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Optimization of the Weck-Cel Collection Method for Quantitation of Cytokines in Mucosal Secretions

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Measurement of immune components in mucosal secretions is important for the evaluation of local immunity at the mucosal surfaces. The Weck-Cel ophthalmic sponge provides a method for the collection of these secretions. The sponge absorbs a relatively large volume of material, therefore allowing for quantitation of multiple immune components. Additionally, it provides a method in which the same device may be used to collect specimens from different mucosal sites, such as the genital tract and oral cavity. This sampling technique has successfully been applied for collection and measurement of antibody in oral and genital tract secretions. The purpose of this work was to optimize the extraction of protein from the sponge matrix. Of particular interest was the recovery of cytokines from the sponge. Satisfactory recovery of the cytokines interleukin 1β (IL-1β), IL-2, IL-5, IL-12, IL-6, IL-8, IL-10, and granulocyte-macrophage colony-stimulating factor was obtained. However, IL-4 and gamma interferon recovery rates remained low. Using an alteration of the published extraction method, cytokine concentrations were measured in cervical secretions from women using oral contraceptives. The data revealed detectable concentrations of IL-6, IL-10, IL-8, and IL-12 on cycle days 9 and 20. The proposed technique provides an easy, practical, and consistent method for collection of nonconventional body fluids, such as cervicovaginal fluids and saliva, for the assay of immunoglobulins and several cytokines.

Monitoring or analysis of humoral immune components in conventional body fluids, such as blood and urine, involves common methods. However, measurement of these factors in nonconventional biological fluids, such as cervical secretions, vaginal fluid, and saliva, is complex. The difficulties lie not only in the analysis of these mucosal fluids but also in obtaining reproducible and unaltered samples. The accuracy and consistency of the sampling procedures can ultimately affect experimental outcomes and quantitation of the individual components. Yet the assessment of such fluids is important because it gives insight into the local immune response, physiological modifications induced by infection, and potential drug profiles at the site of action (11, 12).

There are a number of different methods available for the collection of genital tract secretions, such as cervicovaginal washes, aspiration, and Wick absorption. Each technique has proven its utility but also has a downside. The washes yield a significant amount of material but combine vaginal and cervical secretions. These two secretions have different roles in the protection of the genital tract, and combining them prevents studies of each secretion. Aspiration works well for collection of cervical secretions at midcycle in women who are ovulating but yields little volume at other times in the cycle or when women are using oral contraceptives. Finally, the Wicks collect cervical and vaginal secretions individually but absorb a very small amount of material. The collection volume limits the number of possible analyses that can be performed on each sample. To overcome some of these problems, to standardize the methods of collection across different types of mucosal secretions, and to simplify the collection process in order to incorporate collection of secretions into multicenter clinical trials, the Weck-Cel method using ophthalmic sponges was developed.

Ophthalmic sponges were used successfully for the collection of secretions from the oral and genital tract mucosae to measure antibody levels in response to vaccination (3, 9). The consistency of immunoglobulin (Ig) recovery from the sponges was demonstrated previously (3). This collection technique overcomes some of the limitations encountered when washes or aspiration is used to obtain secretions, and a single device can be used for the collection. The Weck-Cel sponges were designed for the collection of tears and easily absorb fluids without causing trauma to the cervix or local tissue. Also, the nonabrasive collection does not interfere with Pap smear results (6). This method allows for assessment of a dilution factor for each individual secretion collected, therefore reducing the variability induced by unknown dilution of the samples (7). The cellulose fibers in the sponges are highly absorbent and have a low binding affinity for protein. Finally, this technique is simple and the procedure can be completed within 2 min, allowing easy incorporation into clinical trials.

