RESEARCH ARTICLE

Novel Vaginal Microflora Colonization Model Providing New Insight into Microbicide Mechanism of Action

Raina N. Fichorova,[a] Hidemi S. Yamamoto,[a] Mary L. Delaney,[b] Andrew B. Onderdonk,[b] and Gustavo F. Doncel[c]

Laboratory of Genital Tract Biology, Department of Obstetrics, Gynecology and Reproductive Biology,[a] and Department of Pathology,[b] Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA, and CONRAD, Department of Obstetrics and Gynecology, Eastern Virginia Medical School, Norfolk, Virginia, USA[c]

ABSTRACT **Several broad-spectrum microbicides, including cellulose sulfate (CS), have passed conventional preclinical and phase I clinical safety evaluation and yet have failed to protect women from acquiring HIV-1 in phase II/III trials. Concerns have been raised that current preclinical algorithms are deficient in addressing the complexity of the microflora-regulated vaginal mucosal barrier. We applied a novel microflora-colonized model to evaluate CS and hydroxyethylcellulose (HEC), which is used as a "universal placebo" in microbicide trials. Cervicovaginal epithelial cultures were colonized with normal vaginal microflora isolates representing common *Lactobacillus* species used as probiotics (*L. acidophilus* and *L. crispatus*) or *Prevotella bivia* and *Atopobium vaginae*, most prevalent in the disturbed microflora of bacterial vaginosis (BV). At baseline, all strains maintained constant epithelium-associated CFUs without inducing cytotoxicity and apoptosis. CS selectively reduced epithelium-associated CFUs and (to a lesser extent) planktonic CFUs, most significantly affecting *L. crispatus*. Inducing only minor changes in sterile epithelial cultures, CS induced expression of innate immunity mediators (RANTES, interleukin-8 [IL-8], and secretory leukocyte protease inhibitor [SLPI]) in microflora-colonized epithelia, most significantly potentiating effects of bacteria causing BV. In the absence of CS, all bacterial strains except *L. acidophilus* activated NF-κB, although IL-8 and RANTES levels were increased by the presence of BV-causing bacteria only. CS enhanced NF-κB activation in a dose-dependent manner under all conditions, including *L. acidophilus* colonization. HEC remained inert. These results offer insights into possible mechanisms of CS clinical failure. The bacterially colonized cervicovaginal model reveals unique aspects of microflora-epithelium-drug interactions and innate immunity in the female genital tract and should become an integral part of preclinical safety evaluation of anti-HIV microbicides and other vaginal formulations.**

IMPORTANCE **This report provides experimental evidence supporting the concept that the vaginal microflora regulates the epithelial innate immunity in a species- and strain-specific manner and that topically applied microbicides may alter both the bacterial and epithelial components of this homeostatic interaction. Our data also highlight the importance of differentiating the effects of biomedical interventions on epithelium-associated versus conventional planktonic bacterial growth when assessing vaginal mucosal health and immunity.**

Received 19 July 2011 **Accepted** 28 September 2011 **Published** 25 October 2011

Citation Fichorova RN, Yamamoto HS, Delaney ML, Onderdonk AB, Doncel GF. 2011. Novel vaginal microflora colonization model providing new insight into microbicide mechanism of action. mBio 2(6):e00168-11. doi:10.1128/mBio.00168-11.

Editor Peter Gilligan, UNC Health Care System

Address correspondence to Raina N. Fichorova, rfichorova@rics.bwh.harvard.edu.

The healthy cervicovaginal mucosa represents an efficient barrier against sexually transmitted infections (STIs) and dissemination of pathogens. To preserve that barrier, the epithelial cells of the female genital tract have developed tolerance of and mutually beneficial interactions with the constituents of the normal vaginal microflora while maintaining rapid innate immune responses to danger- or pathogen-associated molecular patterns (1–6). Therefore, it is logical that any biomedical intervention strategy designed to prevent STIs must preserve all aspects of the natural barrier in order to be safe and effective for wide use by women at risk. More specifically, in addition to preserving the vaginal microflora, epithelial cell viability, and tissue integrity, anti-HIV microbicides must be especially devoid of proinflammatory activities, since mucosal inflammatory reactions recruit activated CD4-positive HIV-host cells to the mucosa, promote HIV-1 transepithelial penetration, and facilitate viral replication in infected cells (7–10). Current preclinical safety algorithms use sterile *in vitro* models to assess the proinflammatory activity of anti-HIV microbicides; however, a limitation of this traditional approach is the absence of the physiological host-microflora interactions (8, 10). We describe a novel microflora-colonized cervicovaginal epithelial model for safety evaluation of microbicides and other vaginal formulations. We applied this model to gain insights into the mechanisms of cellulose sulfate (CS) failure as a topical vaginal microbicide. Traditional phase I clinical studies have shown that CS exhibits potent anti-HIV activity *in vitro* and have repeatedly demonstrated that CS presents no safety concerns (11–16). However, the first large phase II/III study of CS had to be halted due to failure to prevent and a trend to increase male-to-female HIV-1 transmission (17). Understanding the mechanisms underlying the

unexpected clinical performance of CS and designing preclinical models that are more predictive are essential for further development of successful anti-HIV microbicides.

RESULTS

Characteristics of the vaginal bacterial colonization model in the absence of microbicide test products. The adherence of bacteria to epithelial cells in our model is visualized in Fig. 1A to D. The transmission electron microscopy (TEM) images demonstrate the intimate contact between *Lactobacillus crispatus* (Fig. 1A and B) and *Prevotella bivia* (Fig. 1C and D) and the vaginal epithelial cells, displaying a lack of morphological signs of apoptosis. Similar observations (not shown) were obtained from adherence studies of *Lactobacillus acidophilus* and *Atopobium vaginae*. All bacterial strains studied (*L. acidophilus*, *L. crispatus*, *P. bivia*, and *A. vaginae*) adhered to the epithelial cells in the absence of apoptosis and cytotoxicity, as assessed by microscopy, caspase-3 cleavage (Fig. 1E), and trypan blue exclusion tests (Fig. 1F), and at reproducible CFU rates that were comparable between the bacterial strains (Fig. 1G and H), and the epithelial cell-associated CFU counts were stably maintained at constant rates over the experimental period of 24 to 48 h (Fig. 1G). CFU counts were comparable between the vaginal (Vk2/E6E7) and cervical (Ect1/E6E7) epithelial cell cultures (Fig. 1G and H) as well as between the immortalized epithelial cell (Ect1/E6E7) monolayer model and the polarized primary epithelial cell (VEC-100) tissue model (Fig. 1H).

The direct effects of the different bacterial strains on epithelial cell inflammatory activation under baseline conditions (in the first 24 h of colonization in the absence of CS and HEC) are shown in Fig. 2. The bacterial strains were best distinguished by their abilities to induce NF-κB (Fig. 2A) activation and interleukin-8 (IL-8) production (Fig. 2B). At comparable colonization rates, the BV-associated bacteria induced the most significant NF-κB activation and the greatest increase in IL-8 levels, *L. crispatus* induced weaker NF-κB activation and no IL-8, and *L. acidophilus* did not induce any changes in either NF-κB and IL-8 levels compared to the medium control (no bacteria).

Effects of CS and HEC compounds on vaginal microflora survival and epithelial colonization. Since the primary polarized-tissue model and the immortalized epithelial cell monolayer model showed identical colonization patterns, the immortalized cell line monolayer model, which is significantly cheaper and easier to handle than the polarized-tissue model, was used to repeatedly assess effects of multiple-compound doses and various compound batches on bacterial-epithelial interactions.

