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Evaluation of immunological markers of ovine vaginal irritation: Implications for preclinical assessment of non-vaccine HIV preventive agents

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Abstract

The presence of genital inflammatory responses and a compromised vaginal epithelial barrier have been linked to an increased risk of HIV acquisition. It is important to assure that application of candidate microbicides designed to limit HIV transmission will not cause these adverse events. We previously developed high resolution *in vivo* imaging methodologies in sheep to assess epithelial integrity following vaginal application of a model microbicide, however characterization of genital inflammation in sheep has not been previously possible. In this study, we significantly advanced the sheep model by developing approaches to detect and quantify inflammatory responses resulting from application of a nonoxynol-9-containing gel known to elicit vaginal irritation. Vaginal application of this model microbicide resulted in foci of disrupted epithelium detectable by confocal endomicroscopy. Leukocytes also infiltrated the treated mucosa and the number and composition of leukocytes obtained by cervicovaginal lavage (CVL) were determined by differential staining and flow cytometry. By 18 h post-treatment, a population comprised predominantly of granulocytes and monocytes infiltrated the vagina and persisted through 44 h post-treatment. The concentration of proinflammatory cytokines and chemokines in CVL was determined by quantitative ELISA. Concentrations of IL-8 and IL-1 β were consistently significantly increased after microbicide application suggesting these cytokines are useful biomarkers for epithelial injury in the sheep model. Together, the results of these immunological

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assessments mirror those obtained in previous animal models and human trials with the same compound and greatly extend the utility of the sheep vaginal model in assessing the vaginal barrier and immune microenvironment.

Keywords

sheep; vaginal inflammation; microbicide safety evaluation model; confocal endomicroscopy

1. Introduction

Multiple approaches are being pursued to reduce the spread of HIV infection including behavioral programs promoting monogamy or abstinence, the use of barrier prophylactics and the development of antiviral therapies including HIV vaccines and topically-applied or locally-released microbicide compounds. The benefits of topically-applied microbicides include the potential for female control of preventative products, self-administration, relatively inexpensive and uncomplicated production, and the availability of the microbicide in several different physical application formulations such as films, gels, or intravaginal rings.

Different types of broadly active compounds with disparate physicochemical properties have been developed as candidate microbicides including polyanionic microbicides (e.g. cellulose sulfate, Carraguard, and PRO 2000) (Van Damme *et al.* 2008), surfactants (e.g. nonoxynol-9 [N-9]) (Van Damme *et al.* 2002), dendrimers (for example VivaGel natural antimicrobial peptides (Tanphaichitr *et al.* 2016) and virucidal acid buffering gels (e.g. BufferGel) (Mayer *et al.* 2001). Although preclinical testing of some microbicides was initially positive, results from clinical trials have been disappointing (Van Damme *et al.* 2002, Wilkinson *et al.* 2002, Grant *et al.* 2008, Van Damme *et al.* 2008).

One of the problems identified with some early microbicide failures was the development of inflammation and accompanying damage or disruptions to the vaginal epithelial barrier. This inflammatory response also included the infiltration of inflammatory leukocytes into the cervical and vaginal epithelium including known targets for HIV infection. For example, while N-9 formulations were shown to inactivate numerous sexually transmitted pathogens *in vitro* including *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Treponema pallidum*, and herpes simplex virus (Singh *et al.* 1972, Bolch and Warren 1973, Benes and McCormack 1985) use of a N-9 containing film in a phase 1 trial resulted in an increase in vaginal lesions (Niruthisard *et al.* 1991, Rustomjee *et al.* 1999) and increased infection rates with HIV and *N. gonorrhoeae* (Richardson *et al.* 2001, Van Damme *et al.* 2002). The mechanism responsible for the enhanced infection rates has been linked to N-9-induced injury or irritation of epithelial cells with subsequent release of proinflammatory cytokines and infiltration of innate immune cells (Fichorova *et al.* 2001). Issues with genital irritation or perturbation of genital microbiota have also been associated with use of other types of candidate microbicides (Peterson *et al.* 2007, Carballo-Diequez *et al.* 2012, Keller *et al.* 2012).