To expand our understanding of immunoregulation in the genital tract, a study of cytokine concentrations using the Weck-Cel sponge for collection of cervical secretions from women was undertaken. However, during those studies it was discovered that some cytokines, unlike Ig, bind to the sponges, thereby preventing the diffusion of individual cytokines out of the sponges during the extraction procedure. These studies were conducted to optimize the processing procedure to ensure consistent release of individual cytokines and Ig from the

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sponges. Using this technique, baseline concentrations of cytokines in cervical secretions of women using oral contraceptive pills were determined. The objective of these studies was to demonstrate the utility of this method for collection of genital tract secretions throughout the menstrual cycle and to establish consistency in the volume of material and the recovery of each cytokine obtained from the sponges.

MATERIALS AND METHODS

Immunochemical reagents. Affinity-purified $F(ab')_2$ fragments of goat antibodies specific for human IgG and IgA were purchased from Jackson Immunoresearch (West Grove, Pa.). All secondary antibodies were biotinylated affinity-purified $F(ab')_2$ fragments of goat anti-human IgG or IgA purchased from BioSource International (Camarillo, Calif.). Recombinant cytokine enzyme-linked immunosorbent assay (ELISA) kits were obtained from BioSource International. The kits used were for the cytokines interleukin 1β (IL-1β), IL-2, IL-4, IL-5, IL-8, gamma interferon (IFN-γ, and granulocyte-macrophage colony-stimulating factor (GM-CSF). High-sensitivity ELISA kits were used for detection of IL-6, IL-10, and IL-12.

Cytokine extraction method. Recombinant cytokines obtained from BioSource International as part of the standard curve were utilized for the extraction and recovery experiments. The Weck-Cel sponges were allowed to absorb a solution containing known concentrations of cytokine and Ig calibrator (Binding Site, Birmingham, United Kingdom). The following are the respective cytokine concentrations (in picograms per milliliter) absorbed onto the sponges: IL-1β, 3,000.0; IL-2, 1,562.5; IL-4, 312.5; IL-5, 1,250.0; IL-6, 77.5; IL-8, 2,500; IL-10, 1,250.0; IL-12, 500.0; GM-CSF, 1,500.0; and IFN-γ, 250.0. Cytokine and serum standards were diluted in the standard diluent provided by BioSource International for their ELISA kits, and 1% fetal calf serum (FCS) was added to the final solution. The standard diluent and FCS served as negative controls and were tested for each cytokine. In order to optimize the recovery of cytokines or Ig (see Results), the sponges underwent various treatment. Percent recovery was based upon a comparison of the absolute amount of cytokine in the extracted material compared to a stock solution that was not subjected to the extraction procedure.

The standard Ig extraction method, described previously, was used to optimize this technique for cytokine extraction (3). Briefly, in the original extraction method used, each sponge was weighed to determine the volume of secretions absorbed onto the sponge. The sponge was then equilibrated in 300 μl of phosphate-buffered saline (PBS) with 0.25 M NaCl and 10% FCS for 30 min at 4°C. A Spin-x centrifuge filter unit (Costar, Cambridge, Mass.) was used to separate extracted samples from the sponge matrix. Samples were centrifuged at 12,000 rpm for 20 min.

Total Ig and cytokine concentration analysis. Ig concentrations were determined by quantitative ELISA as previously described (8). Microtiter plates were coated overnight at 4°C with affinity-purified antibodies specific for the appropriate isotype diluted in PBS. Total Ig assays were standardized using an Ig calibrator (Binding Site) of known IgA and IgG concentrations. The extracted samples and the calibrator were diluted in PBS containing 1% FCS (PBS-FCS) and added to the plates in duplicate. After an overnight incubation at 4°C the plates were washed with PBS with 0.05% Tween 20, isotype-specific biotinylated antibodies were added, and the mixture was incubated for 2 h at room temperature. The plates were then washed with PBS–0.05% Tween 20 and incubated for 30 min with horseradish peroxidase-conjugated avidin (Sigma, St. Louis, Mo.), diluted to 0.5 μg/ml, and 0.87% saline containing 0.05% Tween 20. The plates were washed and exposed to a substrate consisting of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) at a concentration of 0.25% (wt/vol) in 0.1 M citrate-phosphate buffer (pH 4.2) containing 0.0075% H_2O_2. Substrate turnover was monitored spectrophotometrically at 415 nm with an automated reader (BioTek, Winooski, Vt.). A standard curve was produced with Delta Soft ELISA analysis software.

