Determination of Cytokine Protein Levels in Cervical Mucus Samples from Young Women by a Multiplex Immunoassay Method and Assessment of Correlates

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Cytokines in cervical mucus are likely to play important roles in controlling pathogens. The cervical mucosal environment is complex, however, with many endogenous and exogenous factors that may affect cytokine levels. We used a multiplex, suspension-array-based immunoassay method to measure 10 proinflammatory (interleukin-1β [IL-1β], IL-6, and IL-8) and immunoregulatory (gamma interferon [IFN-γ], IL-2, IL-4, IL-5, IL-10, IL-12, and IL-13) cytokines in cervical mucus specimens collected via ophthalmic sponge from 72 healthy, nonpregnant women and correlate their levels with biologic and behavioral covariates in a cross-sectional design. Proinflammatory and immunoregulatory cytokines were readily detected, although proinflammatory cytokines were present at markedly higher levels than were immunoregulatory cytokines. Among the covariates examined, the most striking finding was the significant (P ≤ 0.05) association between depressed levels of the cytokines IFN-γ, IL-1β, IL-6, and IL-10 and cigarette smoking. Also, nonsignificant trends toward lower cytokine levels were found in the settings of incident and persistent human papillomavirus infection. The ready detection of proinflammatory cytokines may be reflective of the female genital tract as an anatomic site that is constantly exposed to immunogenic stimulation. Cigarette smoking appears to downregulate cytokine responses in the cervical mucosa, which may help explain the implicated role of tobacco use as a cofactor for cervical cancer development.

A complex interplay of systemic and local immune responses limits the anatomic extent and influences the natural history of sexually transmitted infections of the cervix. At the local level, the study of these responses often includes characterization of the local cytokine milieu. With the microbiologic complexity of the cervical environment, it is likely that numerous cytokines are locally expressed and essential for the proper control of pathogens. Castle et al. showed that cervical concentrations of cytokines do not correlate with plasma concentrations (3), emphasizing the need to examine the mucosal component of the host response using appropriate specimen types.

Clearly, the choice of specimen type is influenced by the immune compartment in question and is often limited by the amount of sample available. The cervix is unique in that it naturally produces cervical secretions by columnar cells, and these secretions appear to be rich in immunoregulatory proteins including cytokines. Various tools for collecting cervical secretions in an undiluted form have been tested; all result in small sample volumes, thus limiting the number of immune markers that can be tested. While enzyme-linked immunosorbent assays have been used to measure up to six markers in secretions, this method is not feasible to measure greater numbers of analytes in small-volume secretions (2). Recently introduced multiplex, suspension-array-based immunoassay methods, such as the Luminex xMAP system, provide a novel platform for the detection of multiple analytes in small volumes (18). Although this technology has been used to measure cytokines in vaginal washes in women with cancer (12) and cervical swabs in pregnant women (17), comparisons between studies can be challenging due to differing specimen collection and recovery methods.

Interpreting measures of cytokine expression is also challenging, as it is likely that factors other than pathogens influence their expression in cervical mucus. Obvious potential influences include the timing of the menstrual cycle, exogenous hormones, and semen. Another possible factor is cigarette smoking, as nicotine and its metabolites are secreted into cervical mucus (9). Consequently, in studies of cytokine responses to pathogens, it is critical to understand the associations between other covariates and cytokines. The purpose of this study, therefore, was to examine the biologic and behavioral correlates of the proinflammatory and immunoregulatory cytokine milieu, measured by a multiplex immunoassay, in cervical mucus samples from healthy (without Chlamydia trachomatis, Neisseria gonorrhoeae, Trichomonas vaginalis, or bacterial vaginosis), nonpregnant women. Samples were collected via ophthalmic sponge to avoid the dilutional effects of lavage specimens. The proinflammatory cytokines studied were interleukin-1β (IL-1β), IL-6, and IL-8. The immunoregulatory cytokines, which were chosen to encompass a range of innate and adaptive immunoregulatory responses, included gamma interferon (IFN-γ), IL-2, IL-4, IL-5, IL-10, IL-12, and IL-13.