Safety testing of new compounds designed to avoid inflammation will require assessment of compounds in appropriate animal models to evaluate potential irritation or toxicity associated with vaginal application. Early phase nonclinical safety testing has primarily been performed in tissue culture, mice, and non-human primates. Candidate compounds in more advanced stages of development have been tested to evaluate chemical toxicity using the rabbit vaginal epithelial model (Eckstein *et al.* 1969, Lard-Whiteford *et al.* 2004). While this species is sensitive to vaginal irritants, the vaginal epithelium of rabbits is comprised of a single layer of columnar epithelial cells rather than the stratified squamous cells found in humans. Traditionally, nonhuman primates have also been used in advanced stages of preclinical testing of microbicides, however cost and limitation in availability are barriers for use in early stages of drug development. Recently, the sheep has emerged as a promising model for evaluating the effects of candidate microbicides on the integrity of the vaginal epithelium. Sheep cervicovaginal tissues are similar to human tissues and the physical size of the genital tract of sheep allows evaluation of the actual products and doses to be used in humans. Additionally, sheep are readily available, docile, and suitable for evaluation with various emerging *in vivo* imaging modalities that can be used noninvasively and repetitively over time to evaluate vaginal mucosal integrity along with pharmacokinetic assessments. We have previously used high resolution optical coherence tomography (OCT) and confocal endomicroscopy (CE) to image cervicovaginal and colorectal epithelial tissues following application of microbicide compounds in this model (Vincent *et al.* 2009, Vincent *et al.* 2013, Vargas *et al.* 2016). However, to date, it has not been possible to assess inflammatory parameters associated with vaginal irritation or microbicide toxicity using the sheep model. In this study, we report the development of methodologies to detect and quantify cellular and molecular markers of vaginal inflammation which will further enhance the utility of the sheep model for the comprehensive preclinical safety evaluation of novel microbicide candidates.

2. Materials and Methods

2.1 Sheep

Virginal Rambouillet Merino sheep weighing 25–35 kg were fasted the evening before treatment with PBS or N-9-containing gel (Gynol II ES vaginal contraceptive gel, Ortho Pharmaceuticals, active ingredient 3% N-9). Groups of 2–4 sheep were sedated intramuscularly with 10 mg/kg Ketamine followed by intravenous administration of 10 mg/kg ketamine and 0.1–0.2 mg/kg Diazepam to allow supine placement on a table for delivery of the gel and collection of lavage fluids. Immediately prior to microbicide treatment, a baseline cervicovaginal lavage (CVL) was taken by delivery of 10 ml phosphate buffered saline (PBS) to the vaginal tract using a syringe with ball-tipped catheter. Fluid was triturated three times before collection. This procedure was performed twice and the CVL was immediately processed for cytokine evaluation and cell differentials (see below). An 8.0 ml bolus of N-9 gel was applied to the vaginal mucosa. Additional CVL samples were collected in sedated sheep at 18 and 44 h post treatment for evaluation of cytokine and cellular constituents. Animal studies conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch.

2.2 Reagents

The ovine-specific reagents used in cytokine ELISA and flow cytometry assays are shown in Table 1.

2.3 Characterization of CVL leukocytes

After allowing debris to settle, viable CVL cells were quantified by trypan blue exclusion. The CVL was centrifuged and the supernatant was removed for detection of cytokines. The cell pellet was re-suspended to 10^6 cells/ml in Hank's balanced salt solution, 5.0 % newborn calf serum and 1.0 % penicillin/streptomycin. Approximately 10^5 cells were transferred to glass slides using a cytospin centrifuge and stained using a Hema3 Stat Pack kit (Fisher Scientific Company L.L.C., Kalamazoo, MI) according to the manufacturer's instruction.