For these experiments, we used a dose range (1 to 1,000 μg/ml) that brackets the anti-HIV 50% effective concentration [EC_{50}] of CS established in various *in vitro* assays (1 to 80 μg/ml) (11, 16, 18). CS and HEC were nontoxic in this dose range, as shown for both compounds in the vaginal monolayer model (Fig. 3A) and for CS in both the monolayer immortalized and the polarized primary ectocervical cell models (Fig. 3B). Microscopic evaluation at the end of each bacterial coculture experiment also confirmed the lack of any morphological signs of cytotoxicity.

While HEC remained invariably innocuous with respect to bacterial growth and colonization rates, CS showed distinct bacterial-strain-dependent effects on epithelial cell-associated and planktonic bacteria (cultured in the absence of epithelial cells). CS reduced most significantly, and in a dose-dependent

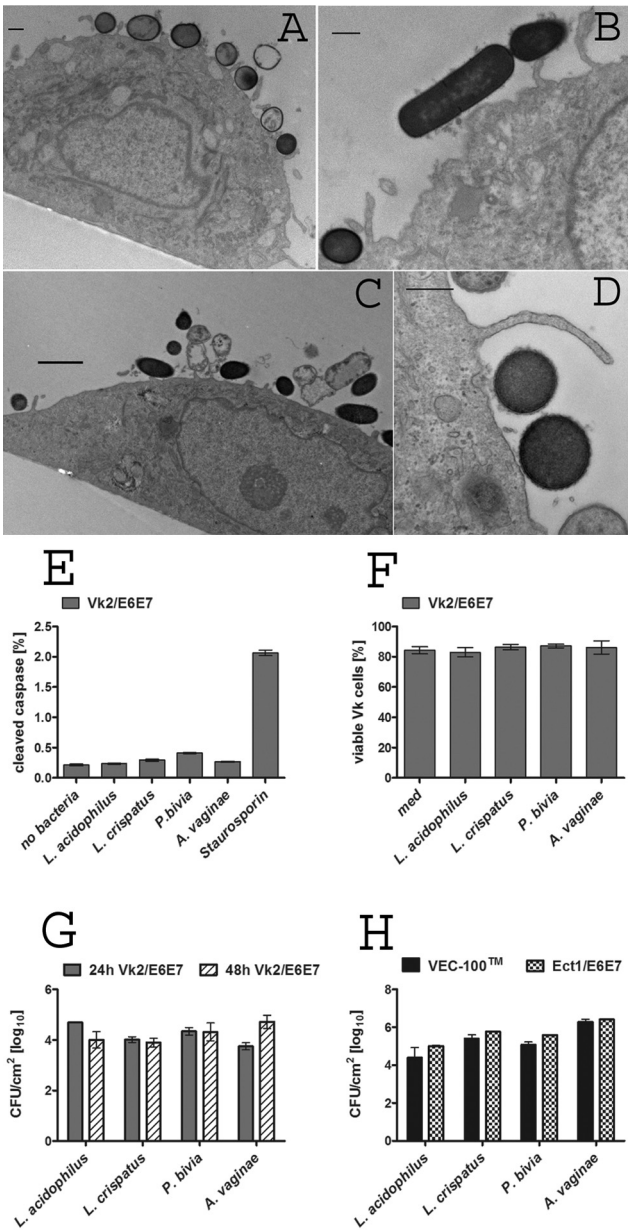

FIG 1 Colonization of human vaginal and cervical epithelial cells by vaginal bacteria showed consistent bacterial association with epithelial cells in the absence of apoptosis and cell toxicity. (A–D) Transmission electron microscopy image showing *L. crispatus* (A and B) and *P. bivia* (C and D) bacteria, visualized as electron-dense bodies, adherent to the surface of vaginal epithelial cells (Vk2/E6E7) with intact morphology after 24 h of colonization. The bars and images represent 500 nm and ×4,800 magnification (A), 500 nm and ×6,800 magnification (B), 2 μm and ×1,900 magnification (C), and 500 nm and ×13,000 magnification, respectively. (E) Caspase-3 cleavage is presented as percentages of cleaved total caspase-3 measured in vaginal epithelial cell lysates at 24 h after bacterial colonization or treatment with 1 μM staurosporine. Bars represent means and standard errors of the means (SEM) of the results determined with duplicate cultures used in two experiments. (F) Viability of vaginal epithelial cells assessed by trypan blue inclusion tests at 5 days postcolonization. Bars represent means and SEM of the results from triplicate culture experiments. (G) CFU counts per square centimeter of epithelial cell surface at 24 h and 48 h postcolonization of Vk2/E6E7 cells. Bars represent means and SEM of the results determined with triplicate cultures used in three experiments. (H) Parallel assessment of CFU counts associated with primary polarized (VEC-100) and immortalized monolayer (Ect1/E6E7) ectocervical epithelial cells at 48 h postcolonization.

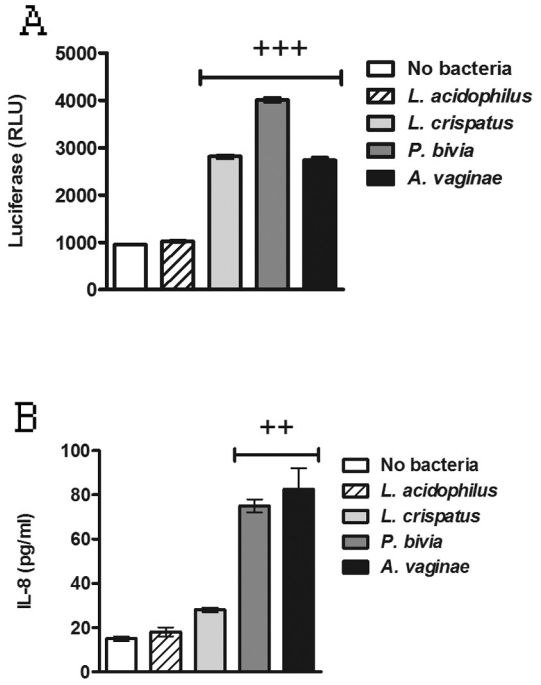

FIG 2 Proinflammatory properties of vaginal microflora strains at 24 h postcolonization of epithelial monolayers in the absence of CS and HEC. (A) NF-κB activation assessed by luciferase activity. Bars represent means and SEM of the results determined with quadruplicate cultures in one of three independent experiments. (B) IL-8 levels measured in the vaginal epithelial cell culture supernatants. Bars represent means of the results determined with duplicate cultures in one of three experiments. For comparisons of the results determined with bacterially colonized cultures to those determined with control cultures without bacteria, ++ represents $P < 0.01$ and +++ represents $P < 0.001$ (ANOVA [Dunnett's multiple-comparison test]).

