Biocompatibility of Solid-Dosage Forms of Anti-Human Immunodeficiency Virus Type 1 Microbicides with the Human Cervicovaginal Mucosa Modeled Ex Vivo

Radiana T. Trifonova, Jenna-Malia Pasicznyk, and Raina N. Fichorova*

Laboratory of Genital Tract Biology, Department of Obstetrics, Gynecology and Reproductive Biology, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts

Received 12 May 2006/Returned for modification 13 July 2006/Accepted 25 September 2006

Topical anti-human immunodeficiency virus (HIV) microbicides are being sought to reduce the spread of HIV type 1 (HIV-1) during sexual intercourse. The success of this strategy depends upon the selection of formulations compatible with the natural vaginal mucosal barrier. This study applied ex vivo-modeled human cervicovaginal epithelium to evaluate experimental solid-dosage forms of the anti-HIV-1 microbicidal cellulose acetate 1,2-benzedicarboxylate (CAP) and over-the-counter (OTC) vaginal products for their impact on inflammatory mediators regarded as potential HIV-1-enhancing risk factors. We assessed product-induced imbalances between interleukin-1α (IL-1α) and IL-1β and the natural IL-1 receptor antagonist (IL-1RA) and changes in levels of IL-6, tumor necrosis factor alpha, IL-8, gamma interferon inducible protein 10 (IP-10), and macrophage inflammatory protein 3α (MIP-3α), known to recruit and activate monocytes, dendritic cells, and T cells to the inflamed mucosa. CAP film and gel formulation, similarly to the hydroxyethylcellulose universal vaginal placebo gel and the OTC K-Y moisturizing gel, were nontoxic and caused no significant changes in any inflammatory biomarker. In contrast, OTC vaginal cleansing and contraceptive films containing octoxynol-9 or nonoxynol-9 (N-9) demonstrated similar levels of toxicity but distinct immunoinflammatory profiles. IL-1α, IL-1β, IL-8, and IP-10 were increased after treatment with both OTC vaginal cleansing and contraceptive films; however, MIP-3α was significantly elevated by the N-9-based film only (P < 0.01). Although both films increased extracellular IL-1RA, the cleansing film only significantly elevated the IL-1RA/IL-1 ratio (P < 0.001). The N-9-based film decreased intracellular IL-1RA (P < 0.05), which has anti-inflammatory intracellular functions. This study identifies immunoinflammatory biomarkers that can discriminate between formulations better than toxicity assays and should be clinically validated in relevance to the risk of HIV-1 acquisition.

Topical microbicides for vaginal application are currently under investigation as a promising strategy for the prevention of human immunodeficiency virus type 1 (HIV-1) infection by killing the virus or inhibiting its uptake and fusion with cells (5, 25, 39). The success of anti-HIV microbicides depends on their ability to maintain or enhance the vaginal and cervical mucosea of the female genital tract, which provides a natural protective barrier against infection, and may account for a relatively low risk of infection in healthy women (14, 33). In contrast, women with inflammatory conditions induced by pathogens or chemical irritants may be at higher risk of acquiring and transmitting HIV-1 due to cytokine-mediated HIV-1 activation, epithelial disruption, and recruitment of HIV-1 host cells (1, 35, 36, 43, 46). Furthermore, the prevalence of proinflammatory cytokines and chemokines may activate HIV-1 replication in submucosal viral reservoirs of women with latent viral infections (24, 26, 28, 34). Therefore, vaginal microbicides and other topical vaginal products used by women at risk for HIV-1 infection must be evaluated for their effects on the vaginal environment.

