

Cervical Concentrations of Interleukin-10 and Interleukin-12 Do Not Correlate with Plasma Levels

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Human papillomavirus (HPV) infects the transformation zone of the cervix and is the primary cause of cervical cancer. The infection is localized to the cervix and mucosal immunity is likely to be an important determinant for viral clearance. Previous studies of immunity to HPV have measured immune markers in the blood, but the relationship of systemic immunity to cervical immunity is poorly understood. In this study of 70 women enrolled in the ASCUS-LSIL Triage Study (ALTS), a clinical trial for management of low-grade cytologic abnormalities of the cervix, we collected paired plasma and cervical secretions to investigate the relationship between cervical concentrations of interleukin-10 (IL-10) and interleukin-12 (IL-12) and plasma levels. Neither IL-10 ($\rho = 0.11$), or IL-12 ($\rho = -0.04$) nor the ratio of IL-12 to IL-10 ($\rho = 0.06$) were correlated between blood and cervical secretions. Except for weak correlations of IL-10 among nonsmokers ($\rho = 0.35$, $P = 0.019$) and those in day 18–27 of their menstrual cycle ($\rho = 0.51$, $P = 0.015$), this lack of correlation persisted in all subgroups defined by genital inflammation or infection, current oral contraceptive use, heme contamination and volume of collected secretions, HPV16 seropositivity, and repeat HPV infection and/or cytologic abnormalities. The lack of correlation and high concentrations in cervical secretions indicate that the cervical IL-10 and IL-12 concentrations exceed what could

be expected from blood as a principle source of IL-10 and IL-12 and suggest that cytokine concentrations in cervical secretions are predominantly the result of local cytokine production.

KEY WORDS: IL-10; IL-12; cervix, plasma, HPV.

INTRODUCTION

A prevailing canon of immunology is that local, mucosal surfaces (e.g., genital tract, gastrointestinal tract, and lung) comprise an immune network (the common mucosal immune system) that is independent of the systemic immune system (1). This view has emerged from the two observations. First, the primary immunoglobulin isotype in mucosal secretions is secretory IgA (sIgA), while the predominant immunoglobulin isotype in sera is IgG. Second, lymphocytes stimulated at one mucosal site disperse and migrate to other mucosal sites, yet systemic responses are rarely detected after mucosal stimulation. The two systems are not entirely divorced, since plasma proteins, including immunoglobulins, bathe mucosal surfaces as the result of transudation across the epithelial barrier. Indeed, cervicovaginal secretions contain more IgG than sIgA (1). However, the exact nature of the relationship between immunity at the cervix and systemic immunity is poorly understood.

We are keenly interested in the interplay of cervical immunity and systemic immunity in response to infection by human papillomaviruses (HPV), the sexually transmitted causal agent of cervical cancer (2, 3). Host immune responses to HPV appear to be an important determinant of whether an HPV infection persists and progresses to high-grade cervical lesions, the precursor to cervical cancer, or is cleared from cervical tissue. Immunosuppressed human immunodeficiency virus (HIV) patients and organ transplantation recipients are at an increased risk of persistent HPV infection and high-

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grade lesions but not of invasive cervical cancer beyond the increased risk of cervical cancer precursors (4), suggesting that immunity to HPV is critical to the early natural history of HPV infection.

It is of note that epidemiologic studies have measured immune markers in blood (e.g., anti-HPV antibodies in serum) but not in local secretions of the cervix, where HPV infection occurs. It seems likely that local immunity will be a stronger determinant of whether HPV infections progress or regress, but there are few data to support this view. From an epidemiological perspective, it is also important to understand whether measuring immune markers in blood is predictive of local immunity.