Collection of biological specimens. Seven healthy women (age range, 19 to 35 years) who had taken OrthoCept oral contraceptive pills for a minimum of 2 months were enrolled in this study, approved by the Institutional Review Board at Magee-Womens Hospital. All women had normal Pap smears and no evidence of infection during a pelvic exam. Saliva, cervical secretions, and vaginal secretions were collected with Weck-Cel sponges. Cervical secretions were collected first after exposure of the cervical os with the speculum. The secretions were collected by placing the ophthalmic sponge directly into the cervical os and allowing it to absorb secretions for approximately 1 min. Vaginal secretions were collected by placing the ophthalmic sponge against the vaginal wall, and in a similar fashion, saliva was collected by placing the ophthalmic sponge over the parotid duct and allowing the sponge to absorb saliva.

Weck-Cel sponges absorb liquid rapidly, reaching a maximum absorption volume of 350 μl in vitro. Table 1 summarizes the volume and dilution factors obtained for material collected when the sponges were allowed to absorb secretions in vivo for 2 min from three mucosal sites. The volumes of vaginal and cervical secretions collected from different women each day throughout the cycle demonstrated a small variability. More notably, the volumes of saliva collected varied dramatically.

TABLE 1. Mean volume of secretions and calculated dilution factors from mucosal sites[a]

Secretion type	Volume (μl)	Dilution
Cervical	29.1 ± 3.0	22.0 ± 3.4
Vaginal	17.3 ± 3.0	37.1 ± 3.7
Oral	105 ± 8.1	7.0 ± 1.3

[a] Volumes and dilutions were calculated based on 40 duplicate samples collected from four women throughout their menstrual cycle. Values are means ± standard errors.

Clinical specimen analysis. To determine the volume of secretions absorbed into the sponges, the final optimized method developed through these studies was used. Each individual sponge was weighed. The sponges were then equilibrated in 300 μl of extraction buffer for 30 min at 4°C. The diluted secretions were separated from the sponge using a Spin-x centrifuge filter unit (Costar) spun at 16,000 × *g* for 15 min at 4°C. Another 300 μl of buffer was added to each spear, and centrifugation at 16,000 × *g* for 15 min was performed immediately. In calculating the final concentration of immune components measured in the secretions, a dilution factor was determined based on the following formula: dilution factor = $[(x-0.06\text{ g}) + 0.6\text{ g of buffer}]/(x-0.06\text{ g})$, where x equals the weight of the sponge after collection and 0.06 g is the weight of the dry spear. The density of the buffer is 1.005 g/ml. Therefore, by conversion, 0.6 ml of buffer is 0.603 g. Given that x is the weight (in grams) of the sponge after collection, the dilution factor can be determined as a dimensionless number. (Note that the weight of the dry sponge is dependent on the lot of the sponges; therefore, the dry weight of the sponge was determined by measuring 10 sponges in each lot. This weight will vary ±3%.) This dilution factor was used to calculate the final concentration of cytokines and Ig in the secretions. Extracted samples were tested for blood content by using Hemastix strips (VWR Scientific Products, Bridgeport, N.J.). Any samples with blood contamination were discarded and not analyzed.

Statistical analysis. Extraction results were analyzed with Statview software. Differences between concentration measurements observed at days 9 and 20 of the menstrual cycle were determined by nonparametric analysis using the Mann-Whitney test. Significance was set at a P value of ≤0.05.