Personal hygiene and lubricating products that are currently available over the counter (OTC) have received little attention in this regard. The inflammatory potential of nonoxynol-9 (N-9) was the first to be clinically tested because of the extensive use of N-9 in chemical contraceptives and sexual lubricants. N-9 was considered a topical microbicide candidate because of its potent anti-HIV-1 activity in vitro, but unfortunately, formulations containing N-9 failed to protect against HIV-1 in clinical phase II/III trials, most likely due to epithelial lesions and inflammatory responses (reviewed in reference 21). These studies showed that neither conventional toxicology nor clinical symptoms routinely monitored in clinical phase I trials could predict the proinflammatory potential of N-9 or its effect on the natural mucosal barrier against HIV-1 and other sexually transmitted disease pathogens. On the other hand, animal and human studies showed that vaginal levels of proinflammatory cytokines and chemokines (interleukin-1α [IL-1α], IL-1β, and IL-8) may better predict the histopathological degree of mucosal inflammation induced by N-9 (13, 15–17).

In this study, we compared epithelial cytokine and chemokine profiles of newly developed solid-dosage formulations of the anti-HIV-1 microbicidal cellulose acetate 1,2-benzedicarboxylate (CAP) to those of OTC vaginal products with and without N-9. CAP belongs to the category of anionic polymer vaginal microbicide candidates (31, 42). Whether in soluble or micronized (Aquateric) form, CAP efficiently inactivates HIV-1 through multiple specific mechanisms (31, 32). With in vitro-reconstructed human vaginal-ectocervical epithelium ex-
pressing a stratified nonkeratinized squamous phenotype (VEC-100; MatTek Corporation, Ashland, MA) (4), we assessed the biocompatibility of CAP and OTC film and gel formulations at concentrations intended for clinical use. Our study revealed favorable profiles for CAP formulations in comparison to OTC products with an established safety record. We identified discriminative product-induced cytokine and chemokine patterns that may be applied as biomarkers in future clinical trials to evaluate microbiode side effects on the vaginal mucosal homeostasis.

MATERIALS AND METHODS

Test agents. The following ingredients and formulations of CAP and their respective placebos were supplied by A. R. Neurath (Lindsey F. Kimball Research Institute of the New York Blood Center, New York, NY): (i) CAP Aquaticer powder (FMC Biopolymers Corporation, Philadelphia, PA) (containing about 67% CAP depending on the lot number) and hydroxyethylcellulose (HEC) universal placebo gel (41), used to prepare CAP Aquaticer/HEC gel containing 185% Aquaticer, and (ii) CAP film with 40% (wt/wt) CAP (32) and placebo film containing 40% (wt/wt) microcrystalline cellulose (FMC Biopolymers). The CAP film and HEC-based gel were evaluated in comparison to the following vaginal products obtained over the pharmacy counter: (i) vaginal cleansing film (VCF) containing 3% octoxynol-9 (both from Apothecus Pharmaceutical Corporation, Oyster Bay, NY); and (iii) K-Y long-lasting vaginal moisturizer (Personal Products Company, Division of McNeil-PPC, Inc., Skillman, NJ). A 1% solution of Triton X-100 in water was provided by MatTek.

Ex vivo treatment protocols. VEC-100 epithelial tissue equivalents in 24-well plate-sized inserts and VEC-100 assay medium were purchased from MatTek. The VEC-100 inserts were placed in 12-well plates over 0.5 ml culture medium per well, creating a dual-chamber system allowing continuous nutrient supply to the basolateral epithelial site from the lower chamber and application of undiluted test agents to the apical epithelial surface in the upper chamber. A Gilson Microman positive displacement pipette M100 (Rainin, Inc., Woburn, MA) was used to apply 100 μl of undiluted gel to the apical surface of the tissue. The CAP/HEC gel was prepared ex tempore by mixing Aquaticer and HEC in a ratio of 1:8.8:2 (wt/wt) so that the final concentration of Aquaticer in the gel was 18%. The CAP and placebo films were cut into disks using biopsy punches (Millex, Inc., Bethpage, NY) matching the diameter of the tissue and then placed onto the epithelial surface, followed by addition of 100 μl assay medium, which converted the film into gel and mimicked the hydration of the film by vaginal secretions in vivo. VCF and VCF+ were not as easily dispersed as the CAP film and therefore were homogenized in prewarmed medium at the same surface/volume ratio before addition of 100 μl of undiluted gel to the epithelial surface. Culture medium alone served as the untreated control. After the desired incubation period, the tissue inserts were removed from the culture plate and the VEC-100 tissues were used for 4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT) assay or protein extraction. The culture-conditioned medium was collected for lactate dehydrogenase (LDH) activity and cytokine immunoassays.