To investigate the relationship of systemic immunity to cervical immunity, interleukin-10 (IL-10) and IL-12 concentrations were determined in paired plasma and cervical secretions collected from 70 women at their first 6-month follow-up visit after enrollment in the ASCUS-LSIL Triage Study (ALTS) (5, 6). ALTS is a multicenter clinical trial designed to compare three different methods of managing mild cervical cytologic abnormalities. These patients represented an ideal cohort because of the extensive screening and availability of clinical specimens, HPV infection status, and cytology analysis. The women enrolled were cytologically abnormal and local immunological determinants have the potential to influence the progression or regression of their lesions. We chose cytokines IL-10 and IL-12 as the immune markers for this study because of their association with Th1 and Th2 responses, respectively; increases in the IL12:IL10 ratio are associated with Th1 responses and increases in IL-10 are associated with the induction of Th2 responses (7). Furthermore, we have validated the collection and measurement of these cytokines in cervical secretions (8).

MATERIALS AND METHODS

Study Population

ALTS (ASCUS-LSIL Triage Study) is a multicenter, randomized clinical trial designed to evaluate three different management strategies (HPV triage, conservative management, and immediate colposcopy) for low-grade squamous intraepithelial lesions (LSIL) and atypical squamous cells of unknown significance (ASCUS) of the cervix (6, 7). Four centers (Pittsburgh, PA; Birmingham, AL; Oklahoma City, OK; and Seattle, WA) were chosen to create a diverse cohort of women with respect to ethnicity, geographic locale, and proportion of

insured clients. Details of the ALTS trial including eligibility criteria and participation rates have been previously described (6, 7).

Subject Selection

Within the ALTS Conservative Management arm, a natural history substudy aimed at evaluating immunological factors associated with disease progression, persistence and regression [ALTS Immunology Study (AIS)] is under investigation. A subset of 216 women enrolled in AIS from three of four sites (Seattle, WA, did not participate in the longitudinal component of AIS) and was followed longitudinally. From this study population, 70 paired plasma-cervical secretion specimens collected from subjects at the first follow-up visit were included in the current analysis. Participants in this study were selected based on HPV type 16 (HPV16) serology findings. Paired specimens were selected from women whose plasma specimens were known to be positive ($n = 32$) and enzyme-linked immunostaining assay (ELISA), negative ($n = 38$) by enzyme-linked immunostaining assay (ELISA) for IgG antibodies specific for HPV16 VLP (9).

From the pelvic examination at the first follow-up visit, a pelvic examination form was generated for each participant and included information on presence of cervical inflammation and concomitant genital infection. A questionnaire was also completed and included information on age, menstrual status and days since last menstrual cycle, oral contraceptive (OC) use within the last 2 years, and smoking status (current, former, never).

Cytology

Women with an ASCUS or a LSIL Papanicolaou smear were referred by a community-based cytopathologist working for a laboratory serving one of the four ALTS clinics and enrolled into the ALTS trial at which time cervical cells in PreservCyt (Cytoc Corporation, Boxborough, MA) were collected for repeat cytologic assessment. Thin-layer cytology specimens were produced from PreservCyt-stored cervical specimens using a semiautomated processor (ThinPrep 2000; Cytoc Corporation). Slides from referral and enrollment were evaluated by clinical center cytologists, re-reviewed by a panel of expert pathologists, and assigned a final diagnosis. The remaining PreservCyt specimen was sent to the clinical centers' HPV laboratory for HPV testing.

HPV Testing

The Hybrid Capture 2 (HC2) (Digene Corporation, Gaithersburg, MD) assay was used to detect HPV DNA in cervical specimens stored in PreservCyt, a liquid-based storage medium, following the manufacturer's instruction. HC2 detects HPV DNA from lysed cervical specimens by hybridization to RNA probes specific for cancer-associated HPV in a 96-well sandwich ELISA format using chemiluminescence. HC 2 tests for any of the following cancer-associated HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68.

