

Cytokine and immunoglobulin concentrations in cervical secretions: reproducibility of the Weck-cel collection instrument and correlates of immune measures

Allan Hildesheim^{a,*}, Lisa M. McShane^b, Mark Schiffman^a, M. Concepcion Bratti^c, Ana C. Rodriguez^{c,d}, Rolando Herrero^{d,e}, Lidia A. Morera^c, Fernando Cardenas^c, Linda Saxon^f, Frederick P. Bowman^g, Peggy A. Crowley-Nowick^g

^a *Interdisciplinary Studies Section, Environmental Epidemiology Branch, DCEG, National Cancer Institute, 6130 Executive Blvd, EPN 443, Bethesda, MD 20892-7374, USA*

^b *Biometric Research Branch, DCTD, National Cancer Institute, Bethesda, MD, USA*

^c *Social Security Administration, San Jose, Costa Rica*

^d *Ministry of Health, San Jose, Costa Rica*

^e *Unit of Field and Intervention Studies, International Agency for Research on Cancer, Lyon, France*

^f *Information Management Services, Rockville, MD, USA*

^g *Dept. of Obstetrics, Gynecology, and Reproductive Biology, Fearing Research Laboratory, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA*

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Abstract

Elucidation of local immune response at the cervix is important for understanding and evaluating STD vaccine approaches currently being proposed. However, no well-validated method exists for the collection of cervical secretions for evaluation of cervical immune response. The purpose of this study was to determine the reproducibility of the Weck-cel sponge used to collect cervical secretions for immunological assessment. Additionally, it was possible to examine correlates of immunity as part of our investigation. Two cervical secretion specimens were collected sequentially from each of 120 women using Weck-cel sponges. Cervical secretions were collected prior to Pap smear sampling to avoid blood contamination. At the laboratory, the duplicate specimens were weighed and tested in replicate wells to determine the concentration of two cytokines (IL-10 and IL-12) and two immunoglobulin isotypes (IgG and IgA). IL-12, total IgG, and total IgA showed a strong correlation between samples from the same woman ranging from 0.78 to 0.84. Kappa coefficients obtained after categorizing assay results ranged from 0.62 to 0.67. Variance components analysis suggested that 69% to 85% of the variance observed was accounted for by between-women variance, with the remaining variability attributed to variation between samples collected from the same woman. IL-10 results were less reproducible than those obtained from the other assays examined, suggesting problems with the assay used to measure this cytokine rather than with the Weck-cel sampling instrument. Various factors were found to significantly correlate with cytokine and immunoglobulin measures at the cervix. Age and reproductive status were associated with all four immune measures; women over 50 years of age and those who were postmenopausal had increased concentrations of IL-10, IL-12, IgG, and IgA. Hemoglobin concentrations were positively correlated with IgG and IL-10 concentrations, but not with IgA or IL-12 concentrations, suggesting local

* Corresponding author. E-mail: hildesha@epndce.nci.nih.gov

production of IgA and IL-12. The concentration of all immune measures decreased with increasing volume of collection. No significant association was observed between time from collection to freezing of specimens and concentrations of cytokines or immunoglobulins. Overall, our data suggest that measurement of immunological parameters in cervical secretions collected using Weck-cel sponges are reproducible. In addition, various correlates of cytokine and immunoglobulin concentrations were identified. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Efforts are underway to develop vaccines that protect against the acquisition of sexually transmitted diseases (STDs), including human papillomavirus (HPV) and herpes simplex type 2 (HSV-2) (Hines et al., 1995; Schiller and Lowy, 1996; Bourns et al., 1997; Jarrett et al., 1997; Rosenthal and Gallichan, 1997; Ashley et al., 1998; Sherman et al., 1998). As part of vaccine development and testing, there is a need to measure local immunological responses at the site of these infections. In the field of HPV research, for example, efforts are underway to develop both prophylactic and therapeutic vaccines that would prevent infection with HPV and/or the development of HPV-related cervical neoplasia (Hines et al., 1995; Schiller and Lowy, 1996; Jarrett et al., 1997; Sherman et al., 1998). A better understanding of local immune response at the cervix is important for understanding and evaluating vaccine approaches currently being proposed. Also, good measures of local immune response are needed as indicators of a successful immune induction by vaccines.

Measurement of local immune responses at the cervix has been hampered, however, by the difficulty in reliably collecting sufficient cervical secretions for use in immunological evaluation. To date, several collection methods have been proposed to collect cervical material for immunological testing, including cervicovaginal lavages, wicks, Sno-strips (Akron, Abita Springs, LA) and Weck-cel sponges (Xomed Surgical Products, Jacksonville, FL), but little data are available that systematically examine the reliability and validity of these various collection methods (Ashley et al., 1994; Bouvet et al., 1994; Haneberg et al., 1994; Belec et al., 1995; Kutteh et al., 1996; Wang et al., 1996; Quesnel et al., 1997). An effective collection method should (1) be reproducible, (2) allow for quantitation of secretion volume col-

lected, (3) collect sufficient material for multiple assays, (4) avoid excessive dilution of specimen, (5) not induce local bleeding, and (6) allow for easy and complete extraction of target proteins from the collection instrument.