Cell viability and cytotoxicity tests. The MTT colorimetric assay was used to assess viability based on mitochondrial enzyme function. In this assay, viable cells convert the MTT into a blue formazan product and the colorimetric reaction is measured by absorbance at 570 nm with a reference wavelength at 630 nm (22). MTT concentration was diluted to 1 mg/ml in VEC-100 assay medium (both provided by MatTek). At the end of each treatment period, the VEC tissue inserts were rinsed twice in phosphate-buffered saline (provided by MatTek) and transferred to 24-well plates containing 0.3 ml MTT solution. After 3 h of incubation at 37°C and 5% CO2, each VEC-100 insert was transferred to 0.5 ml of isopropanol extraction solution (MatTek). The plates were sealed to prevent alcohol evaporation and incubated for 24 h at room temperature before absorbance was read using a Victor2 1420 multilabel microplate counter with Wallac software 2.01 (Perkin Elmer Life Sciences, Boston, MA). Cell viability was presented as a percentage of the average optical density (OD) measured for untreated (medium alone) control tissues.

A Roche LDH assay (Fisher Scientific, Hampton, NH) was used in accordance with the manufacturer’s instructions to quantify cell death based on activity of LDH released from the cytosol of damaged cells. Fifty microliters of LDH reaction mixture was added to 50 μl of VEC-100 cultured medium and incubated for 30 min at room temperature. Absorbance was measured at 490 nm with a reference wavelength at 650 nm by using the Perkin Elmer Victor2 microplate counter. The tissue survival rates (percentages) were calculated by the following formula: 100 − (ODassay − ODcontrol) × 100/(ODtriton X-100 − ODcontrol).

Cytokine assays. Concentrations of major proinflammatory cytokines and chemokines in VEC-100 culture fluids were measured by a multiplex electrochemiluminescent Sector Imager 6000 simultaneously in four-spot (IL-1β, IL-6, IL-8, and tumor necrosis factor alpha [TNF-α]) or in single-spot (gamma interferon inducible protein 10 [IP-10]) assays from Meso Scale Discovery (Gaithersburg, MD). IL-1α, IL-1 receptor antagonist (IL-1RA), and macrophage inflammatory protein 3α (MIP-3α) were measured in the same culture fluids by traditional photometric enzyme-linked immunosorbent assays (Piecer, Rockford, IL, and R&D Systems, Minneapolis, MN) and with the Perkin Elmer Victor2 microplate counter. Since the secreted form of IL-1RA antagonizes the effects of both IL-1α and IL-1β by competing for their cell surface membrane receptor (3), the imbalance in the IL-1 signaling pathway was assessed by changes in the extracellular ratio between IL-1RA (pg/ml) and total IL-1 (IL-1α [pg/ml] plus IL-1β [pg/ml]) in the same culture fluids. Because the intracellular forms of IL-1RA and IL-1α, unlike intracellular IL-1β, have intracellular activities (6, 45), we also measured the intracellular levels of IL-1RA and IL-1α. For this purpose, VEC-100 tissues were lysed in 0.3 ml 25 mM Tris buffer containing 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% Igepal CA-630, protease inhibitor cocktail, and 1 mM phenylmethylsulfonyl fluoride (all from Sigma). The lysates were cleared from cellular debris by centrifugation at 10,000 × g at 4°C for 10 min. The intracellular cytokine concentrations were measured by enzyme-linked immunosorbent assays (IL-1α from Pierce and Quantikine IL-1RA from R&D Systems) and normalized per mg of total protein determined in the lysates by bichromatic acid assay (Piecer). To compare cytokine data from multiple experiments, results were presented as change (n-fold) in cytokine concentrations in treated tissues over the average value obtained from untreated tissues in each experiment.

Statistical analysis. One-way analysis of variance (ANOVA) with Bonferroni’s or Dunnnett’s multiple-comparison tests and Pearson correlation analysis were performed using GraphPad Prism, version 4.00, for Windows (GraphPad Software, San Diego, CA). P values of <0.05 were considered significant.