A modification of the flow cytometry method described previously (Nelson *et al.* 2011) was used to characterize CVL leukocytes. Briefly, approximately 10^6 CVL cells were transferred to wells of a 96- well, V-bottom assay plate. Fc receptors were blocked, cell surface markers were stained with unconjugated primary antibodies followed by secondary staining with PE- or FITC-conjugated rat anti mouse IgG1. Cells were fixed in 1.0 % formaldehyde and data were acquired on a BD FACSCanto II (BD Biosciences) at the UTMB Flow cytometry Core Facility and analyzed using FlowJo software (Tree Star, Ashland, OR).

2.4 Cytokine ELISA

Ninety-six well assay plates were coated with the appropriate anti-cytokine capture antibodies, blocked, and serial dilutions of recombinant cytokine standards for generation of a standard curve and experimental CVL samples were plated and incubated overnight at 4° C. Appropriate anti-cytokine detection antibodies were added and incubated at 37° C for 1 h. Plates were then washed and, for plates containing biotinylated detection antibody, streptavidin peroxidase was added and incubated at 37 °C for 1 h. For plates containing unconjugated detection antibody, HRP-conjugated antibodies specific for the species and isotype of the detection antibody were added and incubated for 1 h at 37° C. Plates were developed and OD₄₉₀ values were obtained as described previously (Johnson *et al.* 2010). Cytokine concentrations were calculated by comparison of the experimental OD₄₉₀ values to the cytokine standard curve using SoftMax Pro software program (Molecular Devices).

2.5 Imaging of the vaginal epithelium by CE

Images of the vaginal surface following 18 hours of treatment with PBS or the N-9 containing gel were obtained using a laser scanning confocal endomicroscope system (Optiscan FIVE1, Optiscan P/L, Notting Hill, Victoria, Australia) as described previously by our group (Vargas *et al.* 2016). Briefly, illumination and collection of fluorescence was through a single 5mm diameter endoscopic probe inserted into the vaginal tract and placed on the vaginal mucosal surface. Illumination was at 488 nm, and fluorescence collected in the spectral range of ~505–750 nm. Collected images had a lateral optical resolution of 0.7 µm, and axial resolution of 10 µm and with a field of view of 475×475 µm. An 8.0 ml bolus of propidium iodide (20 µM) was applied topically to the vagina *in vivo* as performed in previous studies (Vargas *et al.* 2012) and then rinsed after five minutes to label nuclei and

any DNA material. Single *en face* images were taken at the surface at multiple sites on the vaginal mucosa along the dorsal, ventral, and lateral walls.

2.6 Statistics

Statistical differences between groups were tested using a Mann-Whitney test and values for $P < 0.05$ were considered significant. All calculations were performed using GraphPad Prism software version 5.0 (GraphPad Software, San Diego, CA).

3. Results

3.1 Assessment of vaginal epithelium integrity by CE

As an initial validation of candidate microbicide testing in the sheep vaginal model, we utilized an N-9-containing gel as a positive control known to cause local epithelial disruption and inflammatory responses following vaginal application (Niruthisard *et al.* 1991, Fichorova *et al.* 2001, Milligan *et al.* 2002, Vargas *et al.* 2012). CE was used to visualize and assess the integrity of the vaginal epithelium following application of PBS or N-9 gel, following previously established methodology (Vargas *et al.* 2012). Topical staining with PI results in the labeling of DNA material, thus staining the nuclei of cells on the surface of the mucosal epithelium. Figure 1A shows a representative image of the vaginal epithelium 18 hours after PBS application that reveals regularly spaced nuclei having well-defined borders and an intact structure. The organization of nuclei across the vaginal surface is regular, mirroring the pattern reported in our previous study for native or PBS-treated vaginal mucosa (Vargas *et al.* 2012). By contrast, Figure 1B demonstrates the vaginal epithelium at 18 h following application of N-9 gel in which scattered foci of disrupted epithelium and disorganized nuclei are apparent throughout the surface. While the shapes and borders of many nuclei are still apparent, there are areas showing aggregates of cellular material (debris) and the normal regular, widely-spaced pattern of nuclei across the surface is no longer present. Arrowheads denote areas having closely-packed nuclei expected to occur at deeper layers in a normal epithelium, but now exposed due to loss of the surface layer following N-9 application. This is consistent with structural and cellular damage caused by N-9 to the vaginal surface.