To address some of these issues, we have initiated a series of studies to evaluate Weck-cel sponges for use in collecting cervical secretions for immunological assays. Several of these studies are being conducted within the context of a large, 10,000-woman, population-based study of HPV and cervical neoplasia underway in Costa Rica (Herrero et al., 1997). We previously described the collection method (Crowley-Nowick et al., 1997) and also reported that collection by Weck-cel of cervical secretions prior to the collection of the Pap smear does not adversely affect the quality of the smear (Hildesheim et al., 1998). Therefore, collection of such specimens for immunological measurement prior to Pap smear collection (to avoid contamination with blood induced by the Pap smear collection) is a realistic option in natural history and vaccine studies.

In the present report we examine the reproducibility of cytokine (IL-10 and IL-12) and immunoglobulin (total IgG and total IgA) measurements made from sequential, replicate cervical secretion collections from 120 women. In addition, given the information available from subjects in our study, we had the opportunity to examine possible covariates of cytokine and immunoglobulin concentrations at the cervix, including age, time of menstrual cycle, and other factors. We have chosen to measure IL-10 and IL-12, since these cytokine measurements are likely to be used in future studies aimed at correlating type of immune response and cervical disease state. Increasing IL-10 concentrations are commonly used as a marker of a T-helper type 2 (Th2) response that upregulates antibody production and is believed not to be effective at eliminating established viral

infections (Tsukui et al., 1996; Hildesheim et al., 1997). Conversely, an increasing IL-12:IL-10 ratio has been used as an indicator of a T-helper type 1 (Th1) response that upregulates the cytotoxic T-cell response thought to be important in the elimination of viral infections (Scott, 1992; Sieling et al., 1994). As immunoglobulin measures we have chosen total IgG and total IgA because analysis of antibody induction by vaccines will require measurement of both antigen specific and total IgA and IgG concentrations.

2. Methods

2.1. Study subjects

Subjects in the present study are participants in a population-based 10,000-woman natural history study of HPV and cervical neoplasia being conducted in Guanacaste, Costa Rica. Details of this cohort study have been presented elsewhere (Herrero et al., 1997; Hildesheim et al., 1998). In brief, 10,080 women representing one sixth of the adult female population in the region were recruited (93% response rate). Informed consent was obtained from all participants. For the present study, 120 women scheduled for a follow-up Pap smear as part of our longitudinal study were recruited. These 120 women were also participants in our previous study to evaluate the impact of cervical secretion collection on Pap smear quality (Hildesheim et al., 1998).

2.2. Specimen collection and processing

Cervical secretions were collected from participants following speculum insertion using Weck-cel sponges. All material was collected by a single study nurse (L.A.M.). To collect cervical secretions, a Weck-cel sponge was placed gently in the cervical os and allowed to passively absorb cervical effluent for approximately 20–30 s as previously described (Crowley-Nowick et al., 1997; Hildesheim et al., 1998). The study nurse was instructed to avoid touching the vaginal wall and vulva during collection. Two specimens were collected sequentially from each woman prior to Pap smear sampling and stored

in separate prelabeled cryovials. Following collection, specimens were placed in a cooler and kept at 4°C until transfer to a –30°C freezer. Specimens were frozen a mean of 166 min after collection (median = 145 min; Range = 7–409 min). On a weekly basis, specimens were transferred on ice to a –70°C freezer. Every 4–6 weeks, specimens were shipped on dry ice to the study repository located in Rockville, MD. Specimens were relabeled prior to shipment on dry ice to the testing laboratory, so that laboratory personnel would be masked to duplicate specimens collected from the same woman.

2.3. Specimen processing and testing

Upon arrival at the laboratory, specimens were evaluated for visible blood and then weighed to determine the volume of secretions absorbed into the sponges. The sponges were equilibrated in 300 µl PBS + 0.25 M NaCl with 10% Aprotinin (Sigma) for 30 min at 4°C. The secretions were separated using a spin-x centrifuge filter unit (Costar, Cambridge, MA) and centrifuged at 12,000 rpm for 20 min. Following this initial extraction, a second extraction of the sponge was performed in an identical manner and the resultant material from these two extraction steps were combined. Immediately after extraction, 10 µl of diluted cervical secretions were placed on Hemastix (Baxter Scientific) hemoglobin dipsticks to assess the amount of blood present in the specimen. The indicators report on seven levels of red blood cell contamination. For simplicity, we report contamination as no/trace hemoglobin, micro-heme, or macro-heme contamination. After completion of blood measurement, 10% fetal calf serum was added to the extracted sample to stabilize the proteins. A dilution factor for the final extract was determined based on the following formula: dilution factor = $[(x - y \text{ ml}) + 0.6 \text{ ml buffer}] / (x - y \text{ ml})$, where x equals the volume of material collected (mg = ml) and y equals the weight of the dry spear. The dry weight of sponges was determined by measuring the weight of 10 representative dry sponges from each lot of sponges used in this study (dry weight varies by lot number and so each lot of spears was weighed). This dilution factor was used to calculate the final concentration of cytokines and immunoglobulins in the cervical secretions measured in our ELISA assays.

