Use of High-Resolution Confocal Imaging of the Vaginal Epithelial Microstructure to Detect Microbicide Toxicity

Gracie Vargas,1,2 Tuya Shilagard,1 Rebecca Johnston,1 Brent Bell,1 Rachael L. Stegall,3 Kathleen Vincent,3 Lawrence Stanberry,3,a Massoud Motamedi,1,4 and Nigel Bourne3

1Center for Biomedical Engineering and Departments of 2Neuroscience and Cell Biology, 3Pediatrics, and 4Ophthalmology, The University of Texas Medical Branch, Galveston, Texas

Objective. High-resolution optical imaging by confocal reflectance microscopy (CRM) was investigated for its ability to delineate the epithelial microstructure of the vaginal tract and detect alterations that may result from the use of vaginal microbicides.

Methods. The vaginal tracts of Swiss Webster mice treated with medroxyprogesterone acetate were exposed in vivo to a 4% nonoxynol-9 (N-9)–containing gel or saline. The vaginal tract was removed 4 h, 16 h, or 48 h after treatment and imaged by CRM without staining, and biopsy specimens were obtained from the imaged regions and processed for histological analysis.

Results. In control mice, CRM revealed a columnar epithelium and lamina propria with features resembling those observed via histological analysis. CRM revealed an exfoliated epithelium 4 h and 16 h after N-9 treatment, and quantitative measurement of epithelial thickness revealed a mean thickness (± standard error of the mean) of 41.7 ± 1.7 μm in control specimens, compared with 14.9 ± 4.5 μm for specimens obtained 4 h after treatment and 24.4 ± 2.1 μm for specimens obtained 16 h after treatment. Inflammation 4 h after treatment was indicated through detection of inflammatory infiltrates. In samples collected 48 h after treatment, the epithelium was regenerating. The time line of changes in the morphological structure and epithelial thickness detected by CRM closely resembled that of changes revealed by histological analysis.

Conclusions. This study demonstrates that CRM can delineate the epithelial structure and detect indicators of inflammation after treatment with N-9 and that it may be a useful imaging tool for evaluating the effects of vaginal microbicides.

Topical microbicides offer the possibility of a female-controlled means for protection against sexually transmitted infections (STIs). To date, no microbicide examined in phase III trials has been shown to be effective in preventing STIs. Of utmost importance in the development and evaluation of topical microbicides is safety, with recent efforts focused on the action of such agents on epithelial integrity. This issue became important after early microbicide candidates were shown to compromise the cervicovaginal epithelial barrier and to increase susceptibility to STIs, despite early, promising in vitro evidence to the contrary. Among these agents is nonoxynol-9 (N-9), which initially was shown to have effective antimicrobial activity against a number of STI agents in vitro, including human immunodeficiency virus (HIV) [1–3]. However, N-9 failed to act as an effective microbicide in a clinical trial [4], and in 2 efficacy trials frequent use was linked to an increase in the incidence of HIV infection [5, 6]. A trial of another microbicide candidate, cellulose sulfate, was also recently terminated in light of safety concerns [7]. These events have provided the impetus for an increased emphasis on new methods for preclinical assessment of microbicide safety [8–10].
Factors such as epithelial disruption and recruitment of inflammatory infiltrates by topical agents may help increase a person’s susceptibility to STIs [8]. These factors are likely complex, and enhanced understanding of them requires methods that assess the spatiotemporal responses of the vaginal epithelium to topical agents. Colposcopy is an accepted standard for assessing vaginal irritation caused by topical agents. This white light–assisted visualization method provides tissue-level examination of the cervicovaginal surface and can identify gross findings, such as petechiae, erythema, and ulceration [11]. However, endoscopy cannot distinguish deep epithelial disruptions <1 cm in diameter from superficial or less deep disruptions unless there is bleeding [12], nor can it assess microstructural indications that the barrier function of the epithelium has been compromised. Few studies have correlated visual findings with analysis of biopsy specimens, complicating the interpretation of colposcopic findings [12]. Although colposcopy has been used in small-animal models for preclinical studies of microbicidal safety, its ability to detect damage is also limited. For example, in one study, colposcopy failed to detect N-9 toxicity despite evidence of epithelial exfoliation and of inflammation, as indicated by increased cytokine levels and macrophage infiltration [9].