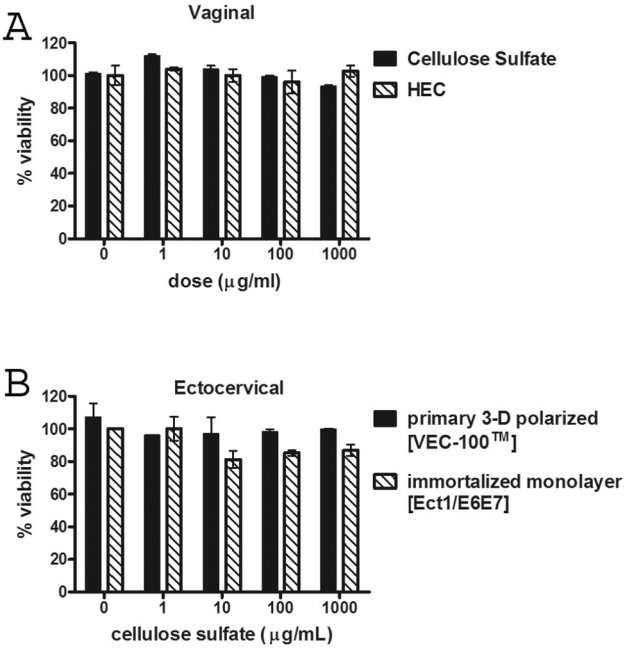

FIG 3 Cell viability determined by the MTT assay after 24 h of compound exposure. (A) Immortalized vaginal epithelial cell monolayers (Vk2/E6E7) exposed to the same dose range of cellulose sulfate and hydroxyethylcellulose; (B) comparison of primary polarized (VEC-100) and immortalized monolayer (Ect1/E6E7) ectocervical epithelial cells exposed to cellulose sulfate. Values represent means and SEM of the results determined with duplicate cultures in one of three experiments.

manner, CFUs of epithelium-associated *L. crispatus* (Fig. 4) followed by planktonic *L. crispatus* (Fig. 5). *P. bivia* CFU levels were lowered by CS in planktonic cultures to a greater degree than in epithelial cell-associated cultures. *A. vaginae* did not survive well in the absence of epithelial cells, and CS reduced its abundant association with epithelial cells the least. Neither planktonic nor epithelium-associated *L. acidophilus* was affected by CS.

Effects of CS and HEC on the immune function of colonized cervicovaginal epithelial cells. The effects of CS and HEC on selected soluble innate-immunity mediators universally triggered by inflammation are shown in Fig. 6. As with the findings seen at 24 h postcolonization (Fig. 2), at 48 h postcolonization both *Lactobacillus* strains maintained a balanced noninflammatory baseline in the absence of added compounds, as evidenced by the lack of significant effects on RANTES (Fig. 6A and B), IL-8 (Fig. 6C and D), and secretory leukocyte protease inhibitor (SLPI) (Fig. 6E and F) production by epithelial cells. In contrast to the lactobacilli, *A. vaginae* significantly increased both RANTES ($P < 0.05$) and IL-8 ($P < 0.01$) production and showed a tendency to decrease SLPI production, while *P. bivia* significantly increased IL-8 production ($P < 0.01$).

In the absence of bacteria, both CS (Fig. 6A, C, and E) and HEC (Fig. 6B, D, and F) showed no significant effects on levels of soluble mediators measured in the cell culture supernatants of ectocervical cells (Fig. 6) and vaginal cells (data not shown). However, CS induced significant changes in IL-8, RANTES, and SLPI levels when applied to bacterially colonized epithelial cells. Exposure to CS (Fig. 6A), but not to HEC (Fig. 6B), across the entire dose range increased RANTES levels nearly 2-fold in the presence of all bacteria ($P < 0.01$), with the highest levels achieved by enhancing the baseline effects of *A. vaginae* (Fig. 6A). Similarly, CS exposure caused a dose-dependent increase in IL-8 levels in epithelial cells colonized by *P. bivia* and *A. vaginae* ($P < 0.01$) and, to a lesser extent, in *L. crispatus*-colonized epithelia ($P < 0.01$ with CS at 1 μg/ml; $P < 0.05$ with CS at 10 and 100 μg/ml) but not in *L. acidophilus*-colonized epithelia (Fig. 6C). HEC exposure again showed no effect (Fig. 6D). SLPI levels were increased in a dose-dependent manner by CS exposure in the presence of all bacterial strains ($P < 0.01$) (Fig. 6E). This trend was not observed with HEC (Fig. 6F).

To assess the effects of CS and HEC on NF-κB activation, we compared epithelial cells grown in the absence of bacteria to epithelial cells colonized by *L. acidophilus*, which had no direct NF-κB activation effects, or by *P. bivia*, which had highest NF-κB activation potency in the first 24 h of bacterial colonization, as shown in Fig. 2. Again, at 48 h after colonization and 24 h after compound exposure, in the absence of CS, *L. acidophilus* showed no effect and *P. bivia* induced NF-κB activation ($P < 0.001$) (Fig. 7A and B). CS exposure induced NF-κB activation in a dose-dependent manner in the absence or presence of bacteria ($P < 0.01$), enhancing the proinflammatory effect of *P. bivia* (Fig. 6A). In contrast, HEC remained inert under all conditions (Fig. 7B).

DISCUSSION

Our report provides experimental evidence supporting the concept that the vaginal microflora regulates the epithelial innate im-

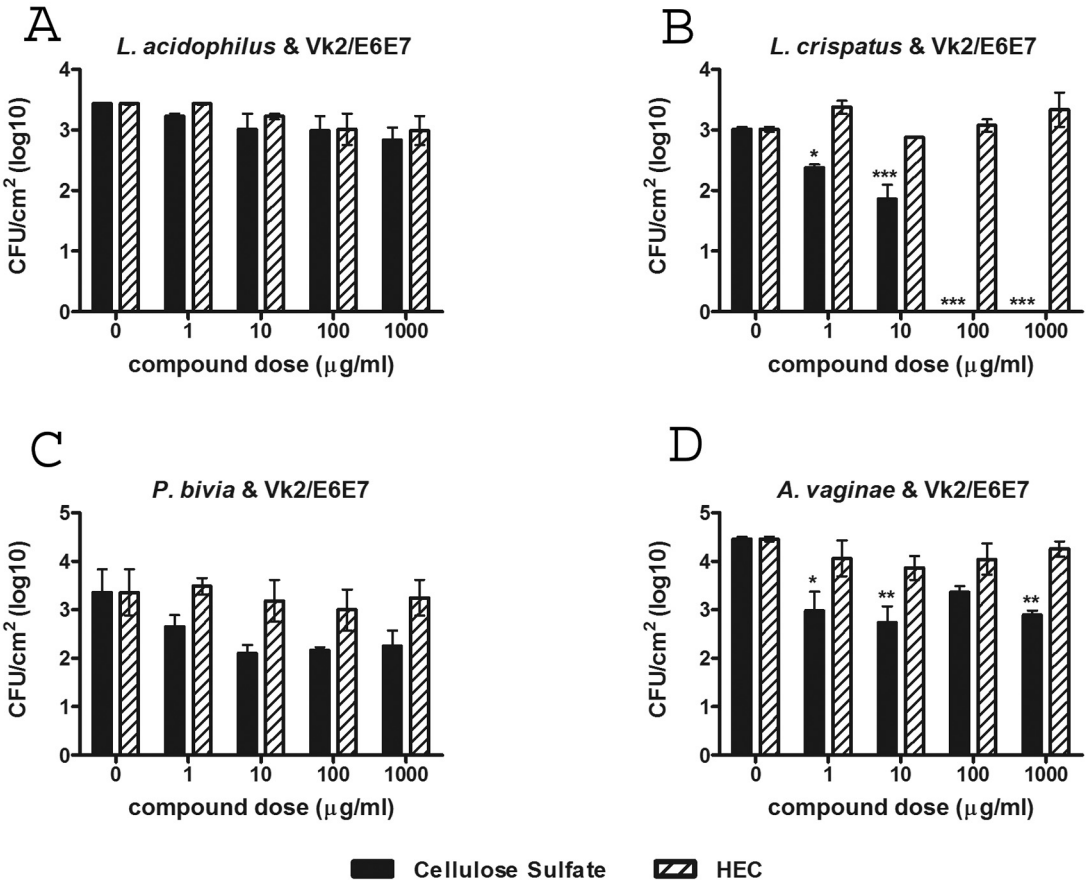

FIG 4 Effects of cellulose sulfate and hydroxyethylcellulose (HEC) on bacterial colonization assessed after 24 h bacterial-epithelial coculture followed by 24h exposure to compound test doses. Bars represent means and SEM of CFUs associated with duplicate vaginal epithelial cell cultures in two experiments with two different batches of each test compound. For comparisons of the results determined with various compound doses to those determined with control medium, * represents $P < 0.05$, ** represents $P < 0.01$, and *** represents $P < 0.001$ (ANOVA [Dunnett's multiple-comparison test]).

munity in a species- and strain-specific manner and that topically applied microbicides may interfere with this homeostatic balance by altering both the bacterial and epithelial components of the interaction.