Specimen Collection, Processing, and Testing for Cytokines

Cervical secretions were collected by passive absorption using Weck-cel sponges (Xomed Surgical Products, Jacksonville, FL) placed at the cervical os as previously described (8). Weck-cel sponges, stored at -70°C , were later processed as previously described (8). Briefly, after inspection for visible blood and weight determination, sponges were extracted and tested for blood contamination as measured by heme levels (Hemastix; Baxter Scientific), which were categorized as no/little heme, microheme, and macroheme. A dilution factor was used to correct the concentrations of the analytes after extraction (8). At the same visit, paired blood samples were collected in a heparin-treated Vacutainer tube as a source of plasma, which was stored at -70°C .

Quantitative ELISA kits (BioSource International, Burlingame, CA) were used to measure IL-10 and IL-12 (p40 form) in cervical secretions and plasma. The analysis was performed per the manufacturers instructions. Assay sensitivities were 0.78–50 pg/ml and 0.80–100 pg/ml for IL-10 and IL-12, respectively, and did not depend on specimen type.

Statistical Analysis

Prior to data analysis, all cytokine concentrations were log transformed and geometric means were calculated. Specimens whose cytokine concentrations were below the lower detection limit (BDL) for the assay were assigned a value of half of the lower detection limit to permit log transformation. Because transformed values were still nonnormal, nonparametric methods were used for statistical analysis. Spearman correlation coefficients were calculated for the correlation of IL-10 and IL-12 concentrations and IL-12: IL-10 between plasma and cervical secretions for all women and for subgroups defined by OC use in the last 2 years, smoking status,

Table I. IL-10 and IL-12 Concentrations (pg/ml) and the Ratio of IL-12:IL-10.

Cytokine	n	Plasma mean; median; range	Cervical Secretions mean; median; range
IL-10 (pg/ml)	70	2.75; 2.68; BDL-98.4	95.4; 160; BDL-2060
IL-12 (pg/ml)	70	114; 120; 25.3-285	124; 208; BDL-2200
IL-12:IL-10	70	41.7; 34.6; 0.899-616	1.30; 1.19; 0.003-964

age, time in the menstrual cycle (≤ 9 , 10–17, 18–27, or 28+ days, including one women in menopause), hemoglobin contamination (no/little, micro, or macro), collection volume ($\leq 18 \mu\text{l}$, $>18 \mu\text{l}$), collection site, indications of genital infection or inflammation, HPV16 antibody status, and repeat HPV infection and/or LSIL, a common manifestation of an HPV infection (7).

RESULTS

The mean plasma IL-10 concentration was 2.75 pg/ml (median = 2.68 pg/ml; range = BDL–98.4 pg/ml) (Table I); 12 women had plasma IL-10 concentrations below the limits of detection. The mean IL-10 concentration in cervical secretions were 95.4 pg/ml (median = 160 pg/ml; range = BDL–2060 pg/ml); 8 women had cervical IL-10 concentrations below the limits of detection. Overall, plasma and cervical concentrations were not correlated ($\rho = 0.11$) (Table II). Nonsmokers ($\rho = 0.35$; $P = 0.019$) and those women who were tested 18–27 days after their last menstrual cycle ($\rho = 0.51$; $P = 0.015$) had weakly correlated plasma and cervical levels.

The mean plasma IL-12 concentration was 114 pg/ml (median = 120 pg/ml; range 25.3–285 pg/ml) (Table I). The mean cervical IL-12 concentration was 124 pg/ml (median = 208 pg/ml; range = BDL–2200 pg/ml); 9 women had cervical IL-12 concentrations below the limits of detection. Overall, plasma and cervical concentrations were not correlated ($\rho = -0.04$) (Table II) and in no subgroups were plasma and cervical IL-12 concentrations correlated.

The mean plasma IL-12:IL-10 was 41.7 (median = 34.6; range = 0.899–616) (Table I). The mean cervical IL-12:IL-10 was 1.30 (median = 1.19; range = 0.003–964). Overall, plasma and cervical ratios were not correlated ($\rho = 0.06$) (Table II) and in no subgroups were plasma and cervical IL-12:IL-10 correlated.