Additional methods for investigating the effects of microbicides include histopathological analysis and cytokine mapping [8, 9, 13, 14]. Histopathological analysis allows visualization of the epithelial microarchitecture and detection of inflammation; however, the time required for ex vivo fixation and processing of tissue specimens can delay results by several days to weeks. Cytokine sampling of vaginal lavage specimens has the potential to provide a molecule-specific indication of inflammation by revealing levels of proinflammatory markers, such as interleukin 1 (IL-1), IL-6, and IL-8, that are believed to play a role in STI transmission [15]. This method does not give an indication of the epithelial microstructure and is not standardized, making interpretation of cytokine profiles difficult and limiting comparison of studies performed by different groups [16]. Thus, although the methods discussed above offer various advantages, their limitations indicate the need for additional and improved monitoring methods to assess epithelial integrity and responses to topical agents. Desired capabilities of such methods include the ability to assess the epithelial microstructure in (or approximately in) real time and to detect the presence of an inflammatory response.

Confocal reflectance microscopy (CRM) measures a reflected signal as a function of depth to provide high-resolution reconstructed images of tissue microstructure without the need for staining. To date, the principle applications of CRM have included the study of the morphological microstructure of normal and diseased tissues, such as skin; the diagnosis of malignancy in vivo; and use as an ex vivo adjunct to surgery [17–22]. CRM, in combination with contrast agents, has been investigated as a noninvasive method for detection of neoplasia in mucosal tissues from the mouth and cervix [23, 24]. We believe that, during preclinical studies of microbicide safety, CRM might be a useful tool for providing information on the epithelium’s integrity and response to microbicide actions, because the technique can provide immediate feedback, owing to its ability to evaluate fresh tissue specimens; because its high resolution can reveal focal and microstructural indicators of damage; and because it can assess the entire epithelium, owing to its depth-resolved capability. Thus, we evaluated the use of CRM as a method for studying the cellular-level morphological characteristics of the vaginal mucosa in mice that were treated with N-9.

**MATERIALS AND METHODS**

**Animal model and intravaginal inoculation.** Female Swiss Webster mice (Harlan) were pretreated with a single subcutaneous injection containing 2 mg of medroxyprogesterone acetate 1 week before receiving N-9. Medroxyprogesterone acetate treatment transforms the keratinizing stratified epithelium of the vaginal mucosa to a diestrous-like state in which the vaginal epithelium is thinned [25]. On the day of microbicide treatment, the vaginal vault was gently swabbed with a premoistened swab, and the animals received 0.03 mL of a vaginal contraceptive gel containing 4% N-9 (Conceptrol; Ortho Options) or phosphate buffered saline (PBS). Studies were approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch and conformed to the recommendations of the US Department of Health and Human Services [26].

**CRM protocol.** Animals were euthanized and their vaginal tracts removed for imaging 4 h (for 4 mice), 16 h (for 5 mice), or 48 h (for 4 mice) after N-9 treatment; 7 mice served as PBS-treated control animals. The excised cervicovaginal tract was opened longitudinally along the posterior side, and vaginal samples were placed on the sample stage with the mucosal surface facing the microscope objective. CRM was performed using a Zeiss 410 confocal laser scanning microscope. A 40×0.8 numerical aperture objective lens was used for delivery and collection of light from the sample. The sample was illuminated with 56-nm light from a krypton/argon ion laser (Melles Griot). Remitted 568-nm light was detected using cooled photomultiplier tubes (Hamamatsu Photonics). The imaging objective provided an x-y field of view of 320 × 320 μm. A pixel dwell time of 15.2 μs was used to obtain images. Z-stacks were collected using a 1-μm increment between z-planes to a depth of 150 μm. Z-stacks of 6 areas of the vaginal tract were obtained. The microscopist was blinded to the treatment conditions. MetaMorph (version 7.1 [Molecular Devices]) was used to process and reconstruct CRM z-stacks into 2-dimensional cross-sectional x-z views and 3-dimensional (3-D) views. Before reconstruction, each image in the z-stack underwent background subtraction of a blank field. Cross-sectional (x-z) views allowed for depth as-
assessment of fresh tissue CRM micrographs in the transverse direction, akin to histological sections.