3.2 Analysis of CVL leukocytes

To provide an immunological assessment of vaginal irritation using the sheep vaginal model, we collected CVL prior to, and at 18 and 44 h after vaginal application of N-9 gel and characterized the collected cell population. As shown in Figure 2A, a small population of cells was present in the vaginas of all sheep prior to N-9 gel application. While epithelial cells were readily detected in CVL at this time (Fig. 2B), small numbers of leukocytes with granulocyte morphology were also observed. By 18 h post-treatment, the number of total viable genital leukocytes increased in individual animals between 50-384-fold and the mean number of leukocytes was significantly higher compared to the pretreatment group ($P < 0.05$, Mann-Whitney test). Large numbers of these cells exhibited granulocyte morphology. The leukocyte infiltrate was still detectable in lavage fluid at 44 h post treatment and the viable cell count in individual animals increased by 10-846-fold compared to the 0 h time point (Fig. 2A). The mean number of CVL leukocytes was significantly higher at 44 h

compared to the 0 h time point ($P < 0.05$, Mann-Whitney test). The composition of the leukocyte population changed slightly during this time with greater numbers of leukocytes with monocyte morphology present in addition to granulocytic cells.

The identity of the inflammatory cells in the vagina following N-9 gel treatment was confirmed by flow cytometry (Fig. 3). CD4⁺ cells were not detected in CVL of any sheep prior to or at 18 h following N-9 gel treatment, however, a small percentage of both CD4⁺ and CD8⁺ cells were detected at the 44 h time point. CD11b is expressed non-covalently with CD18 to form the Mac-1 integrin and is expressed primarily on monocytes, macrophages, granulocytes, dendritic cells and Natural Killer cells. CD11b was expressed on approximately 25% of CVL cells obtained at the 0 h time point. By contrast, the majority of CVL leukocytes collected at 18 and 44 h post-treatment (range 74–96%) were CD11b⁺ inflammatory cells. At 18 h post treatment, this inflammatory population was comprised predominantly of a CD14⁺ intermediate-staining granulocyte population and a smaller CD14⁺ high-staining monocyte population. The frequency of CVL granulocytes generally declined slightly by 44 h post treatment whereas the frequency of CD14 high vaginal monocytes trended slightly higher.

3.3 Detection and quantification of proinflammatory cytokines and chemokines

CVLs were collected from eight sheep prior to and at 18 and 44 h after N-9 -treatment for quantification of selected proinflammatory cytokines. As shown in Figure 4, the mean concentrations of IL-8 and IL-1 β increased following N-9 treatment and were significantly higher at 18 h post treatment compared to baseline whereas the mean IL-6 concentration after N-9 treatment remained near baseline levels. Further, all eight sheep experienced significantly increased IL-8 levels at the 18h and/or 44h time points compared to pre-treatment levels. Similarly, seven of eight sheep experienced significantly higher IL-1 β levels at the 18 h or 44 h time points compared to baseline levels. By contrast, levels of IL-6 did not change significantly from baseline in any sheep following N-9 gel treatment. Post-treatment levels of CXCL-10, IL-17 α , and TNF- α were detected in CVLs from only three or fewer sheep from the eight animals tested (not shown).

4. Discussion

Many of the first generation microbicides were unsuccessful in clinical trials resulting in either a failure to prevent, or occasionally, increasing the risk of HIV infection (Van Damme *et al.* 2002, Van Damme *et al.* 2008). The disruption of the vaginal epithelium, release of proinflammatory cytokines, and induction of inflammatory cell infiltrates including potential targets of HIV infection resulting from application of some of these candidates has been linked to the increased risk of HIV acquisition. In response to the continued need for topical antimicrobial products, new candidate microbicides including co-formulations of microbicides with different modes of action and novel methods of delivery are currently under development. To understand the interaction of these compounds and delivery formats with the genital mucosa and specifically to understand the potential of these compounds for initiating genital inflammation, it is critical to utilize animal models in which both mucosal integrity and potential development of unwanted inflammatory responses can be assessed. A

sheep vaginal model has been previously used to determine the toxicology and pharmacokinetics of a candidate microbicide (Holt *et al.* 2015) and we have developed novel methods for high resolution imaging of the vaginal and rectal mucosa of sheep to assess epithelial damage resulting from detergent-based microbicides (Vincent *et al.* 2009, Vincent *et al.* 2013, Vargas *et al.* 2016).