IL-10 and IL-12 concentrations were determined using high sensitivity quantitative ELISA kits purchased from BioSource International (Burlingame, CA). All cervical specimens were diluted 1:2 in

PBS-1% fetal calf serum (PBS-FCS) added in duplicate to the plates and run according to the manufacturer's instructions. If concentrations were above the highest standard, the samples were rerun at a higher

Table 1
Characteristics of subjects and of duplicate cervical secretion specimens collected from 120 women

Factor	First specimen	Second specimen	<i>p</i> -value ^a
<i>Age (yrs)</i>			
Mean	40	Same as first	
Median	38		
Range	22–90		
<i>Days since last menstrual period^b (%)</i>			
4–7 days	13 (10.8%)	Same as first	
8–15 days	31 (25.8%)		
16–40 days	40 (33.3%)		
> 40 days ^d	16 (13.3%)		
Post-menopausal	20 (16.7%)		
Levels of Hb (%)			0.16 ^e
None/Trace	53 (44.2%)	48 (40.0%)	
Mico	43 (35.8%)	37 (30.8%)	
Macro	24 (20.0%)	35 (29.2%)	
Volume (ul)			0.0001
Mean	48.7	31.4	
Median	35.4	23.6	
Range	3–264	0–153	
IL-10 (pg/ml)			0.08
Geometric mean	38.1	55.1	
Median	84.8	135.6	
Range	BDL ^f -1845	BDL-3134	
IL-12 (pg/ml) ^e			0.003
Geometric mean	135.6	232.8	
Median	254.7	337.0	
Range	BDL ^f -3641	BDL-6311	
Total IgG (ug/ml) ^e			0.006
Geometric Mean	1012	1200	
Median	1200	1466	
Range	28.2–23624	17.1–22471	
Total IgA (ug/ml) ^e			0.84
Geometric Mean	327.0	330.3	
Median	327.0	333.6	
Range	18.5–84120	11.1–32209	

^a*p*-value from paired *t*-test.

^bDefined as days since start of last menstrual period bleeding.

^c*p*-value from Bowker's test for symmetry.

^dDue to pregnancy (*n* = 6), Depo-provera use (*n* = 1), menstrual disorders (*n* = 1), or unknown reasons (*n* = 8).

^eIL-12 results available from 80 subjects. Total IgG results available from 116 subjects. Total IgA results available from 118 subjects.

^fBDL = Below detectable limit. The lower detection limit for the IL10 assay was 0.2 pg/ml of diluted specimen and for the IL12 assay was 0.8 pg/ml of diluted specimen.

Table 2
Spearman correlation between duplicate specimens tested for cytokine and immunoglobulin concentrations

Factor	N	Correlation coefficient (Spearman)
IL-10	120	0.63
IL-12	80	0.78
Total IgG	116	0.84
Total IgA	118	0.82

dilution. The sensitivity of the IL-10 and IL-12 kits were 0.78 to 50 pg/ml and 0.8 to 100 pg/ml for each cytokine, respectively.

Quantitative ELISA assays were used to determine the concentrations of IgA and IgG in cervical secretions. The assays were performed as previously described (Jackson et al., 1991). Microtiter plates were coated overnight at 4°C with affinity purified F(ab')₂ fragments of goat antibodies (Jackson ImmunoResearch, West Grove, PA) specific for the appropriate isotype diluted in PBS. Total immunoglobulin assays were standardized using a pool of normal serum with known concentrations of IgA and IgG (the binding site). The samples and standard were diluted in PBS FCS and added to the plates in duplicate and serially diluted. After a 2-h incubation at room temperature the plates were washed with PBS with 1% Tween 20 (PBS-T), and isotype specific, biotinylated, affinity purified F(ab')₂ fragments of goat anti-human IgG or IgA antibodies (BioSource, Burlingame, CA) were added to the plates and incubated for 2 h at room temperature. The plates were washed with PBS-T and incubated for 30 min with horseradish- peroxidase-conjugated avidin (Sigma, St. Louis, MO), diluted to 0.5 mg/ml in 0.87% saline containing 0.05% Tween 20. After a final wash the plates were exposed to substrate consisting of 2,2' azino-bis(3-ethylbantzthizoline-6-sulfonic acid) (Sigma) at a concentration of 0.25%

Table 3
Agreement levels for cytokine and immunoglobulin concentrations based on quartile cuts

Factor	N	Weighted kappa (95% CI)	Exact agreement (%)	Agreement w/in 1 level (%)	Disagreement of 2 + levels (%)
IL-10	120	0.53 (0.41–0.65)	60.0	23.3	16.7
IL-12	80	0.66 (0.53–0.79)	70.0	18.7	11.3
Total IgG	116	0.67 (0.58–0.76)	62.1	35.3	2.6
Total IgA	118	0.62 (0.52–0.72)	61.0	30.5	8.5

Table 4
Results of variance components analyses-examination of between subject and between specimen (within subject) effects^a

Factor	Between women		Between specimen	
	Variance	ICC ^b	Variance	1-ICC
IL10	2.96	0.51	2.84	0.49
IL12	2.80	0.69	1.29	0.31
IgG	1.35	0.85	0.24	0.15
IgA	0.93	0.80	0.23	0.20

^aVariance estimates obtained from mixed models containing subject as a random effect and sampling order, collection volume, and time to freeze as fixed effects. Analysis performed using log transformed assay results.