**Histological processing.** Immediately after imaging, the entire vaginal tract was immersed in formalin for fixation (duration, 24 h). Punch-biopsy specimens were obtained from imaged regions and embedded in paraffin and sectioned transversely. Serial sections were stained with hematoxylin-eosin (H&E) for histological examination.

**Epithelial thickness determination.** Quantitative determination of epithelial thickness on CRM micrographs was determined by measuring the full depth from the tissue surface to the z-plane where collagen fibers were first visible, which indicates the beginning of the lamina propria. For each field of view, 8–12 randomly chosen regions were measured and their values averaged. H&E-stained sections were examined in the transillumination mode of a standard light microscope, and the distance from the surface to the basement membrane was measured to determine epithelial thickness.

**Statistical analysis.** Epithelial thicknesses were compared by 2-tailed 1-way analysis of variance, followed by Tukey’s post hoc test. All comparisons were 2 tailed.

**RESULTS**

**CRM and histological analysis of vaginal tissue from PBS-treated animals.** Figure 1 shows representative CRM images of freshly excised vaginal specimens from mice treated with PBS. Corresponding H&E-stained sections are also shown in figure 1. In PBS-treated animals, x-y micrographs of the surface showed epithelial cells with bright, highly reflective cytoplasms and dark nuclei (figure 1A). These surface cells had a large cytoplasmic volume, consistent with columnar cells from the mucosal surface. The lamina propria was identified by a fibrillar structure characteristic of the extracellular matrix. The basement membrane separating the epithelium from the lamina propria was defined as the site of the transition between basal cells and the extracellular matrix. The basal cells displayed minimal cytoplasm, with the primary feature being a dark nucleus. Cross-sectional (i.e., x-z) micrographs had a banded appearance composed of a bright region at the surface arising from surface columnar cells, followed by a dark band resulting from the high nuclear density at the basal layer, and a second bright region arising from the lamina propria (figure 1B). Light attenuation with depth eventually resulted in a total loss of signal. The high-resolution, 3-D–reconstructed views showed characteristic round mucosal cells at the epithelial surface (figure 1C). Histological analysis confirmed the presence of features that were identified by CRM. Columnar cells at the surface in H&E-stained sections had a large cytoplasmic volume, and cells near the basal layer had a small cytoplasmic volume relative to nuclear volume, in agreement with CRM findings (figure 1B). Findings of CRM and histological analysis of vaginal tissue from PBS-treated animals were comparable at all times examined.

**Impact of N-9 treatment on CRM and histological analysis.** Figure 2 shows representative CRM images and corresponding H&E-stained sections of freshly excised vaginal specimens at various times after treatment with N-9. CRM micrographs taken 4 h after N-9 treatment revealed substantial disruption of the epithelium (figure 2A). The characteristic surface columnar cells seen in PBS-treated control specimens were absent, and the epithelium had thinned, with only the basal layer remaining.
bright surface band that was seen in control specimens was not observed by CRM in cross-sectional images (figure 2B). In specimens from 2 of the 4 animals, cellular infiltrates resembling polymorphonuclear neutrophils (PMNs) were observed by CRM; features of these cells matched those of PMNs imaged elsewhere by CRM [19]. The surface view in the 3-D reconstruction showed a severely disrupted epithelium without the characteristic round mucosal cells found in control specimens and with surface debris and infiltrates (figure 2B). Corresponding histological analysis of the tissue showed that the majority of the epithelium was thinned and that it was fully denuded on ~20% of the surface. H&E-stained sections also showed evidence of inflammation with the presence of PMNs, thus confirming CRM findings by indicating an inflammatory response in addition to the thinned epithelium.

CRM of specimens from animals euthanized 16 h after N-9 treatment showed that the epithelium was denuded to the basal layer, although no inflammatory infiltrates were seen in these specimens. When viewed in 3-D, the characteristic round mucosal cells observed in the control specimens were absent; instead, the prominent feature was a fibrous structure characteristic of the extracellular matrix of the lamina propria. CRM findings were again confirmed by histological analysis, which showed that, on most of the surface, the epithelium had thinned substantially, down to the basal layer, in all samples. However, as
with CRM imaging, histological analysis showed no evidence of infiltrates in any of the 16-h samples.

