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Correlates of IL-10 and IL-12 Concentrations in Cervical Secretions

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Interindividual variations in host immune responses to HPV infection are thought to be important determinants of viral persistence and progression to cervical intraepithelial neoplasia and cancer. However, few studies have measured local immune markers at the site of infection (e.g., the cervical mucosa). We sought to determine biologic correlates of IL-10 and IL-12 concentrations in cervical secretions. Cervical secretions were passively collected using a WeckCel sponge from 247 women participating in a natural history study of human papillomavirus infection as part of an immunologic ancillary study. IL-10 and IL-12 concentrations were determined using standard ELISA assays. In general, IL-10 and IL-12 levels were significantly intercorrelated (Pearson's correlation coefficient = 0.6) but had somewhat different determinants. Significant increases ($P < 0.05$) in IL-10 concentrations were observed for nonovulatory phases of the menstrual cycle, postmenopausal status, recent use of oral contraceptives (OC), low secretion volume, macrolevels of heme contamination, and high vaginal pH. Increasing IL-10 levels were also observed among smokers, women with increasing numbers of lifetime sex partners, and women who report having less frequent sex (less than once per week), however, these results were not statistically significant. Significantly higher IL-12 concentrations were observed among recent OC users, women with low secretion volume, and women with a high vaginal pH. There was a non-statistically significant observation of increasing IL-12 levels among nonsmokers, women with increasing numbers of lifetime and recent pregnancies, and increasing levels of heme contamination. We failed to observe a significant association between HPV and IL-10 or IL-12 levels in this cross-sectional sample. Future analyses of cervical cytokine levels and HPV infection should control for the inherent variation of local cytokine levels due to hormonal influences, hemoglobin contamination, pH, and cervical secretion volume differences.

KEY WORDS: Mucosal; cervix, secretions.

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INTRODUCTION

Genital human papillomavirus (HPV) is a common, sexually transmitted infection. Most HPV is transient, clearing within 8 months to 2 years after infection (1). However, in a small fraction of women infected with HPV, the infection persists and progresses to more severe lesions known as high-grade squamous intraepithelial lesions (HSILs) and cervical cancer (2, 3).

The host immune response is believed to be an important determinant of whether HPV infections persist or progress. Evidence pointing toward an important role of the immune response in the natural history of cervical neoplasia includes (1) animal studies demonstrating efficacy of prophylactic and therapeutic papillomavirus vaccines (4–6), (2) increased HPV and high grade squamous intraepithelial lesion (HSIL) prevalence among immunosuppressed patients (7, 8), and (3) the repeated observation that specific human leukocyte antigen (HLA) alleles and haplotypes are associated with risk of cervical neoplasia (9–12). Most HPV infections likely involve a balance of Th1 (cellular)- and Th2 (humoral)-type immune responses. It has been proposed that a polarization away from a Th1-type cellular response to a predominately

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Th2-type humoral response may explain the persistence of HPV seen in a minority of infections (13).

Most of the studies designed to test the hypothesis of a switch from Th1 to Th2 predominance have focused on systemic markers of Th1 and Th2 responses. Little is know about responses that occur locally at the cervix, and it is unclear whether systemic measures correlate with local responses. One study that has directly examined matched systemic and local levels of cytokines reported a remarkable lack of correlation between these measures (14).

Our objective in the present pilot study of 247 women was to better understand local immune responses at the cervix for future studies investigating the correlation of local cytokine concentrations and HPV infection. Specifically, we were interested in examining possible biologic correlates of cytokine levels in cervical secretions that should be measured and considered in analyses of association between cervical infections and cervical cytokine levels.

We chose to measure cervical levels of two cytokines, IL-12 and IL-10. These two cytokines were selected for this initial study because IL-12 is believed to correlate primarily with cellular immune responses (Th-1 responses that induce cytotoxic T lymphocytes; CTLs) and IL-10 with humoral immune responses (Th-2 responses that induce antibody production). Furthermore, the measurement of these two cytokines using material obtained from Weck-cel sponges has previously been validated in our population (15, 16). Results from the validation study including a subset of 120 women showed that a low secretion volume, a younger age, a nonovulatory stage of the menstrual cycle, and high levels of hemoglobin contamination were all significant determinants of increases in cervical cytokine levels (16). In the present report we have expanded our study to include 247 women, in which additional potential correlates of cytokine levels are evaluated.