RESULTS

Optimization of the extraction process. The extraction buffer used to separate the protein components of the secretions from the surface and internal matrix of the sponge consisted of PBS, 100 μg of the protease inhibitor aprotinin per ml, 0.1% sodium azide, and 0.25 M sodium chloride. During development of the extraction process, various concentrations of sodium chloride were tested for effects on extraction of Ig. However, increasing the concentration of NaCl beyond 0.25 M did not enhance recovery of cytokines from sponges. Another component of the buffer in the original protocol was FCS, which was initially added to the extraction buffer to aid in release of protein. However, during the analysis of clinical specimens, the FCS interfered with the measurement of blood contamination in cervical secretion specimens. To determine if FCS was necessary for release of protein, a series of triplicate sponges were loaded with IgA and extracted; 3.6 ± 0.2 and 3.5 ± 0.2 μg/ml (mean ± standard deviation) were obtained from the samples with and without FCS in the extraction buffer, respectively. No significant difference between the two buffers was found. In the final protocol FCS was added to the samples after measurement of blood contamination.

Various equilibrium soaking times and soaking temperatures were tested to maximize diffusion of protein from the sponge into the extraction buffer. The sponges containing known quantities of cytokines and Ig were allowed to incubate for 30 min in the extraction buffer at 4°C or room temperature, in order to reach an equilibrium. Table 2 summarizes the results of this analysis. Comparable recoveries were obtained for IL-2, IL-4, IL-5, IL-6, IL-12, GM-CSF, IFN-γ, and IgA at room temperature and 4°C. However, at room temperature, a 50% reduction in IL-10 compared to the recovery at 4°C was noted. In addition, the variability between triplicate samples

TABLE 2. Cytokine recovery from sponges after a 30-min soak at room temperature or 4°C[a]

Protein	% Recovery at:	
	Room temp	4°C
IL-2	60.1 ± 5.2	55.1 ± 4.3
IL-4	0	0
IL-5	93.7 ± 12.34	88.1 ± 10.9
IL-6	34.8 ± 12.6	40.5 ± 4.4
IL-10	29.6 ± 12.6	54.2 ± 7.4
IL-12	83.5 ± 11.4	78.8 ± 5.4
GM-CSF	62.7 ± 10.6	60.0 ± 3.9
IFN-γ	0	0
IgA	99.5 ± 10.3	98.3 ± 5.6

[a] Data are means ± standard deviations from three experiments using one wash with high-salt extraction buffer followed by centrifugation at 16,000 × *g*.

was greater in the specimens equilibrated at room temperature. To ensure the stability of all the cytokines, all subsequent studies used 4°C for equilibrium soaking.

A 4°C incubation combined with the higher concentration of salt in the extraction buffer consistently led to 98 to 100% recovery of Ig and between 80 and 100% recovery for IL-5 and IL-12. However, IL-2, IFN-γ, and IL-4 were problematic, with a consistently low recovery (0 to 30%). In an attempt to improve the extraction of the troublesome cytokines, equilibration times of 30, 60, 90, and 144 min were evaluated. Thirty minutes proved optimal, as no increases in recovery were demonstrated by soaking longer than 30 min. Second and third wash steps were also evaluated with respect to recovery of these cytokines. The extra wash steps did not improve recovery of IL-2 or IFN-γ. Recovery of IL-12, IL-10, IL-5, and GM-CSF was slightly increased by the incorporation of a second wash step (8, 2, 3, and 8%, respectively); the second wash step demonstrated minimal extraction of IL-4. However, recovery of IL-6 was improved by nearly 20% and IL-4, for which no recovery was observed with one wash, was recovered at 6% after the second wash. Furthermore, a third wash step did not enhance recovery. Experiments were also conducted in which the speed of centrifugation was increased from 12,000 to 16,000 × *g*. With the higher speed, recovery of IL-2 was increased from 46.6 to 85.3%, but neither IL-4 or IFN-γ was obtained at a better rate. No increase in recovery was observed when the time of centrifugation was increased to 60 min.