The number of microbicide candidates surpasses the capacity of researchers to test them all in expensive clinical trials. The identification of a successful microbicide product is critical to the rational selection of candidates through the employment of a comprehensive preclinical evaluation algorithm (8). This algorithm includes assays to characterize physicochemical (P/C) properties of active pharmaceutical ingredients and formulations, release rates, specific antiviral activity, toxicity, pharmacokinetics, pharmacodynamics, and efficacy. In addition to the standard endpoints of cell and tissue toxicity, the peculiarities of the mucosal transmission of HIV (19) have highlighted the importance of evaluating inflammatory mediators such as cytokines and chemokines (7, 9, 20). Most of those *in vitro* assays, however, employed cells and tissues from the female genital tract in the absence of bacterial colonization. Clearly, such cells and tissues are not representative of what occurs *in vivo*. With the aim of more accurately replicating *in vivo* conditions, we devised a model based on bacterially colonized cervicovaginal cells that enables the evaluation of the impact of microbicide candidates on resident and pathogenic bacteria and epithelial cells and their interactions.

The BV-associated bacterial species *P. bivia* and *A. vaginae* triggered higher levels of IL-8 and NF-κB activation, consistent with clinical findings, which have associated variations in the vaginal microflora with proinflammatory changes of the vaginal mucosal environment and with changes in the local and systemic cytokine levels in women with bacterial vaginosis (21–23). In agreement with our results, a previous *in vitro* study performed using our immortalized vaginal epithelial cell line (Vk2/E6E7) has shown that short-term (6 h) inoculation with *A. vaginae*, but not with *L. crispatus*, induced proinflammatory pathway activation and upregulation of IL-8 and IL-6 (24). Our data are also consistent with previous observations of *in vivo* strain-specific high-level inflammatory properties of BV-associated microflora and low-level or even anti-inflammatory properties of *Lactobacillus* spp. (22, 25). Our *L. acidophilus* strain is pending further genetic analysis and characterization due to its promising noninflammatory profile.

Additionally, an inverse clinical association has been found between pathogenic Gram-negative bacteria and vaginal levels of SLPI (22). SLPI is a pluripotent bactericidal protease inhibitor essential for the vaginal barrier function, which is reduced in women with BV and lower genital tract infections. Such reductions have been associated with an increased risk of HIV acquisition (22, 26). SLPI has HIV-inhibitory properties (27–35) and has

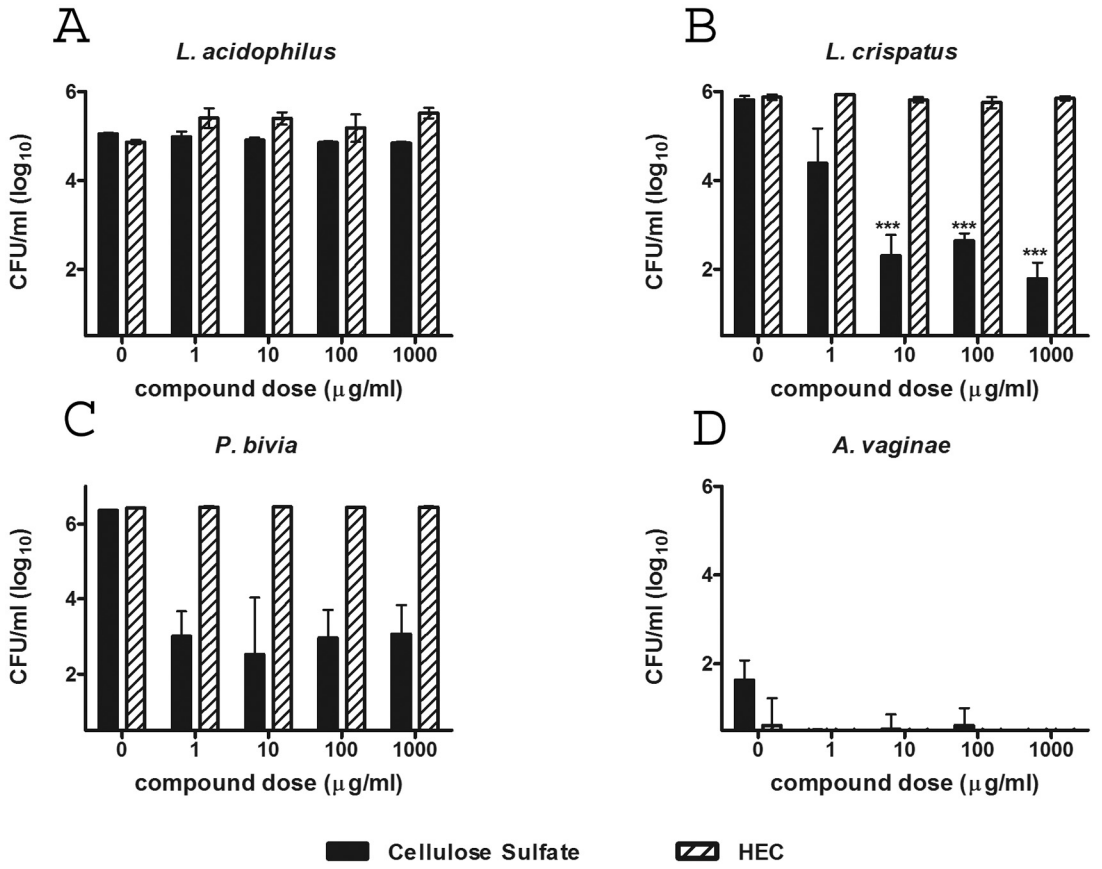

FIG 5 Direct effects of hydroxyethylcellulose (HEC) and cellulose sulfate on planktonic bacterial growth in the absence of epithelial cells. The bacterial suspensions that were used for epithelial colonization were simultaneously mixed with equal volumes of compound doses and incubated under anaerobic conditions for 24 h, followed by agar plating for enumeration of CFUs. Note that *A. vaginae* did not survive well in the absence of epithelial cells, whereas it maintained a stable colonization rate in the vaginal and cervical epithelial cells, as shown in Fig. 1G and H and 4D. Bars represent means and SEM of the results determined with triplicate cultures in two independent experiments performed with two different batches of test compounds. For comparisons of the results determined with various compound doses to those determined with control medium, *** represents $P < 0.001$ (ANOVA [Dunnett's multiple-comparison test]).

also been associated with the mucosal toxicity induced by the failed microbicide candidate nonoxynol-9 (20).

The cervicovaginal cells colonized with bacteria responded differently to CS and HEC. While HEC had no impact on bacterial growth and colonization and produced no significant changes in the selected proinflammatory markers, CS selectively inhibited growth and colonization by *L. crispatus* and *A. vaginae*, reduced growth of *P. bivia*, albeit not significantly, and had no effect on *L. acidophilus*. CS also enhanced bacterially induced NF-κB activation and increased the production of IL-8, RANTES, and SLPI in response to the presence of bacteria.