METHODS

Study Subjects. Subjects in the present study were selected from participants in the follow-up phase of a population-based cohort study of HPV and cervical neoplasia in Guanacaste, Costa Rica (17). A convenience sample of 247 women was selected for participation in a study of the immunology of HPV and cervical cancer. Selection criteria were designed to maximize the number of women with HPV positivity. Participants were enrolled in the cohort between 1993 and 1994, and in the present immunology substudy from October 1996 until June 1997, according to the following criteria: (a) women at high risk for HPV infection at the time of cervical secretion collection; i.e., women with cytology of low-grade squamous intraepithelial lesion (LSIL) and/or a positive HPV test at enrollment ($n = 56$), (b) women who initiated first sexual contact during the follow-up portion of the cohort study (self-reported virgins at enrollment; $n = 53$); and (c) cytologically normal women ($n = 138$).

Specimen Collection and Processing. An interviewer-administered questionnaire was given to collect information regarding demographics, and reproductive and sexual history information in the interim between the enrollment and the follow-up visit, where the samples for this study were collected. A pelvic exam was performed and the samples were collected in the following order: vaginal pH, Weck-Cel secretion for local cytokine measurement, Pap smear for cytologic screening, and cervical swab for HPV DNA testing by PCR. pH measurements were taken using a pHydrion strip and recorded in 0.5-unit increments as described previously (18). Cervical secretions were collected by passive absorption using a Weck-Cel sponge placed at the cervical os as described previously (15, 16). Specimens were kept in a refrigerated cooler and transferred to long-term storage at -70°C within a few hours of collection. Secretion specimens were processed as described previously (16). Extracted specimens were tested for hemoglobin content using Hemastix (Baxter Scientific) and classified as having no/trace hemoglobin, microheme, or macroheme contamination. Specimen volume was measured as the weight of the collected specimen minus the weight of the dry spear (where 1.0 mg specimen weight = 1.0 ml volume) and used to dilution-adjust the IL10 and IL12 concentrations obtained from the ELISA results.

IL-10 and IL-12 concentrations were determined using quantitative ELISA kits (BioSource International, Burlingame, CA). Extracted secretions were diluted 1:2 in PBS–1% fetal calf serum (PBS-FCS) and run in duplicate according to the manufacturer's instructions. The dynamic range of the ELISAs was 0.2 to 50 pg/ml for IL-10 and 0.8 to 100 pg/ml for IL-12. Specimens whose concentrations were above the highest standard were diluted further and reassayed to fall within linear range. Specimens with results falling below the detection limit of the assay were assigned concentrations equal to one-half of the lower detection limit of each assay. The correlation between duplicate specimens was previously reported, with Spearman rank correlations of 0.63 for IL-10 and 0.78 for IL-12 (16).

Cervical cells were collected for Pap smear cytology using a Cervex brush as described (17). Cells were smeared onto a slide for conventional cytology and the residual cells were placed into PreservCyt liquid cytology medium for ThinPrep cytology. Conventional smears were read

in Costa Rica by the local study cytopathologist and the ThinPrep slides were prepared and read by study cytopathologists in the United States. Based on our current understanding of the natural history of cervical neoplasia, Pap results of normal ($n = 179$), reactive changes ($n = 32$), and atypical squamous cells of undetermined significance (ASCUS) with a negative HPV DNA test ($n = 8$) were considered cytologically normal; ASCUS associated with a positive HPV DNA test ($n = 6$), koilocytotic atypia ($n = 2$), and cervical intraepithelial neoplasia I (CIN 1; $n = 13$) results were considered LSIL; and CIN 2 ($n = 5$) was considered HSIL (19, 20). For most analyses, the LSIL and HSIL categories were combined to create a single "abnormal cytology" category. The agreement between the Costa Rican and the Cytyc Pap results was 92.7 %, where Cytyc uniformly graded the 18 discordant results as more severe. We use the Cytyc Pap results in this analysis.