Finally, for samples taken 48 h after N-9 treatment, CRM revealed that the mucosal surface was undergoing reepithelialization. CRM showed that the morphological characteristics of epithelial cells varied, but cells with bright cytoplasm were again present at the surface. Cross-sectional examination revealed that the thickness of the epithelium was greater than that for specimens obtained 4 h or 16 h after treatment and that surface cells appeared bright and round, although they were somewhat different morphologically from the surface cells in PBS-treated animals. The 3-D topographic view also differed markedly from that of the PBS-treated control specimens obtained at this time. In particular, cells at the surface did not have the round shape of the surface cells in control specimens. H&E-stained sections revealed reepithelialization of the vaginal surface, and the epithelium no longer showed evidence of exfoliation in any of the samples. Surface epithelial cells in these samples were not columnar (in contrast to observations for PBS-treated controls), and morphological characteristics of cells differed throughout the depth of epithelium, again mirroring observations made with CRM.

Measurement of changes in epithelial thickness. Cone et al. [9] previously showed a temporal correlation between changes in epithelial thickness and susceptibility to infection by herpes simplex virus type 2 (HSV-2) in mice treated with N-9. Consequently, we examined whether CRM could be used to measure the epithelial thickness of vaginal tissue and to follow the changes in thickness resulting from treatment with N-9 over time. CRM revealed that the epithelial thickness of specimens obtained 4 h ($P < .001$) and 16 h ($P < .05$) after N-9 treatment was significantly less than that of control specimens but that, for specimens obtained 48 h after N-9 treatment, the epithelial thickness had nearly returned to the baseline value (figure 3A). Measurements of epithelial thickness were also made using corresponding histological samples (figure 3B). Again, the results were comparable to those obtained by CRM, with significant epithelial thinning in specimens obtained 4 h or 16 h after N-9 treatment ($P < .001$ for each, compared with control animals) and a return to near-baseline levels in specimens obtained 48 h after treatment.

DISCUSSION

This study evaluated the use of CRM as a means for revealing the subsurface microarchitecture of the vaginal epithelium and for detecting epithelial changes resulting from topical treatment with microbicides. This technique has the potential for use as an adjunct to existing methods of assessment and as a tool to evaluate fresh tissue samples for approximately real-time monitoring of microstructural changes in the epithelium during preclinical studies. Similarly, the method could be useful for noninvasive imaging of explant models in the evaluation of tissue response to microbicides. In evaluating candidate microbicides, there is a real need to identify indicators of epithelial damage or toxicity that may be indicative of an increase in cervicovaginal susceptibility to STI. Because there is evidence to suggest that disruption of the epithelial barrier is linked to increased STI susceptibility, direct imaging of the epithelial microarchitecture could be an important tool during preclinical testing of candidate microbicides. A study by Cone et al. [9] used a mouse model of HSV-2 transmission to correlate the time line of N-9 toxicity with that of susceptibility to HSV-2 infection. The factors examined were the degree of epithelial exfoliation, the number of macrophages in the vaginal lumen, and the levels of inflammatory cytokines. Although there were increased levels of inflammatory cytokines and macrophages at times of increased susceptibility, the time line of changes in epithelial thickness was found to be the factor that best correlated with the course and duration of increased susceptibility. Another interesting result of that study was that colposcopy failed to detect visible signs of N-9 toxicity despite evidence of epithelial exfoliation and inflammation.

During the preclinical evaluation of microbicide safety, it is important to ensure that testing is as rigorous as possible before candidate agents move into the clinical setting, particularly given the unexpected failure of previous candidate agents. It is advantageous to use models in which the risk of epithelial damage is
highest, to facilitate high sensitivity for detecting microbicidelled effects. Medroxyprogesterone acetate pretreatment, as used in our study, results in a diestrous-like state in which the vaginal epithelium is thinned [25]. It is expected that this pre-treatment may result in more pronounced microbicide-elicited damage than may be seen during other phases of the estrous cycle, thus satisfying the desire for a sensitive preclinical model. It will be of interest to evaluate CRM in studies involving naturally cycling mice whose vaginal epithelial structure is constantly changing.