MATERIALS AND METHODS

Study subjects. Subjects for this cross-sectional study were selected from an ongoing prospective study of the natural history of human papillomavirus (HPV)
in a cohort of adolescent and young adult women described previously (15). Informed consent was obtained according to the guidelines set forth and approved by the Committee for Human Research at the University of California, San Francisco. Between September 2000 and October 2003, women aged 13 to 22 years attending either a family-planning clinic or a university health center or friends were asked to participate in the prospective study. All females who were sexually active and who did not have a history of therapy for cervical intraepithelial neoplasia were eligible to participate. Women who were leaving the area within 3 years or who had a history of immunosuppression were excluded. Women were seen every 4 months for study visits that included an interview, Papanicolaou smear, colposcopy, cervical vaginal lavage for HPV testing, and vaginal samples for the determination of pH and microscopic examination for bacterial vaginitis, T. vaginalis, and Candida albicans. Tests for Ch. trachomatis and N. gonorrhoeae were performed annually or if the patient was symptomatic. HPV testing by PCR was performed on cervical washes as previously described (15). Cervical mucus samples for cytokine protein measurement were collected for all visits starting in October 2003.

**Specimen collection.** Oropharyngeal sponges have been used in a number of reports to collect cervical mucus specimens for the study of immune markers (3, 5, 6, 13, 16). Castle et al. previously showed that Merocel oropharyngeal sponges (Medtronic Xomed, Inc., Jacksonville, FL) yield superior recovery for most cytokines tested compared with Weck-Cel oropharyngeal sponges (Medtronic Xomed, Inc.) used in previous studies (2). Notably, they demonstrated good recoveries of IFN-γ and IL-4, which are poorly recovered from Weck-Cel sponges. This was presumed to be due to different sponge compositions (cellulose for Weck-Cel sponges versus hydrolyzed polyvinyl acetate for Merocel sponges). In preliminary experiments using tissue culture supernatants from anti-CD3-activated peripheral blood mononuclear cells, eluted and tested as described below, we confirmed the superiority of Merocel sponges over Weck-Cel sponges as well as Dacron swabs (Fisher Scientific, Pittsburgh, PA) (data not shown). Merocel oropharyngoloscopic sponges had >60% recovery for all cytokines evaluated except tumor necrosis factor, and these were chosen for use in collecting cervical mucus specimens from our cohort. Due to the poor recoveries of tumor necrosis factor, this cytokine was excluded from further study.

For measurement of cervical cytokine levels, cervical secretions were obtained using the Merocel sponges following speculum insertion and before all other cervical samples were collected. The sponge was placed in the cervical os, with care not to touch the vaginal wall and vulva, allowed to absorb secretions for 60 s, placed into a sterile cryovial, and placed immediately on ice following collection. The samples were stored at the clinic site at −20°C for a maximum of 1 week, at which time they were transported on ice to the laboratory and stored at −80°C.

**Cytokine extraction.** After removing the sponges from the sterile cryovials, the sponges were weighed and placed into Eppendorf tubes using forceps cleaned with ethanol after each transfer. Two hundred microliters of ice-cold extraction buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM EDTA, 25 mM EGTA, 0.1 mM sodium orthovanadate, 1 mM NaF, 0.1% Tween 20, and 10% glycerol) was added to each sample, and samples were incubated at room temperature at 4°C. The sponges and extraction buffer were transferred into microcentrifuge tubes with 0.2-μm cellulose acetate filters and centrifuged at 13,000 rpm for 10 min at 4°C. The eluates were tested for hemoglobin levels using Hemastix strips (Fisher Scientific) and then tested immediately for cytokine levels.

**Cytokine testing.** Cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 [p70/p70], IL-13, and IFN-γ) were measured using Protein Multiplex Immunoassay kits (BioSource International, Camarillo, CA) according to the manufacturer’s instructions. Briefly, 25 μl of bead solution was added to each well of a 96-well filter plate and then washed. Samples (50 μl per well) were added, with an equal volume of assay diluent/incubation buffer. The plate was incubated on an orbital shaker for 2 h at room temperature and then washed. A cocktail (100 μl per well) of biotinylated detector antibodies was added, followed by a 1-h incubation on the shaker. After washing, 100 μl of diluted streptavidin R-phycocyanin was added to each well, followed by incubation on the shaker for 30 min. Fluorescence data were acquired using a Luminex 100 instrument (Luminex Corporation, Austin, TX). Each sample was tested in duplicate, and the mean of the two wells was used for analysis. Cytokine concentrations, expressed in picograms per milliliter, were obtained by interpolation from a standard curve run on each plate using stepwise threefold dilutions of protein standards included with each kit. Five-parameter logistic curve fits were performed with MasterMix QT software (MiraBio, Inc., Alameda, CA). The sensitivities of the assays, as reported by the manufacturer, were 3 pg/ml (IL-5, IL-6, and IL-10), 5 pg/ml (IL-4, IL-10, and IFN-γ), 6 pg/ml (IL-2), 10 pg/ml (IL-13), and 15 pg/ml (IL-1β and IL-12).