HPV testing of the specimen collected concurrently with the cervical secretion was performed using L1 consensus PCR (MY09/11 primers) and reverse-blot hybridization, which detects the presence of 27 high- and low-risk HPV genotypes, as described previously (21). Each sample was tested in two laboratories using the same method. Laboratory 1 successfully amplified 99.2% of the samples as determined by β-globin amplification, while laboratory 2 amplified 90.6% of the samples. Among those with sufficient β-globin results in both laboratories, 41 of 220 samples were HPV positive in each laboratory (98.2% agreement; $\kappa = 0.94$). A consensus HPV diagnosis was obtained by considering all samples sufficient for analysis by at least one laboratory. Samples positive for HPV in either or both laboratories were considered to be HPV positive for this analysis.

Statistical Analysis. Each cytokine concentration was normalized by accounting for the differences in volume of secretion collected and the PBS dilution required to perform the ELISA. Normalized cytokine concentrations were ln-transformed to achieve a normal distribution. Means were calculated from the ln-transformed values, and back-transformed geometric mean concentrations are presented. Simple linear regression models were fit to obtain variable-specific mean cytokine concentrations, and global differences in mean cytokine concentration among the categories of a given variable were tested for significance using an ANOVA model F test. Multiple linear regression models were fit to determine significant independent predictors of cervical cytokine concentrations from the variables measured in the study. Variables were selected for the final model based on univariate associations and observed correlations between variables. The most biologically relevant of highly correlated, significant IL-10 or IL-12 determinants were chosen for the final model. Adjusted mean concentrations were calculated by least squares means in SAS (Cary, NC). Tests for trend were computed by modeling categorical variables as continuous variables in multivariate linear regression. All statistical tests, with the exception of the least squares mean, were performed using STATA 6.0 (Stata Corp., College Station, TX).

RESULTS

The mean age of the participants was 37 years (median age = 34 years). Few women reported ever having smoked (4.5 %). Thirty-four women (13.9 %) were postmenopausal (self-reported cessation of menstrual periods not due to pregnancy, lactation, use of Depo-Provera, or unspecified menstrual disorders), and 77 (31.4 %) women reported having recently (within the past month) used oral contraceptives. Eighty percent (80%) of women were parous, with 48% reporting three or more lifetime pregnancies. However, only 9% were recently pregnant (i.e., since study enrollment). Fifty-six percent (56%) report lifetime monogamy, with only 30% reporting having a new sexual partner since study enrollment. Of women who were sexually active during follow-up, 71% reported having intercourse two or fewer times per week. Two hundred nineteen women (89.4%) had a normal Pap result at the time of cervical secretion collection, 21 women were diagnosed with LSIL (8.6%), and 5 women were diagnosed with HSIL (2.0%). The mean volume of secretion collected was 37.1 μl, with 60% of the secretions having detectable heme contamination. Most women (74.3%) had a normal vaginal pH of 4.5. Fifty-eight women reported having had a vaginal yeast infection during follow-up (23.7%). Most (77.6%) of the participants had evidence of vaginal discharge noted by the clinician on their pelvic exam.

The volume of secretion recorded for two participants (0.5 and 861 μl) was considered to be out of a reasonable range. Since the volume of secretion collected for these women could not be verified, they were excluded from the analysis, resulting in a total sample size of 245 women. Of the 245 samples, a total of 51 women (20.8%) had IL-10 results below the detectable limit (BDL) of the assay. After adjustment for sample volume and PBS dilution, the range of IL-10 concentration was below the detectable limit to 2095.2 pg/ml. A total of 179 samples was analyzed for IL-12 levels. The IL-12 measurements were incorporated into the study after initial attempts to measure IL-2 as the representative Th1 cytokine failed, because the IL-2 results from the first 66 samples analyzed were BDL of the assay (15). Sufficient volume from these 66 samples