Given the clinical impracticality of sample collection followed by immediate sample extraction and analysis, it was important to assess the effect of freezing on the various stages of sample handling. The effect of a freeze-thaw cycle on cytokine recovery was assessed. Recovery for sponges extracted and quantitated with no freezing was compared to the recovery for sponges that were frozen prior to extraction and recovery from samples which were extracted and then frozen. No significant decrease was detected in the analysis of cytokines or Ig from samples exposed to a freeze cycle (data not shown).

Using the final extraction procedure, the maximum recovery of each cytokine was determined. A summary of both cytokine and Ig recovery is found in Table 3. The inability of the procedure to increase the recovery of IL-4 or IFN-γ suggests that the molecular properties of the molecules influence the extraction. However, the molecular weight of the cytokines was not found to correlate with the recovery of the particular cytokines or Igs evaluated.

Cytokines in cervical secretions. To establish normal concentrations of cytokines in cervical secretions and demonstrate the usefulness of the described collection method, clinical samples from seven women were analyzed to determine the concentrations of IL-5, IL-6, IL-10, IL-8, IL-12, and GM-CSF. The detectable cytokine concentrations at two time points in the menstrual cycle are summarized in Table 4. No statistically significant differences between cycle days 9 and 20 were found. Although GM-CSF and IL-5 were analyzed, no detectable levels were found.

TABLE 3. Cytokine recovery from sponges using final protocol

Protein	Molecular mass (kDa)[a]	% Recovery[b]
IL-1β	17.5	92.0 ± 5.0
IL-2	15	85.3 ± 6.9
IL-4	15–19	5.0 ± 0.7
IL-5	18	90.3 ± 6.8
IL-6	20.8	50.5 ± 2.1
IL-8	8	58.7 ± 3.7
IL-10	18.7	55.1 ± 4.3
IL-12	75	85.9 ± 3.1
IFN-γ	34–50	0
GM-CSF	18–32	63.3 ± 2.0
IgG	150	104.0 ± 6.3
IgA	160	98.3 ± 5.6

[a] From reference 2.
[b] Determined by comparing ELISA quantitation of extracted cytokine to stock solution of cytokine not absorbed on sponges. Data are means ± standard deviations for three sponges.

DISCUSSION

Our laboratory previously described a technique for the collection of mucosal secretions (3). Initial studies suggested that the extraction procedure used to recover Ig from the sponges was not appropriate for cytokine recovery. IL-4, IL-2, and IFN-γ were not obtained from the sponges and IL-10 and IL-6 were extracted at very low rates using the method previously described. In order to quantitate antibody or cytokine concentrations, an efficient and consistent processing step was necessary to ensure release of protein from the sponges.

Cytokine recovery was increased by altering the existing protocol. The final extraction procedure utilized the standard extraction buffer previously described, but the sponges were incubated at 4°C for 30 min with extraction buffer prior to the first 15-min centrifugation at 16,000 × *g*. A second wash with 300 μl of extraction buffer followed by immediate centrifugation was also incorporated into the final protocol. These changes in the extraction procedure resulted in significant increases in the amount of cytokine obtained without altering the rate of Ig recovery. Of the nine cytokines examined, only IFN-γ and IL-4 bound within the sponges and were essentially impossible to extract. More than 50% each of the IL-6, IL-10, and GM-CSF loaded on the sponges was recovered with the new technique. IL-1β, IL-2, IL-5, and IL-12 had recovery rates greater than 85%. Consistency in the ability to recover cytokines was demonstrated through repeated experiments; variation between extractions was only 3 to 7%. Although 100% of the individual cytokines are not obtained from the sponges, investigators can be confident that this method can extract cytokines from the sponge matrix in a reproducible and reliable manner.