RESULTS

Comparative gel and film evaluation by IL-1α release and cytotoxicity assays. We first ranked CAP and OTC gels and films by their effects on IL-1α and IL-1β release in comparison to a toxic dose of Triton X-100. We used two forms of Triton X-100; we used the 1% Triton X-100 solution provided by the MatTek company as a toxicity control in the VEC-100 model (4), and as a solid-dosage-formulated reference, we applied VCF, which contains 3% octoxynol-9, i.e., Triton X-100. A preliminary time course of 30 min to 30 h demonstrated that plateau IL-1α and IL-1β values were reached within 4 h to 6 h after VCF application in the VEC-100 model (data not shown). The 6-h time point was therefore chosen for ranking of all test formulations.

The changes in the IL-1 levels are presented as increases (n-fold) over levels for the untreated control in Fig. 1A. The IL-1α and IL-1β levels did not change significantly after a 6-h treatment with K-Y gel, CAP film, placebo film, Aquaticer/HEC gel, or HEC placebo gel. In contrast, N-9-containing VCF, which contains 3% octoxynol-9, i.e., Triton X-100. A preliminary time course of 30 min to 30 h demonstrated that plateau IL-1α and IL-1β values were reached within 4 h to 6 h after VCF application in the VEC-100 model (data not shown). The 6-h time point was therefore chosen for ranking of all test formulations.

A strong correlation was observed between the magnitude of IL-1α and IL-1β release and the increase of LDH activity as a measure of cell damage in the same cultures (Pearson r2 = 0.8612 and 0.5014, respectively; P < 0.001). In the LDH cytotoxicity assay, VCF+ ranked similarly to VCF and the 1% Triton X-100 solution while the film and gel CAP formulations
ranked similarly to the untreated control, the placebo controls, and the K-Y gel (Fig. 1B).

The tissue survival rates assessed by the MTT assay correlated well with those of the LDH assay for all film formulations ($r^2 = 0.9250$, $P < 0.01$, Pearson correlation). Both VCF and VCF+ significantly reduced the tissue viability while CAP film and placebo film appeared nontoxic regardless of test method (Fig. 1B). However, all gel formulations, independently of the presence or absence of Aquateric, showed lower tissue survival rates when assessed by the MTT assay than when assessed by the LDH assay (Fig. 1B). The discrepancy between the two assays could be explained by the fact that the MTT assay uses washed tissue and thus depends on the efficiency of the wash step at the end of the treatment period while the LDH assay utilizes the culture fluid collected from the bottom chamber of the dual-compartment system. Gels were difficult to remove from the inside of the culture insert without damaging the tissue, which may have resulted in lower MTT readings.

Ranking of solid-dosage vaginal formulations by proinflammatory versus anti-inflammatory responses in the VEC-100 model. VEC-100 tissues from seven different lots were used to evaluate effects of the test formulations on the release of the chemokines IL-8, IP-10, and MIP-3$\alpha$ and the cytokines IL-6 and TNF-$\alpha$, which have well-defined proinflammatory functions and are known to activate HIV-1 replication (Fig. 3A and B). The variation in the spontaneous releases of these proinflammatory mediators in the VEC-100 tissues is presented in Fig. 2. VCF and VCF+ caused a significant severalfold increase of IL-8 and IP-10 (Fig. 3A). In addition, VCF+ caused a significant increase in MIP-3$\alpha$ levels (Fig. 3B). The CAP film and the Aquateric/HEC gel again ranked similarly to the K-Y gel and the placebo controls, causing no significant changes in the chemokine levels (Fig. 3A and B). None of the test formulations showed a significant change in IL-6 and TNF-$\alpha$ levels (data not shown).