The vaginal epithelium represents a physical barrier to infection by sexually transmitted pathogens and disruption of the epithelium during coitus or as a result of inflammatory processes is thought to increase the risk of infection. Both increased- and no epithelial disruption compared to placebo treatments have been reported by colposcopy-assessment of the vaginal barrier in humans following utilization of N-9-containing microbicides (Rustomjee *et al.* 1999, Richardson *et al.* 2001) suggesting that more sensitive methods are required to accurately assess the integrity of the vaginal mucosa. We previously reported assessment of epithelial damage to rectal and cervicovaginal tissues in sheep in response to the cationic detergent benzalkonium chloride (BZK) by OCT (Vincent *et al.* 2009, Vincent *et al.* 2013). Although BZK has been reported to be more cytotoxic to vaginal cells than N-9 both *in vitro* and *in vivo* (Fichorova *et al.* 2004), we detected epithelial disruptions by CE in the cervicovaginal tissues of sheep following a single vaginal application of N-9 in the present study. Beyond the increased sensitivity of CE, the ability to perform repetitive, non-invasive high resolution imaging studies in sheep highlights an important improvement provided by this microbicide testing model.

To extend the utility of the sheep vaginal model, we developed methods to test for and quantify local vaginal inflammatory responses. The presence of high concentrations of inflammatory mediators, including chemokines such as IL-8 with the potential to recruit HIV target cells, increased the risk of HIV acquisition by women enrolled in the Centre for the AIDS Programme of Research in South Africa 004 phase IIb tenofovir gel trial (Masson *et al.* 2015). Additionally, increased concentrations of IL-1 β and IL-8 were detected over time in CVL after multiple treatments of rabbits with N-9 which correlated with increased rabbit vaginal irritation scores and cumulative inflammatory tissue damage (Fichorova *et al.* 2004). In humans, multiple vaginal applications of N-9 resulted in significant increases in IL-1 β and IL-8 over time (Fichorova *et al.* 2001). The results of the present study are in agreement with these studies as both IL-1 β and IL-8 levels significantly increased in the CVL after a single application of N-9 gel. Additionally, N-9 gel application in sheep resulted in the infiltration of a significant number of leukocytes at 18 h and 44 h after treatment. The population was determined phenotypically and by flow cytometry to be comprised predominantly of granulocytes and monocytes. CD4⁺ and CD8⁺ T cells were detected only at very low numbers at 44h post treatment. Similar cell types have been reported to infiltrate the vaginal mucosa of humans after treatment with N-9 (Fichorova *et al.* 2001). The stage of the estrous cycle was not determined for the ewes used in these studies and it is possible that the type or magnitude of leukocyte populations in the vaginal epithelium may have been altered as animals advanced through the estrous cycle. However, no visual evidence for a change in vaginal epithelium was detected between obtaining the background (0h) sample and the 18h and 44h samples strongly suggesting that the alteration in cell type and number observed at 18h and 44h post treatment reflected a response to the N-9 application. Together, these results show that inflammatory responses can be detected and characterized in the

sheep vaginal model and that these responses reflect with high fidelity those obtained in safety testing studies performed in laboratory animals and humans using the same active compound.

In conclusion, the sheep vaginal model is amenable to the use of repeated, non-invasive imaging modalities for assessment of the integrity of the epithelial barrier. Here, we report a marked advancement in this model and demonstrate the ability to detect and characterize the inflammatory environment arising from chemical irritation of the female genital mucosa. Moreover, the resulting inflammatory milieu that arises in sheep after vaginal application of an N-9 gel mirrors that observed in other experimental animal models and human clinical trials of N-9 application. Together, these results highlight the applicability and utility of the sheep vaginal model for microbicide testing. Utilization of this model should also prove useful in related studies to advance the assessment of genital wound healing and the influence of sex hormones on the dynamics of barrier function and immunologic environment of the vaginal epithelia.