^bICC = Intraclass correlation coefficient = (between women variance)/(total variance), where total variance = (between women variance) + (between specimen, i.e., within women variance).

w/v in 0.1 molar citrate phosphate buffer, pH 4.0 containing 0.0075% H₂O₂. Substrate turnover was monitored spectrophotometrically at 415 nm using an automated reader (Biotech, Burlington, VT). All raw data was analyzed using the Delta Soft curve fitting and integration software.

Results from replicate wells were averaged to determine the cytokine and/or immunoglobulin concentration for each specimen tested. Comparison of results from these replicates revealed that 2.5% of specimens tested for IL-10 had a coefficient of variation (CV) > 20%. The comparable proportion for the IL-12 assay was 6.3%. None of the specimens tested for total IgG and IgA immunoglobulins had CV values > 20%. Examination of the concordance between results obtained from replicate wells for the first 86 specimens collected in our study revealed a correlation coefficient ranging from 0.97 to 0.99. Within plate variability is therefore unlikely to contribute significantly to the observed variability in assay measurements, and was thus not evaluated further as part of our study.

IL-10 results from duplicate sponges were available for all 120 women selected for study. Duplicate IL-12 results were available on 80 women. The remaining 40 women were not tested for IL-12 due to an initial attempt to use the specimens to test for IL-2 concentrations. This initial attempt to test specimens for IL-2 was stopped after examination of specimens from this and other studies revealed that IL-2 concentrations in cervical secretions are on the

edge of assay sensitivity. Duplicate IgG and IgA results were available on 116 and 118 women, respectively.

2.4. Statistical analysis

Analyses were conducted to determine the concordance in assay results between replicate speci-

Table 5
Factors associated with cytokine concentrations at the cervix

Factor	IL10 (pg/ml)				IL12 (pg/ml)			
	N ^a	Crude mean ^b	Adj. mean ^c	p ^e	N ^a	Crude mean ^b	Adj. mean ^c	p ^e
Age ^d				0.05				0.89
≤ 30 years	54	55.6	48.6		40	162.5	165.6	
31–40 years	86	20.1	27.4		50	125.0	148.0	
41–50 years	56	42.0	39.3		41	196.7	215.5	
51+ years	44	202.5	140.7		30	338.3	229.5	
Days since LMP				0.01				0.48
4–7	26	115.9	84.7		16	156.5	136.6	
8–15	62	18.7	20.2		36	112.0	117.2	
16–40	80	28.3	31.5		55	140.3	149.3	
> 40	32	66.0	80.3		24	287.8	429.2	
Post-menopausal	40	198.1	138.4		30	378.1	261.0	
Hemoglobin level				0.04				0.71
None/trace	101	33.6	32.9		67	146.4	163.7	
Micro-heme	80	41.9	67.1		55	178.9	191.8	
Macro-heme	59	88.1	83.6		39	262.0	228.9	
Collection volume				0.0001				0.008
≤ 16 ul	57	178.1	207.9		41	465.7	522.3	
> 16–≤ 30 ul	68	60.9	80.4		47	153.4	164.1	
> 30–≤ 50 ul	49	15.0	19.2		27	142.0	149.5	
> 50 ul	66	24.3	32.8		46	106.0	108.2	
Collection order				0.77				0.23
First	120	38.0	58.8		80	137.3	172.1	
Second	120	55.4	55.1		81	235.6	216.4	
Time to freeze				0.34				0.87
≤ 60 min	50	63.9	56.9		32	177.7	145.0	
61–150 min	78	73.9	92.5		56	191.3	210.0	
151–240 min	46	33.8	36.0		34	193.6	179.7	
> 240 min	66	25.1	55.4		39	158.2	253.5	

^aNumber of specimens.

^bLeast squares mean estimates obtained from a mixed model containing subject as a random effect and the variable of interest as a fixed effect. Analysis performed using log transformed assay results. Back transformed mean estimates presented.

^cLeast squares mean estimates obtained from a mixed model containing subject as a random effect and days since last menstrual period, hemoglobin level, collection volume, collection order, and time to freeze as fixed effects. Analysis performed using log transformed assay results. Back transformed mean estimates presented.

^dAge replaces days since last menstrual period for model described in # above.

^ep-value for model described in # and & above.

mens from the same woman and to determine the effect of age, time in menstrual cycle, collection volume, blood, and time to freeze on cytokine and antibody concentrations present at the cervix. All cytokine and immunoglobulin data were log transformed prior to analysis to normalize the sample distribution. When log transforming data, specimens whose cytokine concentrations were below the detection limit for the assay were assigned a value which

corresponded to half of the lower detection limit for the assay.