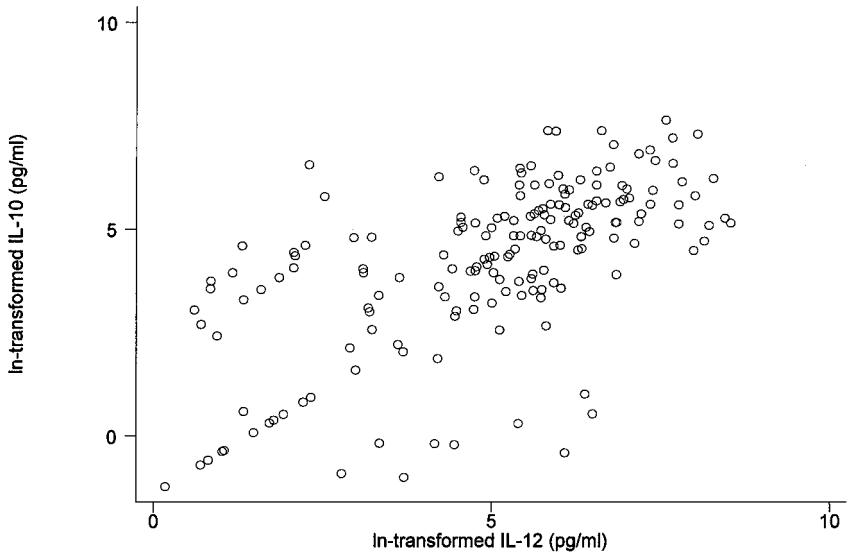

Fig. 1. Scatterplot of ln-transformed IL-10 and IL-12 concentrations in cervical secretions measured by ELISA from 179 women with both measurements.

was not available for further testing of IL-12, resulting in fewer observations for this cytokine. A total of 32 women (17.9%) had IL-12 levels below the detectable limit of the assay. The dilution-adjusted range of IL-12 concentration was below detectable limits to 5071.7 pg/ml. Generally, IL-12 concentrations were higher in cervical secretions than IL-10 levels (median concentrations, 225.3 and 105.6 pg/ml, respectively), but levels of IL-10 and IL-12 were significantly correlated (Fig. 1, Pearson correlation coefficient = 0.6).

We analyzed several covariates as potential determinants of IL-10 and IL-12 concentrations (Tables I and II). The results of both the crude and the adjusted models for IL-10 are presented in Table I. In univariate analysis, low secretion volume, increasing pH, macrolevels of hemoglobin concentration, use of oral contraceptives in the last month, postmenopausal status, increasing number of lifetime sexual partners, and less frequent intercourse were significantly predictive of higher IL-10 levels in cervical secretions ($P < 0.05$). IL-10 concentrations also varied significantly by stage of the menstrual cycle, with the lowest concentrations around ovulation (days 10–17). Women who reported ever smoking cigarettes and women with two or more new sex partners during follow-up showed a nonsignificant increase in IL-10 concentrations as well, however, the numbers in these categories were small. Of note, decreasing IL-10 concentrations with increasing age or number of lifetime pregnancies were significant ($P = 0.04$ and 0.05, respectively) among premenopausal women (data not shown). Other covariates were not affected by menopausal status. After adjustment for stage of the menstrual cycle, recent OC use, secretion volume, and pH, increasing numbers of lifetime sexual partners and less frequent sexual intercourse were no longer significantly associated with IL-10 concentrations. The univariate association of IL-10 concentrations with five or more lifetime sex partners is partially explained by menopausal status and low secretion volume. Although the ANOVA F test was not significant for frequency of sex or lifetime number of sex partners after adjustment, there was a marginally significant trend to lower IL-10 levels with both ($P = 0.058$ and $P = 0.084$, respectively). In this cross-sectional study, we observed an increase in IL-10 among HPV positive women after adjustment, but this effect did not reach statistical significance.