Our data suggest that CS has the capacity to modify cervicovaginal innate immunity and selectively change bacterial survival rates in the context of microflora colonization. Theoretically, reducing the adherence of *A. vaginae* and *P. bivia* may be beneficial for controlling BV. However, this inhibitory effect on *L. crispatus* was more pronounced than the effect on the BV-causing bacteria and previously determined clinical evidence does not demonstrate a significant therapeutic effect of CS on BV (36). The selective reduction of epithelium-associated *L. crispatus* populations may be particularly worrisome, because *L. crispatus* is believed to contribute more than other *Lactobacillus* species to the stability of the normal vaginal microflora (37). Pyrosequencing analysis of the vaginal microbiome of women treated with CS gel for 14 days revealed a prevalence of BV-associated bacterial communities and a reduction of populations of lactobacilli compared to the results seen at baseline and with women treated with HEC-based placebo gel (38). It is also possible that reduction of populations of *L. crispatus* and less-harmful Gram-negative members in the microflora, via a direct effect or inhibition of epithelial adherence, may lead to compensatory overgrowth of other vaginal bacteria. Slightly increased numbers of *Escherichia coli* have been found in women following a 14-day exposure to CS (15). CS was reported to be inhibitory for most or all tested isolates of *Gardnerella vaginalis*, *Peptostreptococcus*, *Prevotella*, *Eubacterium*, and *Fusobacterium* at concentrations of <10 mg/ml in routine microbiological assessments (39), but little is known about the possible mechanism of action by which polyanionic compounds can interfere with the growth of microbes. Previous studies have shown that the mechanism of antimicrobial activity for several STI-causing pathogens by these compounds may involve receptor antagonism or mimicry during cell-cell fusion (11, 40).

The NF-κB activation induced by CS, which peaked at low doses, remains without explanation. Biphasic effects of cellulose sulfate on *in vitro* HIV replication have been reported, with low doses (1 to 3 μg/ml) enhancing and higher doses inhibiting HIV

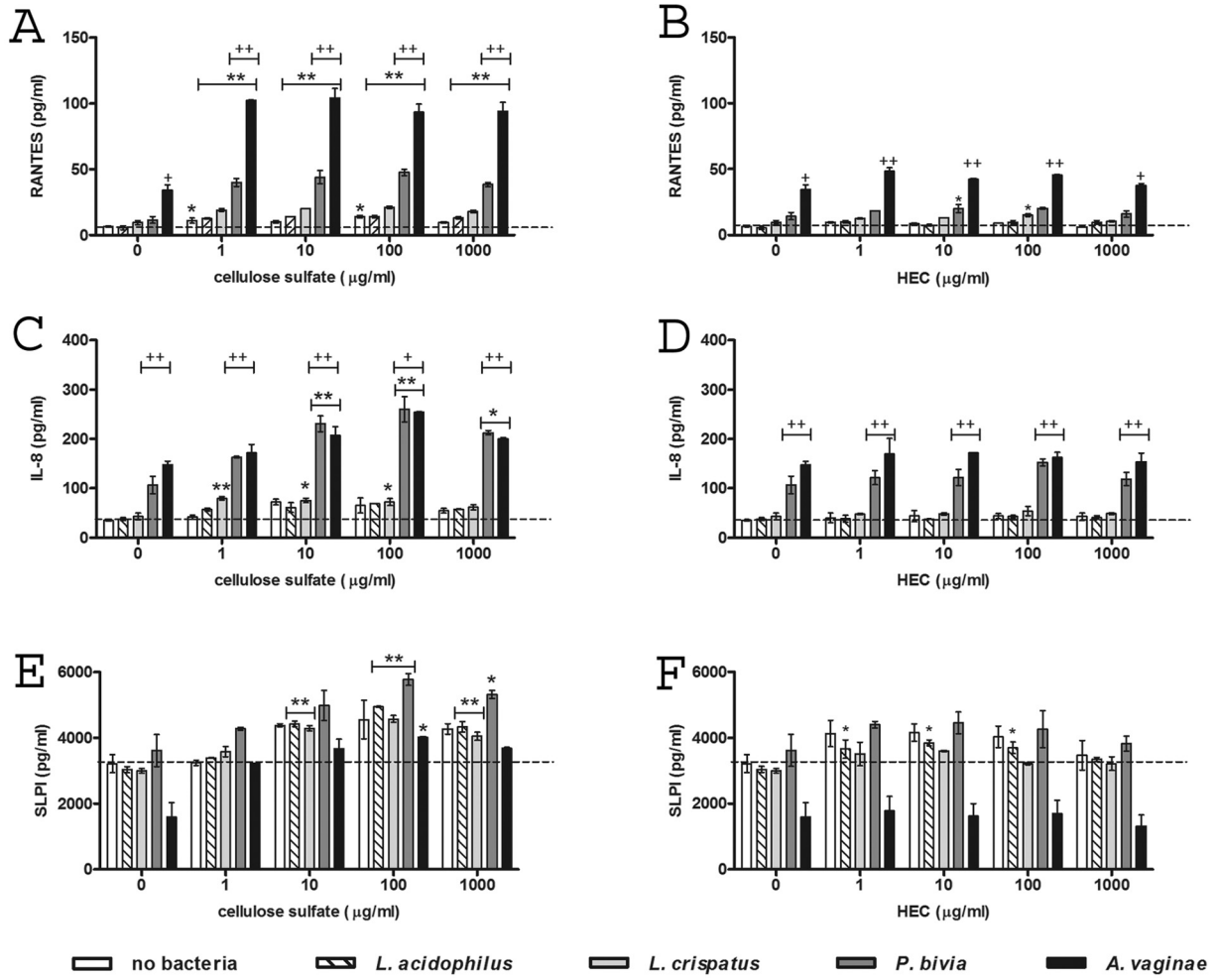

FIG 6 Compound-induced innate immune responses after 24 h of colonization with vaginal bacterial strains followed by 24 h of exposure to cellulose sulfate or hydroxyethylcellulose. Bars represent means and SEM of RANTES (AB), IL-8 (CD), and SLPI (EF) levels measured in supernatants from duplicate ectocervical epithelial cell cultures in one of three experiments with three different batches of cellulose sulfate. The dashed line represents medium-control basal levels in the absence of bacterial colonization. Similar results were obtained with the vaginal epithelial cells (data not shown). For comparisons of the results determined with various compound doses to those determined with control medium within each treatment group (shown at the bottom), * represents $P < 0.05$ and ** represents $P < 0.01$; for comparisons of the results determined with bacterially colonized cultures to those determined with cultures without bacteria for each compound dose, + represents $P < 0.05$ and ++ represents $P<0.01$ (ANOVA [Dunnett's multiple-comparison test]).

infection (41). This effect might be attributable to low-dose-driven activation of NF-κB response elements in the HIV long terminal repeat (LTR), as seen in our cervicovaginal epithelial cell model. NF-κB upregulates inflammatory gene products such as IL-8 that increase the HIV infection risk; at the same time, however, it also upregulates innate immunity gene products, e.g., SLPI and RANTES, that could potentially reduce the risk of HIV. SLPI upregulation induced by high doses of CS may have beneficial effects and may counteract the negative role of NF-κB upregulation. The clinical significance of these phenomena remains to be ascertained in future studies (42). Similarly, the functional significance of the increase in the level of RANTES may be twofold. RANTES is a T-cell chemokine that attracts CD4 and HIV host cells to the epithelial surface, thus increasing the risk of infection (43). At the same time, it can compete with HIV for CCR5 coreceptor usage, thus playing a protective role. CCR5 blockade has been used as a target mechanism for anti-HIV drug development (44–49). Increased levels of RANTES, such as have been observed in the cervicovaginal mucosa of HIV-resistant sex workers, have been interpreted as representing part of the innate immune barrier conferring protection in these women (50); however, they may also represent an unrecognized sign of disturbed microflora balance in these women (26, 51). Interestingly, the level of RANTES was increased most significantly by *A. vaginae*, which is virtually absent from the normal microflora but is found in BV microflora (52).