Mean levels of specimen characteristics were compared for first and second collections using the paired *t*-test (Table 1) (Dixon and Massey, 1983). Geometric means were computed using the log transformed data. When comparing results across studies, it should be noted that the geometric means yielded smaller values than the untransformed means. The

Table 6
Factors associated with immunoglobulin concentrations at the cervix

Factor	IgG (ug/ml)				IgA (ug/ml)			
	<i>N</i> ^a	Crude mean ^b	Adj. mean ^c	<i>p</i> ^e	<i>N</i> ^a	Crude mean ^b	Adj. mean ^c	<i>p</i> ^e
Age ^d				0.08				0.0003
≤ 30 years	53	1245	1216		52	248.9	234.5	
31–40 years	86	791.2	883.6		85	234.8	265.1	
41–50 years	55	1071	1186		56	301.2	345.6	
51+ years	42	2370	2156		44	884.5	760.4	
Days since LMP				0.008				0.0002
4–7	26	1360	1320		26	365.3	376.3	
8–15	60	658.4	705.6		59	216.5	238.4	
16–40	80	933.4	1003		80	224.3	234.9	
> 40	32	1807	1895		32	390.8	405.0	
Post-menopausal	38	2576	2411		40	953.5	821.2	
Hemoglobin level				0.09				0.24
None/trace	99	979.9	1117		100	298.0	340.5	
Micro-heme	78	1181	1382		78	351.5	415.8	
Macro-heme	59	1449	1547		59	328.2	360.2	
Collection volume				0.03				0.0002
≤ 16 ul	56	1417	1608		57	468.1	522.6	
> 16–≤ 30 ul	66	1267	1473		66	360.3	415.2	
> 30–≤ 50 ul	49	1193	1380		49	327.8	366.1	
> 50 ul	65	836.7	976.5		65	207.2	238.0	
Collection order				0.32				0.07
First	117	1050	1289		118	320.8	395.8	
Second	119	1254	1387		119	323.6	347.4	
Time to freeze				0.82				0.88
≤ 60 min	50	1682	1500		50	462.3	426.2	
61–150 min	77	1226	1452		78	320.7	360.6	
151–240 min	45	1124	1275		45	337.7	345.9	
> 240 min	64	808.5	1150		64	236.3	355.6	

^aNumber of specimens.

^bLeast squares mean estimates obtained from a mixed model containing subject as a random effect and the variable of interest as a fixed effect. Analysis performed using log transformed assay results. Back transformed mean estimates presented.

^cLeast squares mean estimates obtained from a mixed model containing subject as a random effect and days since last menstrual period, hemoglobin level, collection volume, collection order, and time to freeze as fixed effects. Analysis performed using log transformed assay results. Back transformed mean estimates presented.

^dAge replaces days since last menstrual period for model described in # above.

^e*p*-value for model described in # and & above.

distribution of categorical variables was compared between first and second specimens using Bowker's test which tests for symmetry in the joint distribution of the two variables of interest (Table 1) (Agresti, 1990). The non-parametric Spearman correlation coefficient was used to calculate the correlation between specimens collected from the same woman (Table 2) (Dixon and Massey, 1983).

Specimen testing results were also categorized into quartiles and the quartile distributions of the first and second specimens were compared to determine overall agreement (Table 3). Approximate quartile cuts were determined using the distribution of all 240 samples tested. The weighted kappa coefficient was used to determine chance-corrected agreement levels (Table 3) (Kelsey et al., 1986). Weights were assigned assuming that the category scores were on a linear scale. When an alternative weighting scheme was used assuming that the category scores were on an exponential scale, nearly identical results were obtained (data not shown).

Variance components analysis using a mixed model was performed to determine the relative contribution of between-women and within-women (i.e., between specimens collected sequentially for the same woman) variance to the total observed variance (Table 4) (SAS Institute, 1992; Searle et al., 1992). A mixed model is a model containing both fixed and random effects. Fixed effects are those attributable to factors that either we control experimentally or we adjust for, for example, woman's age, days since last menstrual period, hemoglobin concentration, collection volume, sample order and time to freeze in our study. In contrast, random effects are those attributable to a factor whose levels represent a random sample from some larger population, for example, women in our study. A mixed model analysis was also conducted to examine the association between selected variables and cytokine and immunoglobulin concentrations (Tables 5 and 6). When trends were observed in the data, they were tested for significance by including the categorical variable in the model as a continuous variable.

Analyses were performed using all results and again after excluding the few specimens with CV > 20%, and results were nearly identical. Therefore, results from analyses which included all available subjects are presented.

3. Results

Characteristics of the study subjects and of duplicate cervical secretion specimens collected from them are summarized in Table 1. Women were on average 40 years of age (median = 38) and ranged in age from 22 to 90 years. 16.7% ($N = 20$) of women were post-menopausal and an additional 13.3% ($N = 16$) were anovulatory due to pregnancy ($N = 6$), Depo-provera use ($N = 1$), menstrual disorders ($N = 1$), or unknown reasons ($N = 8$). By design, women were asked not to visit the study clinic during the first few days following menses.