In univariate analysis for predictors of IL-12 (Table II), decreasing secretion volume, increasing pH, macrolevels of hemoglobin concentration, and increasing numbers of lifetime sex partners were significantly predictive of high IL-12 concentration. IL-12 levels also varied by stage of menstrual cycle and frequency of intercourse and were higher among recent OC users (same trend as with IL-10), however, these associations were not statistically significant ($P = 0.08$, 0.06, and 0.10, respectively). The effect of increased IL-12 levels among women who had used oral contraceptives in the past month was statistically significant ($P = 0.01$) among premenopausal women (data not shown). No other associations varied by menopausal status. Of the covariates with a crude IL-12 association, only secretion volume and pH remained significantly associated after adjustment for stage of the menstrual cycle

Table I. Determinants of IL-10 Levels in Cervical Secretions

Category	*N*	Mean [IL-10] (pg/ml)	*P* value	Adjusted[a] [IL-10] (pg/ml)	*P* value
Age (years)			0.07		0.66
20–29	93	66.3		105.0 (54.5, 202.1)	
30–39	71	31.3		78.0 (40.2, 151.6)	
40+	81	63.5		90.2 (43.5, 187.1)	
Reason for referral to immunology study			0.37		0.57
Random	137	45.8		82.9 (46.1, 149.2)	
Prior LSIL result	55	76.3		115.9 (55.5, 242.3)	
Initiated sexual intercourse during study Follow-up	53	51.0		95.0 (46.5, 194.0)	
Ever smoked cigarettes?			0.12		0.22
No	234	50.0		87.3 (50.6, 150.7)	
Yes	11	149.2		187.6 (52.8, 666.3)	
Day of menstrual cycle			<0.01		<0.01
4–9[b]	53	100.2		108.9 (54.4, 217.9)	
10–17	62	18.2		31.5 (15.5, 64.2)	
18–27	57	38.6		54.1 (26.5, 110.3)	
28+	39	60.4		124.0 (56.1, 273.9)	
Postmenopausal	34	190.5		268.2 (111.5, 645.3)	
OC use within past month			<0.01		<0.01
No	168	37.0		45.7 (26.6, 78.6)	
Yes	77	113.3		180.3 (90.9, 357.7)	
Number of lifetime pregnancies			0.07		0.35
0	50	57.2		95.6 (45.4, 200.9)	
1–2	77	62.3		111.5 (57.4, 216.6)	
3–5	75	31.0		63.2 (32.1, 124.6)	
6+	43	88.5		94.7 (41.7, 214.9)	
Recent pregnancies (number since enrollment)			0.91		0.55
0	224	52.8		88.3 (50.9, 153.2)	
1+	21	49.9		118.3 (42.9, 326.4)	
Lifetime number of male sex partners			0.02		0.21
1	135	36.3		77.1 (43.4, 136.9)	
2–4	75	77.8		115.1 (58.3, 227.1)	
5+	33	98.5		136.3 (58.5, 317.3)	
Number of new sexual partners[c]			0.21		0.64
0	169	47.4		83.0 (46.5, 148.1)	
1	62	57.1		109.7 (54.1, 222.7)	
2+	12	153.3		105.5 (31.5, 353.1)	
Frequency of intercourse (per week)			0.03		0.15
<1	53	95.5		154.3 (74.1, 321.3)	
1–2	102	54.8		97.1 (47.7, 197.6)	
3+	62	32.6		76.4 (36.9, 158.6)	
Cytologic result when secretions collected			0.13		0.34
Normal	219	58.1		92.8 (53.9, 159.9)	
LSIL	21	22.4		63.4 (23.0, 174.9)	
HSIL	5	23.6		30.3 (4.9, 186.2)	
Volume of secretion			<0.01		0.01
<16 μl	61	138.7		146.8 (73.3, 294.0)	
16–30 μl	62	86.9		141.8 (69.9, 287.8)	
31–50 μl	58	29.4		57.6 (29.6, 112.1)	
>50 μl	64	21.7		56.7 (27.1, 118.4)	
Hemoglobin contamination			<0.01		0.04
None	97	39.0		68.0 (36.1, 128.0)	
Microcontamination	82	37.1		76.9 (40.4, 146.6)	
Macrocontamination	66	125.6		150.8 (77.6, 292.7)	
pH			<0.01		<0.01
4.0	10	8.5		18.2 (5.3, 62.4)	
4.5	182	43.1		69.9 (50.8, 96.4)	
5.0	47	135.4		137.5 (75.1, 251.9)	
5.5	6	290.2		388.1 (79.9, 1886.2)	
Self-report of recent yeast infection			0.54		0.94
No	187	50.0		89.4 (45.5, 175.7)	
Yes	58	61.7		91.5 (51.3, 163.1)	
Evidence of vaginal discharge on pelvic exam			0.79		0.36
No	55	48.9		70.2 (32.4, 151.9)	
Yes	190	53.7		93.5 (54.1, 161.5)	
HPV status			0.75		0.19
Negative	194	51.3		84.9 (48.9–147.4)	
Positive	51	57.6		127.9 (60.7–269.3)	