The depressed recovery may be attributed to a number of factors. It may be due to physical entrapment of the cytokine within the sponge matrix or to chemical degradation of the cytokines. In particular, IFN-γ and IL-4 have inherent stability problems, suggesting that the molecules are unstable in the

TABLE 4. Mean cytokine concentrations in cervical secretions

Cytokine	Assay detection limit[a] (pg/ml)	Cytokine concn[b] (pg/ml)		No. of samples (n = 14) below detection limit[c]
		Cycle day 9	Cycle day 20	
IL-5	4.0			14
IL-6	0.104	6,898.4 ± 3,156.6	3,255.4 ± 1,198.6	0
IL-8	10.0	138,297.1 ± 52,503.6	54,415.4 ± 11,208.3	0
IL-10	0.028	342.7 ± 284.2	975.7 ± 864.8	1
IL-12	0.8	322.7 ± 123.2	387.1 ± 262.1	1

[a] Level of detection was determined based on product information.
[b] All final concentrations were calculated by using a dilution factor and are means ± standard errors for seven women.
[c] IL-10 was below the assay detection limit on cycle day 9 in one patient, while IL-12 was below the detection limit on cycle day 20.

extraction buffer, or have altered conformation such that the quantitation by ELISA methods is inaccurate. An alteration in the structure of IL-4 or IFN-γ may also expose residues in the molecule that react with the polymer matrix of the sponge, resulting in binding and entrapment of cytokine within the sponge matrix (13). The only structural observation that corresponded to recovery rate involved the number of disulfide bonds present in the cytokine structure. IL-2, IL-5, and IL-12 each contain only one disulfide bond (2). Their recovery rates were 85, 90, and 86%, respectively. Cytokines with two disulfide bonds include IL-6, IL-8, IL-10, and GM-CSF (2), and the recovery rates for these cytokines were 50, 59, 55, and 63%, respectively. IL-4, with three disulfide bonds, had only a 5% recovery rate. This trend suggests that percent recovery decreases with increases in the number of disulfide bonds. However, the trend did not hold true for IFN-γ, which has no disulfide bonds. Why IFN-γ is lost on the sponges is unclear.

The ophthalmic-sponge technique was used to analyze cytokine concentrations in the cervical secretions of women using oral contraceptive pills. Of all the cytokines analyzed, IL-6, IL-8, IL-10, and IL-12 demonstrated consistent detectable levels in the secretions. This result differs from those of previous studies examining cervical mucus (5), where only low levels of IL-6 and IL-10 were demonstrated and the cytokines in samples from most subjects were below the level of assay detection. This difference may be accounted for by the different collection method and ELISA kits used for the concentration determinations. IL-8 concentrations in users of oral contraceptive pills were higher than concentrations of other cytokines both early and late in an oral-contraceptive-mediated menstrual cycle (138.3 ± 52.5 and 54.4 ± 11.2 ng/ml, respectively). No statistically significant differences were noted in the concentrations measured at day 9 or 20 of the menstrual cycle for any of the observed cytokines. This is unlike IL-8 or IL-6 concentrations in healthy cycling women, who demonstrate significant variability in the concentration of each cytokine over the menstrual cycle (4, 5). Similar findings were obtained by using other collection methods and for ovulating women (1, 10). The measurement of various cytokines in the cervical secretions demonstrates the complexity of the microenvironmental milieu of the cervix. These data represent an assessment of cytokine levels in healthy women and can be used to compare changes induced by infection or by the use of intravaginal products.

In summary, the ophthalmic-sponge technique is a useful and simple method for isolation of mucosal secretions. Consistent recovery of both Ig and cytokines allows for analysis of secretions from various mucosal sites. A limitation of this technology is the fact that at least two tested cytokines, IL-4 and IFN-γ, are not recoverable from the sponges. This suggests that investigators should test for binding to the Weck-Cel prior to initiating studies of a new cytokine or protein. However, the impact from data generated using this technique in vaccine and drug trials will significantly increase our knowledge of mucosal immunity and should far outweigh the limitations of the method.

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