Because the volume of sample collected could not be standardized, all values were corrected using a previously reported dilution factor ([x − 0.064 g] + 0.21 g)/x − 0.064 g, where x is the weight of sample with sample, 0.064 g is the average weight of 10 dry sponges (same lot as that used for clinical specimen collection), and 0.21 g is the weight of 200 μl of buffer added for elution) (2, 13). Within-run reproducibility (between duplicates) of the cytokine measurements was evaluated using 16 randomly chosen cervical samples eluted and tested as described above. Of the cytokines that were above detection limits, mean coefficient of variation values ranged between 6% and 11%, except for IL-1β (15%) and IL-2 (20%).

**Data analysis.** Because we were interested in examining healthy women, those who were found to be positive for cervical infections including Ch. trachomatis, N. gonorrhoeae, or T. vaginalis or who were pregnant were excluded from this analysis. Women with yeast present in KOH wet mounts were not excluded since these cases were considered to be vaginal infections, and many women were asymptomatic. We adopted two different approaches to model the effects of covariates on cytokines: (i) a Box-Cox regression model with power transformation, treating cytokines as continuous variables, and (ii) a multinomial logistic regression, treating cytokines as categorical variables ( quartiles, plus a fifth category for undetectable levels). Because of our relatively small sample size and the nonnormal distributions of cytokine levels, the more conservative logistic regression method, which is more robust to the influence of very high and low observations, is reported here. Comparing the results from the two approaches, the Box-Cox regression model captured all of the statistically significant variables presented in the logistic regression model.

For comparisons of cytokine levels by HPV status, women were categorized into three groups based on the subject’s HPV status at the study visit for which the cervical secretion sample was available for cytokine testing and the change from the prior visit, (i) persistent infection, defined as a positive result for the same HPV type at both the cytokine test visit and the study visit 4 months prior; (ii) incident infection, defined as an HPV-positive result at the cytokine test visit but an HPV-negative result at all visits prior to the cytokine test visit; and (iii) negative HPV controls, defined as HPV-negative results at the cytokine test visit and all prior visits including the study entry visit. Because the cytokine data were not normally distributed, the Wilcoxon rank sum test (with samples in which cytokine was undetectable included as the lowest rank) was used to compare different HPV groups.

**RESULTS**

**Study population and distribution of cytokines in samples.** Samples collected from 72 women were used in this cross-sectional study. Subject demographics and covariate frequencies are shown in Table 1. Distributions of the concentrations of proinflammatory and immunoregulatory cytokines are shown in Table 2. Except for the T-helper type 2 (Th2) cytokines IL-4 and IL-13, cytokines could be detected in 97 to 100% of samples. The most notable finding, however, was that the cytokines were markedly higher than levels of proinflammatory those of immunoregulatory cytokines.

**Covariates of cytokine levels in cervical mucus.** Covariates of cytokine levels in cervical mucus samples were examined in a multinomial logistic regression model (Table 3), controlling for the variables shown as well as for race and time since last menstrual period (LMP). Among the associations shown, yeast infection was significantly associated with elevated levels of IFN-γ, IL-5, and IL-12, and the presence of white blood cells (>5 white blood cells per high-power field) on wet mount examination was significantly associated with elevated levels of IL-8 and IL-13. In contrast, smoking one or more cigarettes within the preceding 24 h was significantly associated with lower levels of IL-1β, IL-6, IFN-γ, and IL-10, although no associations were found for the number of cigarettes (either as a continuous variable or categorical variable [0, 1 to 5, or >5]) smoked during that period. No significant (P ≤ 0.05) associations for age, total lifetime number of sexual partners, intercourse within the prior 24 h, history of anal intercourse, cur-
correlates of cytokine levels in cervical mucus

Continuous variables
- Age (yr)
  - Mean: 19.9
  - SD: 1.9
- Age at first intercourse (yr)
  - Mean: 16.0
  - SD: 1.8
- No. of days since last intercourse
  - Mean: 32.9
  - SD: 50.3
- Total lifetime no. of sexual partners
  - Mean: 6.4
  - SD: 8.4

Categorical variables [no. of patients (%)]
- Race
  - Asian: 18 (25)
  - Black: 5 (7)
  - Latina: 15 (21)
  - White: 29 (40)
  - Other: 5 (7)
- LMP
  - 0–8 days: 12 (17)
  - 9–17 days: 23 (32)
  - 18–30 days: 16 (22)
  - >30 days: 21 (29)
- HPV
  - −/+: 15 (21)
  - +/+: 18 (25)
  - −/−: 34 (47)
  - +/−: 5 (7)
- Yeast infection, current
  - Yes: 7 (10)
- Intercourse within last 24 h
  - Yes: 16 (22)
- >5 WBCs/HPF on wet-mount examination
  - Yes: 13 (18)
- Positive hemoglobin in sample
  - Yes: 46 (64)
- History of anal intercourse
  - Yes: 6 (8)
- OCP use, current
  - Yes: 47 (65)
- Medroxyprogesterone use
  - Yes: 5 (7)
- Cigarette smoking within last 24 h
  - Yes: 20 (28)

