cpm (−/−) and 423 cpm (+/+); 5 days, 918 cpm (−/−) and 309 cpm (+/+). *P*s shown were determined using a Kruskal-Wallis test.

that the decrease in immune response in persistently infected women is a generalized effect that includes HPV-specific responses. Surprisingly, we did not find evidence that the presence of multiple HPV infections would lead to weaker proliferative responses than for subjects with single infections.

Although few studies have evaluated whether reduced responsiveness to PHA is associated with HPV infection or associated disease, our findings are in agreement with a previous study where similar patterns of lymphoproliferative and cytokine responses to HPV16 antigens and PHA for patients with CIN III or cervical cancer were reported (7) and also with a lower interleukin 2 response to HPV antigens in individuals with more advanced cervical dysplasia (9).

The mean magnitude of immune responsiveness to PHA observed in our study was well within what could be considered the reference range, and there was no evidence of severe functional immunologic impairment among the majority of our study participants. Despite this fact, clear associations were still discernible between modest down-regulation of immune response levels and persistent infection.

The association we observed between reduced proliferative responsiveness and HPV persistence was strongest in analysis that focused on individuals with HPV persistence over a 9- to 10-year period (median of 108 months). Although the possibility does exist that women in our study with evidence of long-term HPV positivity have repeated viral acquisition over time rather than true viral persistence, our findings suggest that weaker cellular immune function may be strongly associated with length of HPV persistence.

Stratification of our data by age indicated that the primary effect was evident among women 65 years of age and older, an age group where immune senescence has been previously reported (28–40). Surprisingly, however, we did not observe evidence of age-related immune decline in our HPV-negative control group. Given that HPV is a very common exposure among sexually active individuals, one possible explanation for our finding is that as women age, those with a weaker immune system are more likely to become persistently infected and are therefore underrepresented in our HPV-negative control group. This possibility is supported in our study by the observation of an age-related immune impairment among women with evidence of persistent HPV infection. However, further studies are necessary to formally address this possibility.

Although defining the specific immune mechanisms that explain the associations we observed is beyond the scope of our study, the fact that associations persisted after adjustment for lymphocyte subsets (CD3, CD4, CD8, CD45, CD19, CD16/CD56, and CD14) measured by flow cytometric analysis indicate that our results are not explained by a loss of proliferating lymphocytes in PBMCs. Further analysis will be necessary to determine whether alterations in other immune cell subpopulations could be associated with decreased immune responsiveness and thus with viral persistence. In particular, the levels of naive or memory T cells as well as markers of T-cell activation and regulatory T cells could prove of interest. These analyses are currently in progress in our group.

Another interesting, albeit speculative finding was the observation that women who acquired an HPV infection during our study despite evidence that they were not sexually active during that period had reduced levels of immune responsiveness to PHA, Flu, and VLP compared with the sexually active group. The effects observed, whereas considerable in magnitude, were not statistically significant given the small number of women involved. It is possible that our observations were due to chance, or that differences observed are due to the age differential between women in our study who acquired HPV infection despite lack of sexual activity and those who acquired infection while sexually active. Nonetheless, one possible explanation for our observations is that HPV infections that occur in the absence of sexual activity could represent reactivations of existent, latent HPV infections that are allowed to resurface with the weakening of the ability to control the virus immunologically.

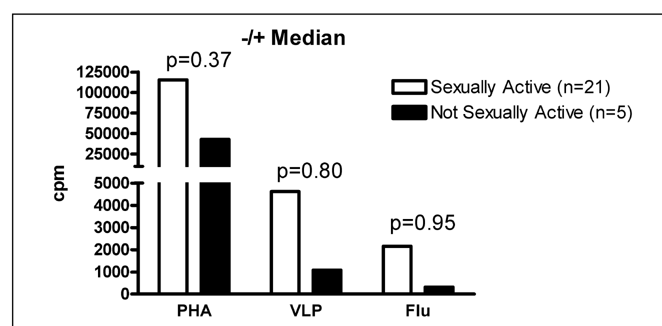

Figure 2. Median lymphoproliferative responses to PHA, Flu, and VLP in women who acquired an HPV infection, stratified by sexual activity during the study time period. PBMCs were cultured with media, recall antigens (HPV-16 L1 VLP, 10 μg/mL or influenza virus/Flu, 1:100), or a mitogen (PHA, 1:100) and incubated for 3 days (media, PHA) or 5 days (media, VLP, Flu). T-cell proliferation was assayed by [^{3}H]thymidine incorporation. Median net cpm calculated by subtracting cpm from media cultures. Median cpm for media (sexually active): 3 days, 596 cpm; 5 days, 767 cpm. Median cpm for media (not sexually active): 3 days, 775 cpm; 5 days, 723 cpm. *P*s shown were determined using a Kruskal-Wallis test.

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Our study is limited by the fact that measurement of immune responses was done at a single time point, at the end of follow-up. Because immune variables were evaluated after viral clearance or persistence, it is not possible to determine whether the observed association between reduced immune responsiveness and HPV persistence is causal or resultant from persistent viral infection. Future studies will be needed to establish whether decreases in immune responsiveness lead to persistent infection or whether HPV infection is capable of down-regulating host immune responses.

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