In conclusion, the findings in our physiologic model of microflora-epithelial cell interactions provide experimental proof for the role of bacterial colonization in regulating the vaginal immune environment. This model should be further explored to better define its value in predicting clinically undesirable mucosal alterations by vaginal products.

The findings of this study further constitute an example of how a microbicide may interfere with microbial-epithelial interactions. Taken together, they allow us to put forth a hypothesis to explain why administration of CS failed to protect women from

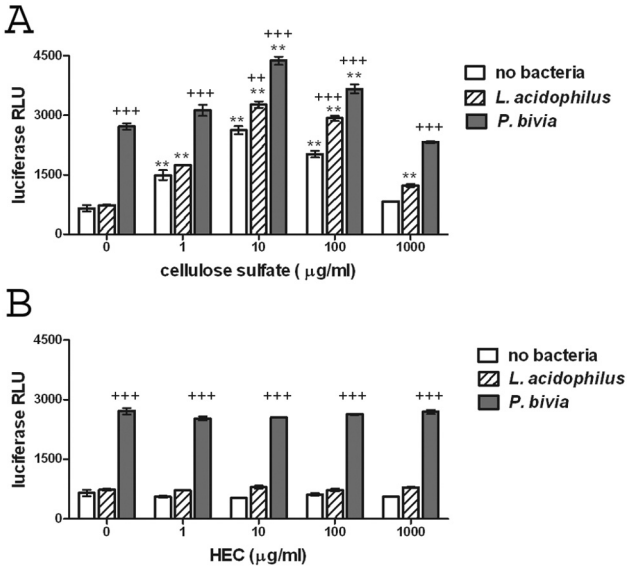

FIG 7 Compound-induced NF-κB activation after 24 h of bacterial colonization followed by 24 h of exposure to cellulose sulfate (A) or hydroxyethylcellulose (B). Bars represent means and SEM from luciferase activity determinations performed with duplicate cultures in one of three experiments with three different batches of each test compound. For comparisons of the results determined with various compound doses to those determined with control medium within each treatment group (shown at the right), ** represents $P < 0.01$; for comparisons of the results determined with bacterially colonized cultures to those determined with cultures without bacteria for each compound dose, ++ represents $P < 0.01$ and +++ represents $P < 0.001$ (ANOVA [Dunnett's multiple-comparison test]).

acquiring HIV and may have even increased the risk if frequently used. Through the comparisons of CS to HEC, a nonsulfated cellulosic derivative that is the main polymer of the "universal" placebo used in microbicide trials, the data provide an insight into possible mechanisms by which CS may disturb the vaginal microbiome and the physiological interactions of these bacteria with the cervicovaginal mucosa, thereby increasing its susceptibility to HIV-1 infection.

In a more general aspect, our data highlight the importance of differentiating effects of biomedical interventions on epithelium-associated versus conventional planktonic bacterial growth when assessing mucosal health and immunity.

MATERIALS AND METHODS

Test agents. Cellulose sulfate was obtained from CONRAD (Arlington, VA; manufactured by Patheon, Research Triangle, NC), and hydroxyethylcellulose (HEC) was purchased from Hercules, Hopewell, VA. Endotoxin contamination of each reagent and compound was ruled out using the Endosafe system (Charles River Laboratories, Charleston, SC) based on the Limulus amoebocyte lysate (LAL) test (53) at a sensitivity of 0.05 endotoxin units (EU)/ml. A negative endotoxin test result was a requirement for reagent use.

Epithelial cell culture. Human immortalized endocervical (End1/E6E7), ectocervical (Ect1/E6E7), and vaginal (Vk2/E6E7) epithelial cell lines, whose differentiation patterns and immune responses closely resemble those of their normal tissues of origin (1, 2, 4, 20, 54–56), were grown as monolayers in antibiotic-free keratinocyte serum-free medium (KSFM) (Invitrogen, Carlsbad, CA) supplemented with bovine pituitary extract, epidermal growth factor, and calcium chloride as described previously (55). The physiologic properties of the monolayer cultures were compared to those of polarized three-dimensional (3-D) VEC-100 tissues derived from primary human ectocervical epithelial cells grown using permeable-membrane support (MatTek Corporation, Ashland, MA), which were previously shown to resemble the bactericidal and immune properties of normal tissues of origin (57, 58). The VEC-100 tissues were maintained in antibiotic-free medium (MatTek).

Bacterial strains and colonization assays. The *Lactobacillus acidophilus*, *Lactobacillus crispatus*, and *Prevotella bivia* isolates were originally collected from vaginal swab samples from healthy women participating in a vaginal microflora research study (59). *Atopobium vaginae* (BAA-55) was acquired from the American Type Culture Collection (ATCC, Manassas, VA). We chose the *L. acidophilus* strain as a representative of the broader *L. acidophilus* homology group of commensal lactic acid-producing bacteria because of its known probiotic properties (60) and because *L. acidophilus* is one of very few taxa that have thus far shown promising randomized clinical trial evidence for microbiological and clinical cure of BV (61). *L. crispatus* was chosen for its prevalence in the healthy human vaginal microflora and its pharmaceutical use as a probiotic (37, 60, 62), and *P. bivia* and *A. vaginae* were chosen for their prevalence as BV-associated microflora (37, 52, 63). The bacterial isolates were identified using phenotypic characteristics and established criteria (64), and the identifications were confirmed using the Microbial Identification System for long-chain fatty acid analysis (MIDI Inc., Newark, NJ). For epithelial colonization, bacterial suspensions were prepared in antibiotic-free KSFM and added at 2.2×10^6 CFU/cm^2 to confluent epithelial surfaces at a 10:1 bacterial cell/epithelial cell ratio. This multiplicity of infection (MOI) was the best approximation we could make based on the average values determined for recovery of vaginal bacteria per gram of vaginal fluid (59, 65, 66). Our preliminary setup included comparisons of doses administered over a range of MOIs at 4-h incubation intervals, and we saw consistent adherence rates that did not increase when higher CFU numbers were added to the vaginal epithelial surface (67). The bacterial-epithelial cocultures were then incubated using an orbital shaker at 35°C under anaerobic conditions and an AnaeroPack system (PML Microbiologicals, Wilsonville, OR).

After 24 h, the loosely attached bacteria were removed by two washes in sterile Dulbecco's phosphate-buffered saline (PBS) (Invitrogen by Life Technologies, Carlsbad, CA), followed by 24-h exposures to various doses of test compounds in antibiotic-free KSFM. At the end of this period, supernatants were collected for soluble mediators and the epithelial cells were washed two times with sterile PBS and used for assessment of epithelial viability, CFU counts, caspase-3 cleavage, and NF-κB activation as described below.

Transmission electron microscopy. To visualize bacterial attachment to epithelial cells in our model and to assess morphological signs of apoptosis, Vk2/E6E7 cells were seeded on Aclar embedding film (Ted Pella Inc., Redding, CA). After 24 h of bacterial colonization with 2.2×10^6 bacteria/cm^2, the epithelial monolayers were washed with cold PBS and fixed for 3 h at room temperature in 2% formaldehyde–2.5% glutaraldehyde–0.1 M sodium cacodylate buffer (pH 7.4) followed by 30 min of incubation in 1% osmium tetroxide–1.5% potassium ferrocyanide, washing in PBS, and 30 min of incubation in aqueous uranyl acetate (all from Electron Microscopy Science, Hatfield, PA). The cells were next washed in PBS and dehydrated in 50%, 70%, 95%, and 100% alcohol for 5 min per gradient. Cells were embedded in plastic by inverting an Epon-araldite gelatin capsule over the sample and then polymerized at 60°C for 24 h. Ultrathin sections were cut using a Reichert Ultracut-S microtome (Lecia Microsystems, Buffalo Grove, IL), applied to lead citrate-stained copper grids, and examined using a TecnaiG2 Spirit BioTWIN transmission electron microscope (FEI Company, Hillsboro, OR) at a primary magnification of ×1,000 to ×30,000. Images were recorded with an AMT 2k charge-coupled-device (CCD) camera (Advanced Microscopy Techniques, Woburn, MA).