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>No. of samples in which cytokine was detected</th>
<th>Median cytokine level, pg/ml (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proinflammatory</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>70 (97)</td>
<td>187 (96, 481)</td>
</tr>
<tr>
<td>IL-6</td>
<td>72 (100)</td>
<td>2,348 (1,093, 4,107)</td>
</tr>
<tr>
<td>IL-8</td>
<td>71 (99)</td>
<td>13,338 (6,139, 26,936)</td>
</tr>
<tr>
<td>Immunoregulatory</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>70 (97)</td>
<td>4 (1, 7)</td>
</tr>
<tr>
<td>IL-2</td>
<td>72 (100)</td>
<td>23 (14, 35)</td>
</tr>
<tr>
<td>IL-4</td>
<td>57 (79)</td>
<td>11 (6, 17)</td>
</tr>
<tr>
<td>IL-5</td>
<td>71 (99)</td>
<td>5 (2, 7)</td>
</tr>
<tr>
<td>IL-10</td>
<td>72 (100)</td>
<td>37 (15, 70)</td>
</tr>
<tr>
<td>IL-12</td>
<td>72 (100)</td>
<td>99 (60, 180)</td>
</tr>
<tr>
<td>IL-13</td>
<td>28 (39)</td>
<td>30 (13, 85)</td>
</tr>
</tbody>
</table>

a Number of samples, out of 72 tested, in which cytokine was detected.

b Median (interquartile range [IQR]) of samples in which cytokine was detected.

do Hemoglobin in the eluates and cytokine levels; no significant associations were found, although examinations were scheduled at times when menses was not expected.

Cytokine levels in incident and persistent HPV infection.
We compared the levels of proinflammatory and immunoregulatory cytokines in women with incident or persistent HPV infection to those of a control group without HPV infection (Table 4). While none of the differences were significant (P ≤ 0.05), a clear trend of the association of depressed levels of several cytokines (IL-1β, IL-6, IL-2, and IL-13) in women with incident infection, compared to levels in women with no infection, emerged. A similar trend was observed for IL-1β and IL-13 in women with persistent infection compared to those with no infection.

DISCUSSION

rent oral contraceptive pill (OCP) or medroxyprogesterone use, or days since LMP were found. Since it might be expected that blood from the endometrium or peripheral blood from trauma would influence cytokine levels or inhibit their measurements, we examined the association between the presence of hemoglobin in the eluates and cytokine levels; no significant associations were found, although examinations were scheduled at times when menses was not expected.

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DISCUSSION

In the present study, we found that both proinflammatory and immunoregulatory cytokines were easily detected in cervical mucus using a multiplex immunoassay and that their levels correlated with several biologic and behavioral factors. The latter is not surprising since it is expected that the cervical mucosal immune complex will be influenced by environmental factors such as infectious agents and carcinogens. We also noted that several proinflammatory cytokines, IL-1β, IL-6, and IL-8, were present at high levels in cervical mucus, with median concentrations 2 to 3 orders of magnitude higher than those of the immunoregulatory cytokines. These findings could not be explained by the presence of endocervical infections such as C. trachomatis or N. gonorrhoeae since these were excluded from our analysis. Nguyen et al. previously reported a similar observation (12), with high levels of IL-1β, IL-6, and IL-8 but low or undetectable levels of other cytokines, by use of a multiplex immunoassay, in vaginal washes from patients with and those without cervical cancer. The ready detection of these proinflammatory cytokines in cervical secretions may be reflective of the female reproductive tract as an anatomic site that is constantly exposed to immunogenic stimulation.

Interestingly, most of the proinflammatory and immuno-
regulatory cytokines were not upregulated by any of the factors examined, suggesting a relative steady state. Among the proinflammatory cytokines, only IL-8 was increased in the presence of white blood cells, the traditional marker of inflammation, and none of the proinflammatory cytokines were associated with the clinical detection of yeast. In contrast, detection of yeast was associated with the elevation of three of the immunoregulatory cytokines, reflecting a combination of TH1 (IL-12) and T H2 (IL-5) responses.