One-hundred and eighty-one (75.4%) of the 240 specimens included in the present study showed no or only very low levels of hemoglobin in the specimen, and no significant difference was noted in the proportion of samples with macro levels of hemoglobin when specimens collected first and second were compared. However, there was a non-significant tendency for the second specimen to contain macro levels of hemoglobin more often than the first (29.2% vs. 20.0%, respectively; $p = 0.16$). The specimen collected first had a larger average volume than that collected second from the same woman. Among the 120 women in our study, the mean specimen volume was 48.7 ul for the first specimen and 31.4 ul for the second ($p = 0.0001$). In contrast, we observed that the concentration of cytokines and antibodies was higher in the second specimen relative to the first. This was true for all measurements with the exception of total IgA.

Cytokine and immunoglobulin concentrations obtained from the first and second specimens collected from participants were compared and Spearman correlation coefficients computed (Table 2). The correlation between measurements was 0.63 for IL-10, 0.78 for IL-12, 0.84 for total IgG, and 0.82 for total IgA. Interestingly, for both cytokine measurements, we observed several instances where one of the duplicate specimens tested positive for the cytokine of interest, while the other specimen was below the detectable limit of the assay for the same cytokine. This occurred among specimens from 26 (21.7%) women for IL-10 and 13 (16.3%) women for IL-12. Among these discordant specimen pairs, only 3 pairs were found to be discordant for both the IL-10 and the IL-12 assays. The specimen pairs from the 26

women with discordant IL-10 concentrations and 13 women with discordant IL-12 concentrations were examined further with respect to specimen volume and cytokine concentrations. For both IL-10 and IL-12 we observed comparable volumes between the specimens which tested positive and their paired sample which was below the detectable limit of the assay (mean difference in volume between pairs was 1.1 μ l for IL-10 and 0.17 μ l for IL-12). We also noted for both IL-10 and IL-12, that the geometric mean concentration of cytokine for the positive specimens in the problematic pairs was lower than the comparable geometric mean obtained from the remaining positive specimens in our set (126.6 pg/ml vs. 209.6 pg/ml, respectively for IL-10; 294.3 pg/ml vs. 520.6 pg/ml, respectively for IL-12). When the 26 women with positive IL-10 results for one specimen and below detectable limit results for its paired specimen were excluded, the Spearman correlation coefficient increased from 0.63 to 0.84, as one would expect. Similarly when the 13 women with positive IL-12 results for one specimen and below detectable limit results for its paired specimen were excluded, the Spearman correlation coefficient increased from 0.78 to 0.89.

Next, specimen results were categorized into quartiles and the distribution of the first and second specimens collected from participants were compared. Results are presented in Table 3. Exact agreement between specimens based on this quartile distribution was 60% for IL-10 and 70% for IL-12. The comparable exact agreement rates for total IgG and IgA were 62.1% and 61%, respectively. In contrast to the large proportion of specimens with exact agreement, disagreement by more than one category (e.g., specimen 1 classified into quartile 1 and specimen 2 classified into quartiles 3 or 4) was observed among 16.7% of subjects for IL-10, 11.3% for IL-12, 2.6% for total IgG, and 8.5% for total IgA. Again, when subjects who tested positive for cytokines on one of the two specimens and below the detection limit on the other were excluded, the rates of exact agreement increased to 75.5% for IL-10 and 82.1% for IL-12. For disagreements of more than one category, the rate decreased to 3.2% (IL-10) and 4.5% (IL-12) following this exclusion. Weighted kappa statistics, which express the overall agreement between readings after controlling for chance agree-

ment, are also presented in Table 3. Kappas were 0.53 for IL-10, 0.66 for IL-12, 0.67 for total IgG, and 0.62 for total IgA. This suggested moderate to good rates of agreement between duplicate specimens collected using the Weck-cel sponges.

To determine what proportion of variability in cytokine and immunoglobulin concentrations can be attributed to between-women and between specimens variability (i.e., within-woman variability), variance components analyses were performed using log transformed assay results (Table 4). The between specimens variability is the random variation which cannot be explained by systematic (fixed) effects of sampling order, collection volume, and time from collection to freezing of specimens. The intraclass correlation coefficients (ICC) obtained from these analyses suggest that 51% (IL-10), 69% (IL-12), 85% (total IgG), and 80% (total IgA) of the variability in cytokine and immunoglobulin concentrations observed in our study can be accounted for by variability which exists between women, while 15% (total IgG) to 49% (IL-10) of the variability is accounted for by variability which exists within participants (i.e., between the two specimens collected from each woman).