To further investigate the mechanisms underlying the distinct proinflammatory potentials of the different vaginal film formulations, we evaluated the balance in the IL-1 signaling pathway by measuring intracellular IL-1RA and IL-1$\beta$ levels and the ratio between extracellular levels of IL-1RA and total IL-1 (IL-1$\alpha$ plus IL-1$\beta$). The intracellular levels of IL-1RA were significantly reduced in cultures treated with VCF+ compared to levels in untreated control cultures or CAP- and placebo-treated cultures ($P < 0.05$) (Fig. 4A). No significant differences in intracellular levels of IL-1$\alpha$ were observed between untreated control tissues and CAP-, placebo-, and VCF-treated tissues (data not shown). Additional differences between VCF and VCF+ were revealed when the extracellular IL-1RA and the IL-1RA/IL-1 ratios were analyzed. The extracellular levels of IL-1RA were significantly ($P < 0.001$) increased by both VCF and VCF+ compared to all other conditions; however, this change was significantly lower in the VCF+ treatment than in the VCF treatment ($P < 0.001$) (Fig. 4B). The extracellular ratio of IL-1RA to total IL-1 (IL-1$\alpha$ plus
IL-1β was weakly elevated in tissues treated with VCF+ and significantly increased in VCF-treated tissues compared to ratios in untreated control tissues and CAP+ and placebo film-treated tissues ($P < 0.001$) (Fig. 4C).

**DISCUSSION**

The establishment of molecular endpoints providing quantitative data reflecting the extent of vaginal inflammation has become critical in the safety evaluation of vaginal microbicides that are being developed for the prevention of sexually transmitted infections. The predictive value of proinflammatory cytokines and chemokines has been explored by human and animal studies. Increased levels of IL-1β and IL-8 in vaginal secretions of women have been associated with influx and activation of inflammatory cells in the context of bacterial vaginosis or N-9 treatment (10, 16). A rabbit model demonstrated correlation between IL-1β levels in vaginal secretions and the classic histologic signs of inflammation, i.e., epithelial erosion, edema, erythema, and inflammatory leukocyte infiltration (15).

The present study demonstrated the spontaneous expression and product-induced change of an array of proinflammatory cytokines and chemokines in the VEC-100 tissue model. The induction of IL-1 release by the N-9-containing film in the VEC-100 model agreed with previous results obtained with N-9 solutions or gels in other experimental models and in human subjects (15, 16). Both IL-1 cytokines are synthesized and stored in the epithelial cytoplasm as precursor proteins. IL-1β becomes biologically active only after it exits the cells as a proteolytically cleaved mature form (3). In contrast, both the precursor and mature forms of IL-1α are biologically active, with the intracellular precursor being capable of nuclear translocation and activation of proinflammatory gene expression via NF-κB and AP-1 (3, 45). IL-1α and IL-1β released from damaged or activated epithelial cells can trigger cascades of inflammatory events leading to activation of viral replication and recruitment of HIV-1 target cells, such as CD4+ T cells and macrophages (16, 27). Some of the multiple biological effects
of IL-1 that augment HIV-1 replication include stimulation via the long terminal repeat enhancer region and induction of glucocorticoid hormones that stimulate the virion infectivity factor (Vif) (reviewed in reference 12). Thus, the increase of extracellular IL-1 levels by VCF and VCF+ could be interpreted as a risk factor for HIV-1 infection common for all toxic agents. However, it is important to take into account that epithelial injury also triggers the release of IL-1RA (16), which is capable of reducing the IL-1β and IL-1α proinflammatory properties by competitively binding their type I IL-1 receptor (19). IL-1RA is part of the early monocytic response to HIV-1 infection (47) and blocks IL-1-induced HIV-1 replication in chronically infected cells (20). On the other hand, HIV-1 may downregulate the protective IL-1RA response to exogenous stimuli in HIV-1-infected individuals (12, 37). In the VEC-100 model, we observed distinct changes in IL-1α/IL-1β total IL-1 (IL-1α plus IL-1β) in response to treatment with VCF and VCF+. The clinical significance of the IL-1RA/IL-1 ratio as a net measure of the mucosal inflammatory reaction has been demonstrated previously by its correlation with clinical severity of inflammatory bowel disease (2, 9). In our study, the homeostatic IL-1RA/total IL-1 (IL-1α plus IL-1β) ratio in untreated VEC-100 tissues was within the ranges found in cervicovaginal lavage specimens collected from healthy female volunteers (16) and was less potently increased after VCF+ treatment than after VCF treatment. On the other hand, the intracellular levels of IL-1RA, which downregulates proinflammatory epithelial responses in an intracrine manner (6, 18), were significantly reduced only by N-9-containing VCF+. These data suggest that the concomitant assessment of intracellular and extracellular IL-1RA and IL-1 levels may contribute to a better understanding of the proinflammatory properties of vaginal OTC products and candidate microbicides.