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Highlights

1. Methods were developed to detect and measure inflammatory responses in the ovine vagina.
2. Altered epithelial integrity after N-9 application was detected by confocal endomicroscopy.
3. Neutrophils and monocytes infiltrated the ovine vaginal mucosa following N-9 gel application.
4. IL-8 and IL-1 β are potential markers of epithelial injury in the ovine vaginal irritation model.

Fig. 1.

In vivo confocal endomicroscopy of the ovine vaginal epithelium. (A). Image of vaginal epithelium 18 h after treatment with PBS control shows regularly-spaced nuclei (arrowheads) labeled with propidium iodide showing the typical mature surface epithelium. (B). The vaginal epithelium 18 hours following treatment with 3% N-9 gel shows injury with both aggregates of cellular debris (long arrows) and closely-spaced nuclei (arrowheads) that are consistent with loss of the superficial, more widely-spaced surface cell layer.

Fig. 2.

Inflammatory leukocytes infiltrate the vaginas of sheep following treatment with N-9 vaginal gel. (A) Viable cell counts in CVL of treated sheep. Results shown represent the cell counts from four individual sheep. * $P < 0.05$ compared to 0 h, Mann-Whitney test. (B) Differential analysis of CVL cells. CVL were collected at the indicated times and stained with a differential cell stain. Magnification is at 40 $\times$.

Fig. 3.

Flow cytometric identification of ovine CVL leukocytes. CVL were collected from sheep at the indicated times relative to N-9 gel treatment. Cells were stained as described in Methods for CD4, CD8, CD11b and CD14. Representative results from one animal of seven animals analyzed are shown. A single isotype control was prepared for the 18 h and 44 h time points.

Fig. 4. Increased levels of IL-8 and IL-1 β serve as markers of epithelial damage in N-9 gel-treated sheep. CVL were collected from sheep at the indicated times relative to N-9 gel treatment. Lavages were processed and analyzed for the indicated proinflammatory cytokines as described in Methods. Results shown represent the mean of eight individual animals. * Denotes $P < 0.05$ compared to the pre-treatment value, Mann-Whitney test.

Table 1

Ovine-specific reagents used in these studies

	Specificity	Vendor	Vendor Location
Ovine ELISA Reagents			
Cytokine Capture Antibodies	IL-6, IL-8	EMD Millipore Corporation	Temecula, CA, USA
	IL-17 α , CXCL10	Kingfisher Biotech, Inc.	Saint Paul, MN, USA
	TNF α	LifeSpan BioSciences, Inc.	Seattle, WA, USA
	IL-1 β	United States Biological	Salem, MA, USA
Cytokine Detection Antibodies	IL-6, IL-8	EMD Millipore Corp.	Temecula, CA, USA
	IL-17 α , CXCL10 (biotinylated)	Kingfisher Biotech, Inc.	Saint Paul, MN, USA
	TNF α , IL-1 β (biotinylated)	United States Biological	Salem, MA, USA
	Rabbit IgG (HRP)	EMD Millipore Corp.	Temecula, CA, USA
Recombinant Protein Standards	IL-6, IL-8, IL-17 α , CXCL10, IL-1 β	Kingfisher Biotech, Inc.	Saint Paul, MN, USA
	TNF α	United States Biological	Salem, MA, USA
Ovine Flow Cytometry Reagents			
	Ovine CD4, CD8, CD14	Accurate Chemical & Scientific Corp.	Westbury, NY, USA
	Ovine CD11b	Research Technology Innovation (RTI)	Brookings, SD, USA
	Mouse IgG1 isotype control, Anti-mouse IgG1--PE, Anti-mouse IgG1--FITC	BD Pharmingen	San Diego, CA, USA