A mixed model analysis was also performed to examine possible correlates of cytokine and immunoglobulin concentrations at the cervix (Tables 5 and 6). Both crude and adjusted models are presented. As shown in the tables, older age was associated with higher concentrations of both cytokines and immunoglobulins. For all four immune measures examined, women 51 years of age and older had higher concentrations than younger women, although for IL-12 and total IgG the difference observed was not statistically significant (for IL-12, $p = 0.89$, $p_{\text{trend}} = 0.52$; for IgG, $p = 0.08$). Among women ≤ 50 years no trend was observed between age and mean cytokine or immunoglobulin concentrations ($p_{\text{trend}} > 0.10$ for all assays). In concordance with the observations made among older women, postmenopausal women had consistently higher concentrations of all four cytokines and immunoglobulins examined, when compared to premenopausal women. Among premenopausal women, the lowest concentrations of cytokines and immunoglobulins were generally observed at days 8–15 of the menstrual cycle (late follicular/mid cycle). The number of women

who were premenopausal but anovulatory (i.e., > 40 days since last menstrual period) was too small to allow for further evaluation of this heterogeneous group of women. Hemoglobin concentrations were found to be positively associated with IL-10 ($p = 0.04$) and total IgG ($p = 0.09$; $p_{\text{trend}} = 0.03$) concentrations, but not as strongly linked to IL-12 ($p = 0.71$; $p_{\text{trend}} = 0.41$) or total IgA ($p = 0.24$) concentrations. Consistent with our findings within women (Table 1), cytokine and immunoglobulin concentrations were found to be negatively associated with specimen collection volume. Furthermore, this association of low volume with high immune measures was found to be independent of age or menopausal status, as significant effects remained in analyses controlling for these factors. Also consistent with our findings from Table 1, IL-10, IL-12, and IgG concentrations tended to be higher for the second compared to first specimens collected, in analyses which did not control for other factors of interest. Following adjustment for the various factors listed on the table, however, no significant association was noted between sampling order and concentrations of IL-10, IL-12, and IgG; the increased concentrations observed among second specimens was explained largely by the fact that these specimens had lower collection volume and that lower volume was strongly associated with higher cytokine and immunoglobulin concentrations. For IgA, a tendency, albeit non-significant, was observed, after adjustment, for concentrations to be lower in the second relative to the first specimens collected from women ($p = 0.07$). No significant association was observed between interval from specimen collection to freeze and concentrations of cytokines or antibodies, either when overall associations (shown in Tables 5 and 6) or linear trends were evaluated (data not shown).

4. Discussion

Despite the increased interest in measuring local immunity at the cervix, there has been little effort to validate methods of collection (Quesnel et al., 1997; Ashley et al., 1994; Belec et al., 1995). This has hampered research to elucidate local immune response to STDs. To our knowledge, the present study represents the first large effort to systematically quantitate the reliability of a cervical secretion

collection instrument for use in immunological assays. In one previous study among 27 women, Belec et al. (1995) proposed the use of cervical lavages using phosphate-buffered saline containing lithium chloride to allow for the quantitation of collection volume. However, a subsequent study of eight women which compared cervicovaginal lavage collection with collection methods which employed Sno-strips or wicks found the immunoglobulin and protein concentrations in lavage samples to be 100 times lower than in secretions collected from the same women using either of the other two collection methods (Quesnel et al., 1997).

By using Weck-cel sponges for the passive absorption of secretions at the cervix from 120 women, we were able to collect sufficient material to allow for multiple assays (four assays were run, each in duplicate, using a single sponge). In addition, we were able to collect cervical secretions without excessive specimen dilution. Furthermore, our findings suggest good reproducibility of results when testing sequential duplicate specimens collected from the same woman. With the exception of the IL-10 results, Spearman correlations were moderate to high (range: 0.78–0.84), kappas were in the moderate range (range: 0.62–0.67), and variance components analyses suggested that 69–85% of variance is due to between women variability while only 15–31% of variance was accounted for by between specimen differences within a woman. These results are reassuring and suggest that as collection and assay methods improve in future years, highly reliable cervical immune measures can be attained. The fact that IL-10 was the single assay whose results demonstrated poor reproducibility suggests that this may be due to problems with the IL10 assay itself, rather than with the Weck-cel collection instrument used.

For the cytokine measurements which, on average, were less reliable than the total IgG and IgA measurements, we observed that duplicate testing followed by exclusion of subjects with one specimen positive and the other negative resulted in considerable increase in reliability. Provided these discrepant findings are due to collection and/or assay variability rather than true biological differences in the duplicate specimens from these women, study designs which exclude these subjects could increase measurement accuracy without introducing bias. Our

observations (1) that the volume collected from specimens which tested positive and those which tested below the detectable limit of the assay were comparable, and (2) that little overlap was observed between those samples with discrepant results for IL-10 and IL-12, suggest that these discrepancies were due to problems related to the cytokine assays themselves rather than with the Weck-cel collection instrument. These observations are supported by the fact that such problems were noted only for the cytokine measurements and not for the immunoglobulin measurements. However, since immunoglobulin assays were conducted at much higher dilutions than cytokine assays, the observation that discrepant findings were seen for the cytokine assays alone might point to the presence of inhibitors or cross reactive factors present at high enough levels to affect cytokine results. Also, our observation that the mean concentrations of IL-10 and IL-12 were lower in those specimens whose paired specimen was below the detectable limit of the assay compared to the remaining specimens which tested positive in our set suggest that these discrepancies might be arising preferentially among specimens collected from women whose cytokine concentrations are low. Exclusion of these specimens from analysis should therefore be performed with caution.