DISCUSSION

Overall, neither IL-10 and IL-12 concentrations, nor their ratio (IL-12:IL-10), were correlated between

Table II. Correlations of Plasma and Cervical Secretion IL-10 and IL-12 Concentrations, and the Ratio of IL-12 to IL-10 (IL-12:IL-10)

	<i>N</i>	%	IL-10 ρ^a	IL-12 ρ	IL-12:IL-10 ρ
Overall	70		0.11	-0.04	0.06
Collection volume					
$\leq 18 \mu\text{l}$	35	50.0	0.22	-0.11	0.16
$> 18 \mu\text{l}$	35	50.0	-0.05	0.11	-0.08
Blood in cervical secretions					
No heme	21	30.0	-0.04	-0.02	0.24
Microheme	28	40.0	0.24	0.09	0.01
Macroheme	21	30.0	0.13	0.07	-0.01
OC use in the last 2 years					
Yes	29	41.4	0.17	-0.01	0.03
No	41	58.6	0.08	-0.10	-0.07
Days since menstrual period					
≤ 9 days	15	21.4	-0.11	-0.21	0.30
10-17 days	14	20.0	-0.34	0.11	-0.05
18-27 days	22	31.4	0.51	-0.02	0.05
28+ days	19	27.1	-0.05	-0.10	0.13
Age					
≤ 21 years	24	34.2	0.36	-0.17	0.38
22-29 years	27	38.5	-0.01	-0.13	0.02
30+ years	19	27.1	-0.04	0.35	-0.04
Smoking					
Never	44	62.9	0.35	-0.05	0.19
Former	20	28.5	-0.36	-0.02	0.05
Current	6	8.5	0.28	-0.54	-0.54
Repeat HPV infection/SIL					
Yes	42	60.0	0.19	0.04	0.09
No	28	40.0	-0.25	-0.04	0.00
HPV 16 seropositive					
Yes	32	45.7	0.08	0.11	0.08
No	38	54.2	0.17	-0.18	0.17
Genital infection or inflammation					
Yes	10	14.3	-0.13	0.27	0.02
No	60	85.7	0.14	-0.06	0.09

^aSpearman correlation coefficient. Boldface indicates a significant Spearman correlation ($P < 0.05$).

plasma and cervical secretions. These measures of immunity in plasma and cervical secretions also were not correlated in those women who were HPV16 seropositive. These data suggest that, despite the presence of antibodies in blood suggesting a link between local and systemic immunity, measurements of systemic immunity may not completely represent local immune response to an HPV infection. Nor were the levels correlated in women with indications (DNA or SIL) of a current HPV infection, suggesting that HPV infection does not cause gross changes in the permeability of the epithelial barrier, such as those that can occur with inflammation, resulting in an influx of blood constituents.

Relatively few studies have evaluated correlations of cervical and serum cytokines directly. Two previous studies of fertile and infertile women found that tissue necrosis factor- α but not interferon- γ , IL-6, or IL-8 were correlated in sera and cervical secretions (10, 11).

Only IL-10 concentrations in nonsmokers and women tested 18-27 days after their last menstrual cycle had weakly correlated plasma and cervical levels. Aside from

chance alone, it is unclear why women who do not smoke or who are early in the luteal phase of the menstrual cycle would have correlated levels or the significance of such a finding. These results must be confirmed within a larger study population.

The mean cervical IL-10 concentration found in this study was twice the concentration of cervical IL-10 found in cervical secretions reported in a previous study in Costa Rican (8). It is not clear whether population differences or the collection method contributed to this difference. By contrast, the mean cervical IL-12 concentration in this study was similar to that of women in Costa Rica. Plasma IL-10 concentrations were similar to previously reported serum values (12, 13). The IL-12 level was within a wide range (14-150 pg/ml) of reported values for mean serum IL-12 concentrations in normal subjects (13, 14).