[a] Adjusted for stage of menstrual cycle, recent OC use, secretion volume, and pH.
[b] Women were asked not to schedule a pelvic exam during menstruation, precluding evaluation of days 1–3 of the menstrual cycle.
[c] Number of new partners since enrollment.

Table II. Determinants of IL-12 Levels in Cervical Secretions

Category	*N*	Mean [IL-10] (pg/ml)	*P* value	Adjusted[a] [IL-10] (pg/ml)	*P* value
Age (years)			0.52		0.92
20–29	72	141.5		165.6 (83.6, 328.2)	
30–39	47	118.6		178.7 (87.3, 365.7)	
40+	60	183.2		150.2 (74.6, 302.6)	
Reason for referral to immunology study			0.59		0.76
Random	96	151.9		169.0 (92.5, 308.7)	
Prior LSIL result	44	175.2		186.7 (88.6, 393.2)	
Initiated sexual intercourse during study follow-up	39	112.5		138.6 (66.3, 289.8)	
Ever Smoked Cigarettes?			0.81		0.21
No	171	148.5		173.5 (99.9, 301.3)	
Yes	8	125.2		76.3 (20.5, 283.8)	
Day of menstrual cycle			0.08		0.46
4–9[b]	41	167.1		170.9 (84.2, 346.8)	
10–17	40	94.6		155.5 (71.3, 338.9)	
18–27	39	103.4		101.7 (47.4, 218.0)	
28+	32	165.1		205.2 (93.2, 451.8)	
Postmenopausal	27	341.4		208.3 (90.0, 482.4)	
OC use within past month			0.10		0.04
No	120	123.9		138.2 (78.8, 242.4)	
Yes	59	209.6		266.3 (130.7, 542.7)	
Number of lifetime pregnancies			0.16		0.13
0	37	79.4		90.4 (42.0, 194.3)	
1–2	61	150.9		174.2 (87.5, 347.1)	
3–5	51	176.8		226.3 (110.1, 464.9)	
6+	30	220.5		186.4 (81.8, 425.1)	
Recent pregnancies (number since enrollment)			0.22		0.13
0	163	139.0		149.5 (85.9, 260.2)	
1+	16	265.7		320.0 (114.2, 896.2)	
Lifetime number of male sex partners			0.02		0.32
1	97	103.6		138.2 (76.8, 248.7)	
2–4	56	222.0		216.6 (109.1, 430.1)	
5+	25	272.4		198.2 (84.4, 465.6)	
Number of new sexual partners[c]			0.59		0.49
0	122	164.1		182.4 (102.5, 324.8)	
1	46	116.7		148.3 (71.8, 306.1)	
2+	10	175.4		93.9 (28.1, 313.8)	
Frequency of intercourse (per week)			0.06		0.43
<1	37	287.8		198.1 (95.5, 411.0)	
1 or 2	84	142.5		128.7 (62.0, 267.1)	
3+	42	106.7		123.4 (56.7, 268.5)	
Cytologic result when secretions collected			0.61		0.81
Normal	161	154.5		166.7 (96.1, 289.3)	
LSIL	14	89.0		121.6 (41.7, 355.0)	
HSIL	4	127.6		132.8 (19.6, 899.1)	
Volume of secretion			<0.01		<0.01
<16 μl	48	290.1		276.3 (135.6, 562.9)	
16–30 μl	46	274.4		331.5 (157.8, 696.5)	
31–50 μl	39	108.1		126.3 (62.0, 257.1)	
>50 μl	46	50.7		61.2 (29.7, 126.3)	
Hemoglobin contamination			0.01		0.11
None	67	90.7		115.9 (59.8, 224.6)	
Microcontamination	60	150.5		163.3 (85.3, 312.7)	
Macrocontamination	52	268.6		248.9 (124.1, 499.3)	
pH			<0.01		0.01
4.0	7	14.7		18.7 (4.9, 71.1)	
4.5	133	124.5		134.5 (98.1, 184.4)	
5.0	34	341.2		250.5 (133.8, 469.1)	
5.5	5	1091.6		1124.0 (231.6, 5455.3)	
Self-report of recent yeast infection			0.20		0.67
No	136	132.3		179.2 (89.1, 360.4)	
Yes	43	206.8		156.0 (87.1, 279.6)	
Evidence of vaginal discharge on pelvic exam			0.23		0.35
No	41	204.2		216.2 (96.8, 482.6)	
Yes	138	133.7		157.8 (91.2, 273.3)	
HPV status			0.77		0.70
Negative	142	150.6		167.6 (95.5–294.3)	
Positive	37	135.3		147.0 (68.8–313.9)	