In our study, older age was a strong correlate of cytokine and antibody concentrations at the cervix. Women over the age of 50, most of whom were postmenopausal, had consistently higher concentrations of both the cytokines and immunoglobulins measured. Previous studies which have examined the effect of aging on systemic immunoglobulin concentrations have shown an increase in immunoglobulin concentrations with age (Paganelli et al., 1992). Among younger, premenopausal women we also noted that the lowest concentrations of cytokines and antibodies were generally observed at days 8–15 of the menstrual cycle (late follicular/mid cycle), the period in which estrogen levels peak (Adashi et al., 1996). Whether either of these effects is related to estrogen levels or to other factors remains to be determined. Studies are currently ongoing to examine the relationship between hormones and mucosal immunity (Crowley-Nowick et al., 1995a,b).

The observed effect of hemoglobin levels on cytokine/immunoglobulin concentrations and of col-

lection on hemoglobin levels are also of interest. The finding that hemoglobin levels correlated positively with concentrations of total IgG is not surprising, given that sera is thought to contribute significantly to total IgG concentrations in cervical secretions (Brandtzaeg, 1997). The serum contribution in these specimens may be more significant than IgG produced locally by IgG plasma cells (Crowley-Nowick et al., 1995a,b). The lack of correlation between hemoglobin and total IgA is also not surprising and confirms previous suggestions that IgA present at the cervix originates in large part from local production (Mestecky et al., 1994; Kutteh et al., 1996). In our study, hemoglobin levels also correlated positively with IL-10 but not with IL-12 concentrations suggesting local production of IL-12 but systemic transudation of IL-10. This is somewhat surprising because serum concentrations of IL-10 are relatively low in healthy women. In addition epithelial cells are a major secretor of IL-10 and are known to express IL-10 mRNA in the cervix of healthy women (Olaitan et al., 1998). Therefore, local production of IL-10 would be expected to contribute significantly to the local pool of IL-10 in genital tract secretions. Further studies are necessary to evaluate the origins of cytokines in cervical secretions.

Presence of macro levels of hemoglobin was only observed in a minority of specimens in our study (24.6%), confirming that the Weck-cel sponge is a gentle collection procedure unlikely to induce bleeding. Although the effect was modest and not statistically significant, our data was suggestive of levels of hemoglobin being higher in the second compared to the first specimen collected (29% of second specimens compared to 20% of first specimens had macro-heme levels; $p = 0.16$). This finding raises the possibility that even gentle collection of cervical secretions using Weck-cel sponges might induce bleeding in a subset of women. It should be noted, however, that the presence of blood in cervical secretion specimens per se does not necessarily reflect contamination due to the collection instrument, since blood 'spotting' is often observed naturally between menses. In the future, it will be important to discern between these two sources of blood in secretion collections, since induced bleeding is to be avoided while bleeding which is not induced by the collection instrument is a natural phenomenon at the cervix

and therefore an important route of exposure of the cervix to immune-related factors.

Our finding that cytokine and immunoglobulin concentrations decreased with increasing collection volume is of note. One possible interpretation for this observation is that the flow of cervical secretions and the production of these immune factors are independent and that a higher secretion volume along with constant production of cytokines/immunoglobulins result in decreased concentration. This phenomenon was described previously in studies examining menstrual cycle variation in immunoglobulin concentrations (Kutteh et al., 1996).

Time to freeze did not significantly impact the concentration of cytokines or immunoglobulins detected at the cervix. This has important implications for the ability of collecting such specimens in large population-based field studies, where it is not always possible to have immediate access to freezers. It should be noted, however, that our study was not designed to evaluate degradation in the initial minutes following collection. Although specimens were kept at 4°C until they could be frozen and Aprotinin was added to the extraction buffer, to avoid possible degradation, we cannot rule out the possibility of cytokine and/or immunoglobulin degradation between the time of collection and placement into the -30°C freezer (which in our study occurred 7 min after collection, at the earliest). In our studies, immunoglobulin concentrations remain constant after a 7-h incubation at room temperature, suggesting that immunoglobulins are fairly stable. However, stability of individual cytokines has not been defined. We have found that IL-6 is stable at room temperature for an extended time, but TNF-alpha is not. Therefore, each cytokine may differ. These issues are best examined in the context of controlled experiments of carefully spiked specimens in a laboratory setting, which were not incorporated into the present study.

Limitations of our study should be mentioned. First, our study was not designed to evaluate plate-to-plate variability of the cytokine and immunoglobulin assays examined. Therefore, to the extent that significant plate-to-plate variability exists, we would not have been able to control for it. Second, our measure of number of days since start of last menstrual period is only a crude measure of phase of menstrual cycle and we were therefore not able to

carefully examine menstrual cycle fluctuations in cytokine or immunoglobulin concentrations. Third, due to the substantial portion of specimens that tested below the detectable limit of the assay for IL-10, the normalcy assumption required for the mixed model analysis may be in question. Hence, the mixed model analysis results for IL-10 should be viewed cautiously and in combination with the quartile-based analysis that does not depend on normally distributed data.

In summary, results from our study demonstrate that Weck-cel sponges can be used to collect cervical secretions in a reproducible manner. In addition, numerous factors which are associated with immune response markers were identified in our study, including age, menopausal status, hemoglobin level, and specimen volume.

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