In summary, the absence of correlation of cervical IL-10 and IL-12 concentrations with plasma levels suggests that these cytokines are derived from local cellular secretion rather than serum transudation. It is of interest

that neither IL-10 nor IL-12 concentrations in blood and secretions were correlated even among women who had a cervical infection with HPV or other pathogens, which may impair the integrity of the epithelial barrier and allow for accumulation of serum proteins in cervical secretions. Furthermore, IL-10 and IL-12 concentrations were not correlated even among those women with macro heme contamination in the cervical secretions. These results emphasize the independence of mucosal immunity in the cervix and suggest that local production of cytokines is important for regulation of genital tract immunity.

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REFERENCES

- Mestecky J, Fultz PN: Mucosal immune system of the human genital tract. *J Infect Dis* 179:S470-474, 1999
- Bosch FX, Manos MM, Munoz N, Sherman M, Jansen AM, Peto J, Schiffman MH, Moreno V, Kurman R, Shah KV: Prevalence of human papillomavirus in cervical cancer: A worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. *J Natl Cancer Inst* 87:796-802, 1995
- Walboomers JM, Jacobs MV, Manos M M, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ, Munoz N: Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol* 189:12-19, 1999
- Frisch M, Biggar RJ, Goedert JJ: Human papillomavirus-associated cancers in patients with human immunodeficiency virus infection and acquired immunodeficiency syndrome. *J Natl Cancer Inst* 92:1500-1510, 2000
- The Atypical Squamous Cells of Undetermined Significance/Low-Grade Squamous Intraepithelial Lesions Triage Study (ALTS) Group: Human papillomavirus testing for triage of women with cytologic evidence of low-grade squamous intraepithelial lesions: Baseline data from a randomized trial. *J Natl Cancer Inst* 92:397-402, 2000
- Schiffman M, Adianza ME: ASCUS-LSIL Triage Study. Design, methods and characteristics of trial participants. *Acta Cytol* 44: 726-742, 2000
- Spellberg B, Edwards JE Jr: Type 1/type 2 immunity in infectious diseases. *Clin Infect Dis* 32:76-102, 2001
- Hildesheim A, McShane LM, Schiffman M, Bratti MC, Rodriguez AC, Herrero R, Morera LA, Cardenas F, Saxon L, Bowman FP, Crowley-Nowick PA: Cytokine and immunoglobulin concentrations in cervical secretions: reproducibility of the Weck-cel collection instrument and correlates of immune measures. *J Immunol Meth* 225:131-143, 1999.
- Strickler HD, Kirk GD, Figueroa JP, Ward E, Braithwaite AR, Escoffery C, Drummond J, Goebel B, McClimens R, Manns A: HPV 16 antibody prevalence in Jamaica and the United States reflects differences in cervical cancer rates. *Int J Cancer* 80:339-344, 1999
- Naz RK, Butler A, Witt BR, Barad D, Menge AC: Levels of interferon-gamma and tumor necrosis factor-alpha in sera and cervical mucus of fertile and infertile women: Implication in infertility. *J Reprod Immunol* 29:105-117, 1995.
- Naz RK, Butler A: Interleukins-6 and -8 levels in sera and cervical mucus of fertile, idiopathic infertile, and immunoinfertile women: Implication in infertility. *Am J Reprod Immunol* 35:534-540, 1996.
- Sarris AH, Kliche KO, Pethambaram P, Preti A, Tucker S, Jackow C, Messina O, Pugh W, Hagemester FB, McLaughlin P, Rodriguez MA, Romaguera J, Fritsche H, Witzig T, Duvic M, Andreeff M, Cabanillas F: Interleukin-10 levels are often elevated in serum of adults with Hodgkin's disease and are associated with inferior failure-free survival. *Ann Oncol* 10:433-440, 1999.
- Phillips TM, Krum JM: Recycling immunoaffinity chromatography for multiple analyte analysis in biological samples. *J Chromatogr B Biomed Sci Appl* 715:55-63, 1998
- Tamaru M, Matsuura B, Onji M. Increased levels of serum interleukin-12 in Graves' disease. *Eur J Endocrinol* 141:111-116, 1999