[a] Adjusted for stage of menstrual cycle, secretion volume, and pH.
[b] Women were asked not to schedule a pelvic exam during menstruation, precluding evaluation of days 1–3 of the menstrual cycle.
[c] Number of new partners since enrollment.

and pH or stage of the menstrual cycle and secretion volume, respectively. Even though the association for macrolevels of hemoglobin was not significant after adjustment, a significant trend of increasing IL-12 with increasing heme contamination remained ($P_{trend} = 0.04$). After adjustment for secretion volume, stage of the menstrual cycle, and pH, the association of recent use of oral contraceptives strengthened ($P = 0.04$), a result largely explained by the stage of the menstrual cycle. Furthermore, ever smokers showed a modest decrease in IL-12 concentrations ($P = 0.21$), as did women with fewer lifetime pregnancies ($P_{trend} = 0.06$) and no recent pregnancies ($P = 0.13$). There was no difference in IL-12 levels by HPV status in crude or adjusted models. Further adjustment did not significantly change the results from the final model presented in Tables I and II.

DISCUSSION

In our previous report from this population, we examined the reproducibility of the Weck-cel collection instrument and the correlates of IL-10 and IL-12 in cervical secretions. From this analysis of duplicate samples from 120 participants, we concluded that the Weck-cel was a reliable method of secretion collection and found that increasing age, postmenopausal status, macrolevels of hemoglobin concentration, nonovulatory stages of the menstrual cycle, and low secretion volume were positively associated with increased levels of cervical IL-10 and IL-12 (16). In the present report, we extended the sample size to a total of 245 participants and examined additional covariates as potential correlates of cervical IL concentrations, including recent use of oral contraceptives, smoking status, lifetime pregnancies, recent pregnancy, lifetime number of sexual partners, recent sexual partners, frequency of sexual intercourse, cytology, concomitant yeast infection or vaginal discharge, and vaginal pH. We selected as participants in our immunology substudy women likely to be most informative for adequate analysis of local immune response to HPV infection (i.e., ensured a higher than average proportion of HPV-positive women), which may bias the results of determinants of IL-10 and IL-12 levels at the cervix. However, no association was observed between the study selection group and IL-10 or IL-12 levels, suggesting that our selection criteria did not bias our findings. Furthermore, when we restricted the analysis to the cytologically normal random sample of women, the results were essentially the same (data not shown). The only exception was the positive association of recent OC use and IL-12 concentrations, which was much stronger among the women who initiated intercourse during the study. Timing of OC use may therefore be an important factor in this observation.

Approximately 20% of the cervical samples demonstrated undetectable levels of either IL-10 or IL-12. The strongest determinant of both IL-10 and IL-12 concentrations was secretion volume, as was demonstrated in an earlier report from our group. Several of the other associations were weakened by adjustment for secretion volume, suggesting that their effect was due in part to an effect on levels of secretion (the denominator of the cytokine concentration value) rather than on the actual production of cytokines (the numerator of the concentration value).