Tests of epithelial cell viability. To assess compound cytotoxicity, we used the CellTiter 96 MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-

2H-tetrazolium bromide] assay (Promega, Madison, WI) for the immortalized cell lines and the VEC-100 model as previously described (20, 54, 56, 58). In this assay, mitochondria in viable cells convert the yellow MTT into a blue formazan product. The colorimetric reaction is measured by determining absorbance at 570 nm with a reference wavelength at 630 nm. Absorbance was read using a Victor2 counter with Wallac 2.01 software (PerkinElmer Life Sciences). Viability was quantified as the percentage of the total cell numbers seen under each set of conditions of compound treatment versus the average optical density (OD) measured for untreated (medium alone) control cells.

In addition, we used the trypan blue assay to enumerate viable epithelial cells in the bacterially colonized epithelial cultures and for a quantitative assessment of cleaved versus total caspase-3 amounts as a marker of epithelial cell apoptosis (Meso Scale Discovery [MSD], Gaithersburg, MD).

For the trypan blue assay, epithelial cells were dislodged with 0.1% trypsin–0.01% EDTA (Invitrogen by Life Technologies, Carlsbad, CA) as described previously (55) at 5 days after bacterial colonization. Upon neutralization with 10% fetal calf serum in DMEM-F12 medium (Invitrogen), the epithelial suspensions were mixed with equal volumes of 10% trypan blue (Fisher Scientific, Pittsburgh, PA) followed by enumeration of viable cells (no color) and dead cells (blue color due to dye uptake) by the use of a standard hemocytometer microscope.

For the cleaved and total caspase-3 assays, epithelial cultures were incubated with medium control or bacteria for 24 h. The proapoptotic agent staurosporine (Sigma Aldrich, St. Louis, MO) was added at 1 μM to serve as a positive control. At the end of this period, epithelial cell monolayers were lysed in MSD-provided Tris lysis buffer and protease inhibitor cocktail per manufacturer's instructions and 25 μl of each lysate was loaded into the MSD 2-spot assay plates for simultaneous measurement of total and cleaved caspase-3 levels. Caspase-3 is a downstream proapoptotic effector caspase, regulating multiple proteins with key functions in apoptotic signaling (68).

CFU enumeration. Viable bacteria associated with epithelial cells as well as planktonic bacterial growth in epithelial cell-free medium were assessed by CFU counts after 24 h of epithelial colonization with bacteria and 24 h of incubation with test compounds. For assessment of epithelial cell-associated CFUs, at the end of each incubation period, epithelial cells were washed twice with ice-cold PBS and hypotonically lysed in ice-cold HyPure water (Fisher Scientific, Pittsburgh, PA) for 15 min, followed by adjustment of osmolarity with PBS (concentrated 2-fold) and plating on *Brucella* anaerobic agar with 5% sheep blood (BD, Franklin Lakes, NJ). The plates were incubated in an anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, MI) containing an atmosphere of 10% hydrogen, 10% carbon dioxide, and 80% nitrogen at 37°C for 24 to 72 h (until visible colonies were formed), followed by CFU counting. For the assessment of direct effects of compounds on planktonic bacteria, the same solutions of compounds that were tested in the colonized model were mixed with bacterial suspensions in the absence of epithelial cells and incubated under the same anaerobic conditions for 24 h followed by agar plating and CFU counts performed as described above.

NF-κB luciferase assay. Epithelial cells seeded at 1×10^4 cells/well in 96-well plates were transfected with pHTS–NF-κB firefly luciferase reporter vector (Biomyx Technology, San Diego, CA) by the use of a genejuice transfection protocol as described previously (4). After supernatant removal at the end of the test compound treatment period, epithelial cells were lysed in GloLysis buffer and luciferase activity was determined using a Bright-Glo luciferase assay system per manufacturer instructions (Promega, Madison, WI). Luminescence signal was measured using a Victor2 1420 multilabel microplate counter with Wallac 2.01 software (PerkinElmer Life Sciences, Boston, MA).

Quantitation of innate immunity mediators. Concentrations of the chemokines interleukin-8 (IL-8) and RANTES in cell culture supernatants were measured using electrochemiluminescence (ECL) assays and a Sector Imager 2400 reader (MSD, Gaithersburg, MD). SLPI levels were measured using a Quantikine enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN) and the Victor2 reader. Compound interference with the cytokine detection assays was ruled out by spiking known amounts of recombinant cytokine standards in compound solutions prepared in cell culture medium and measuring percentages of cytokine recovery from compound-supplemented medium versus the percentages determined for the plain medium control as described previously (9).

Statistical analysis. One-way analysis of variance (ANOVA; Bonferroni or Dunnett's multiple-comparison analyses) was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). P values of <0.05 were considered significant.

ACKNOWLEDGMENTS

This work was supported by grant PPA-09-034 to R.N.F. from CONRAD, Eastern Virginia Medical School, under a Cooperative Agreement (GPO-A-00-08-00005-00) with the U.S. Agency for International Development (USAID). The development of the vaginal colonization model was partially supported by a Connor's Seed Grant for Gender Biology, Center for Women's Health, Brigham and Women's Hospital (R.N.F.), and by grant NICHD 5R21HD054451-02 (R.N.F.).

The views expressed by the authors do not necessarily reflect the views of CONRAD, Eastern Virginia Medical School, or USAID.

REFERENCES

1. **Fichorova RN, Anderson DJ.** 1999. Differential expression of immunobiological mediators by immortalized human cervical and vaginal epithelial cells. Biol. Reprod. **60**:508–514.
2. **Fichorova RN, Cronin AO, Lien E, Anderson DJ, Ingalls RR.** 2002. Response to *Neisseria gonorrhoeae* by cervicovaginal epithelial cells occurs in the absence of Toll-like receptor 4-mediated signaling. J. Immunol. **168**:2424–2432.
3. **Fichorova RN, Desai PJ, Gibson FC, III, Genco CA.** 2001. Distinct proinflammatory host responses to *Neisseria gonorrhoeae* infection in immortalized human cervical and vaginal epithelial cells. Infect. Immun. **69**:5840–5848.
4. **Fichorova RN, et al.** 2006. *Trichomonas vaginalis* lipophosphoglycan triggers a selective upregulation of cytokines by human female reproductive tract epithelial cells. Infect. Immun. **74**:5773–5779.
5. **Wira CR, Fahey JV, Sentman CL, Pioli PA, Shen L.** 2005. Innate and adaptive immunity in female genital tract: cellular responses and interactions. Immunol. Rev. **206**:306–335.
6. **Wira CR, Grant-Tschudy KS, Crane-Godreau MA.** 2005. Epithelial cells in the female reproductive tract: a central role as sentinels of immune protection. Am. J. Reprod. Immunol. **53**:65–76.
7. **Doncel GF, Chandra N, Fichorova RN.** 2004. Preclinical assessment of the proinflammatory potential of microbicide candidates. J. Acquir. Immune Defic. Syndr. **37**(Suppl. 3): S174–S180.
8. **Doncel GF, Clark MR.** 2010. Preclinical evaluation of anti-HIV microbicide products: new models and biomarkers. Antivir. Res. **88**(Suppl. 1): S10–S18.
9. **Fichorova RN.** 2004. Guiding the vaginal microbicide trials with biomarkers of inflammation. J. Acquir. Immune Defic. Syndr. **37**(Suppl. 3): S184–S193.
10. **Lard-Whiteford SL, et al.** 2004. Recommendations for the nonclinical development of topical microbicides for prevention of HIV transmission: an update. J. Acquir. Immune Defic. Syndr. **36**:541–552.
11. **Anderson RA, et al.** 2002. Preclinical evaluation of sodium cellulose sulfate (Ushercell) as a contraceptive antimicrobial agent. J. Androl. **23**: 426–438.
12. **Doh AS, et al.** 2007. Safety and acceptability of 6% cellulose sulfate vaginal gel applied four times per day for 14 days. Contraception **76**:245–249.
13. **El-Sadr WM, et al.** 2006. Safety and acceptability of cellulose sulfate as a vaginal microbicide in HIV-infected women. AIDS **20**:1109–1116.
14. **Mauck C, et al.** 2001. Single and multiple exposure tolerance study of cellulose sulfate gel: a phase I safety and colposcopy study. Contraception **64**:383–391.