The most striking finding was the association between depressed levels of cytokines (IFN-γ, IL-1β, IL-6, and IL-10) and cigarette smoking. Tobacco use has been implicated as a cofactor in cervical cancer development (1, 9), and we previously showed that smoking is also a risk factor for the development of low-grade squamous intraepithelial lesions of the cervix in adolescents infected with HPV (10). Interestingly, there was no clear pattern for proinflammatory, T H1, or T H2 cytokines, but rather, there was a more general downregulation of cytokine levels. There was also no association with the number of cigarettes smoked, suggesting that even small amounts of nicotine exposure (one cigarette) are sufficient for the effects observed. By a real-time PCR method, we previously found a depression of cytokine mRNA expression in

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**TABLE 3. Significant covariates of cytokine levels in cervical mucus**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Continuous variables</th>
<th>Categorical variables</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Age at first intercourse</td>
<td>Days since last intercourse</td>
</tr>
<tr>
<td>Proinflammatory</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td>1.52** (1.13, 2.04)</td>
<td></td>
</tr>
<tr>
<td>Immunoregulatory</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
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<tr>
<td>IL-2</td>
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<td>IL-10</td>
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<td>IL-12</td>
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<td>IL-13</td>
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</tbody>
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**TABLE 4. Cytokine levels in incident and persistent HPV infections compared with negative control subjects**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Negative control groupa (n = 34)</th>
<th>Incident HPV infection groupb (n = 15)</th>
<th>Persistent HPV infection groupc (n = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median cytokine level, pg/ml (IQR)d</td>
<td>No. of samples in which cytokine was undetectablee</td>
<td>Median cytokine level, pg/ml (IQR)d</td>
</tr>
<tr>
<td>Proinflammatory</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>263 (117, 609)</td>
<td>2</td>
<td>171 (102, 566)</td>
</tr>
<tr>
<td>IL-6</td>
<td>3,117 (1,111, 4,957)</td>
<td>0</td>
<td>1,699 (977, 2,315)</td>
</tr>
<tr>
<td>IL-8</td>
<td>13,338 (6,294, 24,165)</td>
<td>1</td>
<td>16,666 (5,783, 28,439)</td>
</tr>
<tr>
<td>Immunoregulatory</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5 (2, 9)</td>
<td>0</td>
<td>3 (2, 4)</td>
</tr>
<tr>
<td>IL-2</td>
<td>29 (15, 39)</td>
<td>0</td>
<td>19 (12, 35)</td>
</tr>
<tr>
<td>IL-4</td>
<td>11 (5, 26)</td>
<td>9</td>
<td>8 (5, 17)</td>
</tr>
<tr>
<td>IL-5</td>
<td>6 (2, 9)</td>
<td>0</td>
<td>4 (2, 7)</td>
</tr>
<tr>
<td>IL-10</td>
<td>43 (15, 81)</td>
<td>0</td>
<td>46 (29, 73)</td>
</tr>
<tr>
<td>IL-12</td>
<td>121 (71, 301)</td>
<td>0</td>
<td>95 (52, 123)</td>
</tr>
<tr>
<td>IL-13</td>
<td>44 (20, 91)</td>
<td>20</td>
<td>13 (5, 30)</td>
</tr>
</tbody>
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a HPV negative at the cytokine test visit and all prior study visits.
b HPV positive at the cytokine test visit but HPV negative at all study visits prior to the cytokine test visit.
c HPV positive (same type) at both the cytokine test visit and the study visit 4 months prior.
d Median (interquartile range [IQR]) cytokine levels (picograms per milliliter) of samples in which cytokine was detected.
e Number of samples in which cytokine was undetectable (included as lowest rank in Wilcoxon rank sum test).
exfoliated cervical cells associated with tobacco use (15); aside from IFN-γ, the exact cytokines were different, possibly reflecting compartmental differences in immune responses (secreted mucus versus exfoliated endocervical cells) as well as the more limited cytokine panel examined in that study. The current study adds further support for a model in which nicotine or its metabolites downregulate immune responses at the cervical mucosa, with consequences for the development of HPV-related cervical disease. Effects of smoking on cytokines in cervical secretions are far from simple, however. Simhan et al., who employed a multiplex immunoassay, recently showed significantly increased levels of IL-4, IL-10, and IL-13 in cervical secretions in gravid (first-trimester) smokers compared with gravid nonsmokers (17). The most likely explanation for the seemingly discrepant findings is a complex interplay between hormonal and other physiologic correlates of pregnancy and the effects of tobacco. None of the subjects in the present study, in contrast, were pregnant. An alternate possibility that cannot be ruled out, however, is methodologic differences between the studies. Simhan et al. used Dacron swabs and also used a different method for recovering cytokines from the collection device.

An increased number of days since last intercourse was associated with the decreased expression of IL-5, suggesting that cytokines from the collection device.

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