In the present study, CRM allowed specific epithelial structures, including surface mucous columnar cells, basal cells, and the fibrillar extracellular matrix of the lamina propria, to be identified in fresh unstained tissue to depths of 100–125 μm. Corresponding histological analysis served as a means to corroborate these microstructural findings through visual comparison of cross-sectional (x-z) CRM micrographs with cross-sectional histological sections. For example, the morphological characteristics of the large mucous columnar cells present at the surface of the PBS-treated control tissue in H&E-stained sections were similar to the characteristics revealed in CRM micrographs. CRM was also shown to successfully follow changes in epithelium after single-dose treatment with N-9, as CRM characterization of these changes mirrored those detected by histological analysis. Changes revealed by both CRM and histological analysis included a decreased number of surface columnar cells and epithelial thinning up to 16 h after treatment, partial reepithelialization 48 h after treatment, and inflammatory infiltrates 4 h after treatment.

The time line of changes in the vaginal epithelium revealed by CRM is consistent with data from published studies in which N-9 was shown to lead to epithelial disruption, including sloughing and complete epithelial loss, in uterine columnar epithelium [27] and cervicovaginal epithelium [14]. A study by Dayal et al. [27] reported that exposure to a gel containing 3.5% N-9 resulted in disruption of uterine columnar epithelium after 24 h, in contrast to exposure to a control gel or water, with epithelial regeneration detected 48 h after N-9 treatment and complete restoration by 72 h after treatment. An interesting finding was that the regenerated epithelium consisted of cuboidal cells instead of the normal columnar cells. This emulates the findings we obtained via CRM and histological analysis, which revealed that the regenerating epithelium had nearly recovered its baseline thickness but consisted of morphologically altered cells.

The ability to observe indicators of inflammation is an important characteristic of this imaging method, because an inflammatory response may be linked to increased susceptibility to STIs caused by certain pathogens, including HIV. Infiltrating inflammatory cells were observed by CRM 4 h after N-9 treatment. Infiltrates were also identified in H&E-stained sections at this time. Inflammatory infiltrates were not observed by CRM 16 h after treatment, a finding also confirmed by histological analysis. Published studies have reported temporal modulations in levels of inflammatory markers that coincide with the trend we observed. For example, Catalone et al. [14] noted that a single dose of N-9 elicited an inflammatory response within 2–4 h that accompanied columnar epithelial disruption and that, although the epithelium remained denuded 8 h after treatment, the inflammatory response was largely gone and remained absent 24 h after treatment. Similarly, in the current study, inflammatory infiltrates were identified by CRM in samples obtained 4 h after treatment but not in those obtained 16 h or 48 h after treatment.

Although all studies described here were conducted using ex vivo tissue specimens, there is the potential for in vivo imaging in the future. With the advancement of emerging technologies such as confocal microendoscopy, in vivo preclinical and clinical assessment will be possible. CRM has been used in vivo in the past to examine more-accessible tissues, such as skin and human oral mucosa, to depths of >300 μm [18–24]. Recent developments in optical technology offer the promise of endoscopic in vivo imaging as well. Such advances include the development of fiber-optic confocal reflectance microscope systems with miniature imaging components [28–31]. Fluorescence-based confocal systems with endoscopic imaging capabilities and imaging probes small enough to fit in the lumen of cervicovaginal tracts of small animals have also recently been developed [32]. These emerging technologies will also make possible repeated in vivo imaging during longitudinal studies.

In summary, CRM was successfully used to detect subsurface microarchitectural changes in cervicovaginal mucosa treated with a contraceptive gel containing N-9. CRM followed changes in the epithelial structure (i.e., exfoliation, regeneration, and cellular morphological characteristics) and revealed an inflammatory response through detection of inflammatory infiltrates. High-resolution imaging by CRM or similar optical microscopy techniques may be useful adjuncts to traditional histological and colposcopic analyses in the assessment of the effects of microbicides on cervicovaginal tissue.

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References