Despite the significant increase of extracellular IL-1RA, both VCF and VCF+ induced differential increases of chemokines, which could be explained by inefficient IL-1 receptor blocking, altered chemokine mRNA stability due to reduced intracellular IL-1RA (44), or chemokine upregulation through IL-1 receptor-independent proinflammatory pathways. Our study is the first to identify IP-10 and MIP-3α as potential biomarkers of product-induced vaginal inflammation. Interestingly, while both VCF and VCF+ increased IP-10 and IL-8 to similar degrees, only N-9-containing VCF+ caused a significant change in MIP-3α levels. Since both VCF and VCF+ caused similar levels of toxicity and IL-1 release, these results suggest that the MIP-3α chemokine may serve as a proinflammatory marker independent of cell death and membrane disruption. The relative clinical significance of the severalfold change in IL-8, IP-10, and MIP-3α levels remains to be determined in future microbicide safety trials. However, clinical and experimental evidence suggests their importance in the establishment of HIV-1 infection. IL-8 plays a critical role in the formation of inflammatory infiltrates of neutrophils and monocytes (29) and may contribute to enhanced HIV-1 replication in infected monocytes (30). The major T-cell chemoattractant IP-10 is overexpressed in the context of HIV-1 infection, with persistently elevated IP-10 levels correlating with antiretroviral therapy failure (38, 40). IP-10, like IL-1β, IL-6, TNF-α, IL-8, and other proinflammatory mediators, stimulates HIV-1 replication in peripheral blood monocytes and monocyte-derived macrophages (23). Although IP-10 may contribute to accumulation of HIV-1-specific cytotoxic T cells, the response may not be efficient in controlling the spread of HIV-1 through the genit al tract mucosa. Therefore, the induction of IP-10 by vaginal irritants may be regarded as an HIV-1-enhancing risk factor to be monitored in preclinical and clinical microbicide safety studies. The role of MIP-3α in HIV-1 pathogenesis is less studied; however, MIP-3α recruits dendritic cells to the site of inflammation, thus possibly increasing the risk of HIV-1 transmission (11).

This study demonstrates that solid-dosage vaginal formulations can be tested in the VEC-100 model at concentrations intended for clinical use and ranked based on cytokine and chemokine responses. Our results extend the safety record of CAP, which has been used for many years as a general pharmaceutical excipient for oral tablets with no systemic toxicity (7). The CAP Aquateric reduced simian immunodeficiency virus/HIV-1 transmission and appeared safe on colposcopic examination in a rhesus macaque model (8). In the present study, the CAP film and Aquateric/HEC gel ranked similarly to the K-Y vaginal moisturizer, which is available as an OTC product, and to the universal HEC placebo, which has been designed to be biologically inert and shown to be innocuous to the vaginal mucosa in several animal models (41). Our data also suggest that the CAP film has lower proinflammatory potentials than VCF and VCF+, which are available as hygiene or contraceptive products in the U.S. OTC market.

Future clinical studies should confirm the safety of the products tested in this study for use by women at risk of HIV-1 infection and the clinical relevance of the cytokine changes detected in the VEC-100 tissue model.

ACKNOWLEDGMENTS

This work was supported by NIH/NICHD grant 1P01HD041761. We thank A. Robert Neurath and Nathan Strick from the Laboratory of Biochemical Virology at the New York Blood Center, New York, for providing the CAP formulations and placebo controls for our study.

REFERENCES


Downloaded from http://aac.asm.org on September 7, 2017 by guest