Increasing vaginal pH was positively associated with IL-10 and IL-12 concentrations. Vaginal pH is known to increase with chronic infections, such as with bacterial vaginosis, where a pH>5.0 is a diagnostic criteria (22). Chronic infections are likely to increase local lymphocyte infiltration that could satisfactorily explain the observed increase in IL-10 and IL-12 levels with increasing pH.

Macrolevels of hemoglobin contamination in the secretions were also significant predictors of both high IL-10 and high IL-12 concentrations, as seen previously (16). This may be reflective of increasing contribution of serum cytokine levels in the heme-contaminated secretions. However, recent data from our group (14) show that the correlation between serum and cervical cytokine levels is poor, with much higher local than systemic concentrations, which argues against serum cytokine contamination explaining higher local levels in heme-contaminated samples.

There was some evidence to suggest a possible role for hormones in the regulation of local IL-10 and IL-12 that is independent of secretion volume. Levels of IL-10 and IL-12 were lowest around days 10–17 (periovulation) and among postmenopausal women. Additionally, we found that women who reported recent use of oral contraceptives had a higher mean IL-10 and IL-12 concentration than women who did not report recent OC use. While the menstrual cycle association was not statistically significant for IL-12 levels, the direction of the association was similar for IL-10 and IL-12, and the smaller sample size for IL-12 may explain the discrepancy in the level of statistical significance. These data are consistent with animal studies that showed a down-regulation of immune markers at the cervix in response to changes in female reproductive hormones throughout the menstrual cycle (23).

Finally, women who reported ever having smoked cigarettes (4.5%) had higher mean IL-10 but lower mean IL-12 levels at the cervix. Because of the few smokers in this population, neither of these observations was statistically significant. However, the magnitude and direction of the differences of the representative Th1 and Th2

cytokines observed are intriguing and should be investigated in a larger study including more women with smoke exposure.

We did not observe an association between local cytokine concentration and prevalent HPV infection. A recent study of high-risk female adolescents found increased levels of IL-10 among women coinfected with HIV and HPV (24). In contrast to our results, however, IL-12 levels were elevated in HPV-positive women who were either coinfected with HIV or another sexually transmitted infection (STI; includes laboratory-diagnosed *T. vaginalis*, *C. trachomatis*, or *N. gonorrhoeae*). We are unable to reproduce these findings since we did not directly measure other sexually transmitted infections. However, it is interesting to note that a high pH (indicative of *T. vaginalis* infection) was associated with increasing levels of both IL-10 and IL-12, consistent with the prior report.

Clearly, the elucidation of important local immune markers in the natural history of HPV and of other sexually transmitted infections will involve more than the two cytokines measured in this study. For example, the cytokines studied in this analysis are primarily produced from activated immune cells such as macrophages (IL-10 and IL-12), dendritic cells (IL-12), and Th2 CD4+ cells (IL-10) and are known to participate in cross-regulatory functions that define a tightly controlled immune response. Therefore, the investigation of just one or a few cytokines may not best reflect the immune environment acting in the early, innate response to viral infection that may play a major role in driving the course of the adaptive response. Recent data suggest the possibility that cytokines/chemokines such as IL-4, IL-2, IL-5, GM-CSF, MIP1α, and MIP1β may play an important role in maintaining the Th1/Th2 balance at the site of infection (25, 26), which may be quite informative in understanding an individual's general local immune environment. However, our goal was both to assess the feasibility of measuring local immune markers from cervical secretions and to assess more general predictors of cytokine concentration so that we can appropriately adjust for confounders when assessing HPV-specific changes. Future studies will incorporate additional immune markers and will include repeated measures of HPV infection to assess the effect of the local immune environment not only on the detection of prevalent infection, but on the course of infection (e.g., normal clearance vs persistence). In these studies it is clear that factors such as day of the menstrual cycle, vaginal pH, exogenous hormone use, and hemoglobin contamination should be considered when analyzing data and making biologic interpretations. Especially important to consider are the correlation of secretion volume with total cytokine concentration and the assessment of whether determinants affecting total concentration act biologically through increases in protein expression and/or secretion or more indirectly through changes in secretion volume.

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