15. **Schwartz JL, et al.** 2006. Fourteen-day safety and acceptability study of 6% cellulose sulfate gel: a randomized double-blind phase I safety study. Contraception **74**:133–140.
16. **Scordi-Bello IA, et al.** 2005. Candidate sulfonated and sulfated topical microbicides: comparison of anti-human immunodeficiency virus activities and mechanisms of action. Antimicrob. Agents Chemother. **49**: 3607–3615.
17. **Van Damme L, et al.** 2008. Lack of effectiveness of cellulose sulfate gel for the prevention of vaginal HIV transmission. N. Engl. J. Med. **359**: 463–472.
18. **Cheshenko N, et al.** 2004. Candidate topical microbicides bind herpes simplex virus glycoprotein B and prevent viral entry and cell-to-cell spread. Antimicrob. Agents Chemother. **48**:2025–2036.
19. **Hladik F, Doncel GF.** 2010. Preventing mucosal HIV transmission with topical microbicides: challenges and opportunities. Antiviral Res. **88**(Suppl. 1):S3–S9.
20. **Fichorova RN, Tucker LD, Anderson DJ.** 2001. The molecular basis of nonoxynol-9-induced vaginal inflammation and its possible relevance to human immunodeficiency virus type 1 transmission. J. Infect. Dis. **184**: 418–428.
21. **Hedges SR, Barrientes F, Desmond RA, Schwebke JR.** 2006. Local and systemic cytokine levels in relation to changes in vaginal flora. J. Infect. Dis. **193**:556–562.
22. **Nikolaitchouk N, Andersch B, Falsen E, Strömbeck L, Mattsby-Baltzer I.** 2008. The lower genital tract microbiota in relation to cytokine-, SLPI- and endotoxin levels: application of checkerboard DNA-DNA hybridization (CDH). APMIS **116**:263–277.
23. **Witkin SS, Linhares IM, Giraldo P.** 2007. Bacterial flora of the female genital tract: function and immune regulation. Best Pract. Res. Clin. Obstet. Gynaecol. **21**:347–354.
24. **Libby EK, Pascal KE, Mordechai E, Adelson ME, Trama JP.** 2008. *Atopobium vaginae* triggers an innate immune response in an *in vitro* model of bacterial vaginosis. Microbes Infect. **10**:439–446.
25. **Fichorova RN, et al.** 2011. Maternal microbe-specific modulation of inflammatory response in extremely low-gestational-age newborns. mBio **2**(1):e00280-10. doi:10.1128/mBio.00280-10.
26. **Novak RM, et al.** 2007. Cervicovaginal levels of lactoferrin, secretory leukocyte protease inhibitor, and RANTES and the effects of coexisting vaginoses in human immunodeficiency virus (HIV)-seronegative women with a high risk of heterosexual acquisition of HIV infection. Clin. Vaccine Immunol. **14**:1102–1107.
27. **Farquhar C, et al.** 2002. Salivary secretory leukocyte protease inhibitor is associated with reduced transmission of human immunodeficiency virus type 1 through breast milk. J. Infect. Dis. **186**:1173–1176.
28. **McNeely TB, et al.** 1995. Secretory leukocyte protease inhibitor: a human saliva protein exhibiting anti-human immunodeficiency virus 1 activity *in vitro*. J. Clin. Invest. **96**:456–464.
29. **Moutsopoulos NM, Greenwell-Wild T, Wahl SM.** 2006. Differential mucosal susceptibility in HIV-1 transmission and infection. Adv. Dent. Res. **19**:52–56.
30. **Semba RD, et al.** 1999. Mastitis and immunological factors in breast milk of human immunodeficiency virus-infected women. J. Hum. Lact. **15**: 301–306.
31. **Shugars DC, Sauls DL, Weinberg JB.** 1997. Secretory leukocyte protease inhibitor blocks infectivity of primary monocytes and mononuclear cells with both monocytotropic and lymphocytotropic strains of human immunodeficiency virus type I. Oral Dis. **3**(Suppl. 1):S70–S72.
32. **Shugars DC, Wahl SM.** 1998. The role of the oral environment in HIV-1 transmission. J. Am. Dent. Assoc. **129**:851–858.
33. **Skott P, Lucht E, Ehnlund M, Björling E.** 2002. Inhibitory function of secretory leukocyte proteinase inhibitor (SLPI) in human saliva is HIV-1 specific and varies with virus tropism. Oral Dis. **8**:160–167.
34. **Wahl SM, et al.** 1997. Secretory leukocyte protease inhibitor (SLPI) in mucosal fluids inhibits HIV-I. Oral Dis. **3**(Suppl. 1):S64–S69.
35. **Wahl SM, et al.** 1997. Anatomic dissociation between HIV-1 and its endogenous inhibitor in mucosal tissues. Am. J. Pathol. **150**:1275–1284.
36. **Halpern V, et al.** 2008. Effectiveness of cellulose sulfate vaginal gel for the prevention of HIV infection: results of a phase III trial in Nigeria. PLoS One **3**:e3784.
37. **Verstraelen H, et al.** 2009. Longitudinal analysis of the vaginal microflora in pregnancy suggests that *L. crispatus* promotes the stability of the normal vaginal microflora and that *L. gasseri* and/or *L. iners* are more conducive to the occurrence of abnormal vaginal microflora. BMC Microbiol. **9**:116.
38. **Zeichner SL.** 2011. HIV human vaginal microbicide effects on the vaginal microbial community. Keystone Symposia: "Protection from HIV: Targeted Intervention Strategies," Whistler, BC, Canada.
39. **Simoes JA, et al.** 2002. Two novel vaginal microbicides (polystyrene sulfonate and cellulose sulfate) inhibit *Gardnerella vaginalis* and anaerobes commonly associated with bacterial vaginosis. Antimicrob. Agents Chemother. **46**:2692–2695.
40. **Herold BC, et al.** 2000. Poly(sodium 4-styrene sulfonate): an effective candidate topical antimicrobial for the prevention of sexually transmitted diseases. J. Infect. Dis. **181**:770–773.
41. **Tao W, Richards C, Hamer D.** 2008. Enhancement of HIV infection by cellulose sulfate. AIDS Res. Hum. Retroviruses **24**:925–929.
42. **Van Damme L, Taylor D.** 2008. Cellulose sulfate for prevention of HIV infection. N. Engl J. Med. **359**:2066–2068.
43. **Kaul R, et al.** 2008. Genital levels of soluble immune factors with anti-HIV activity may correlate with increased HIV susceptibility. AIDS **22**: 2049–2051.
44. **Capobianchi MR, et al.** 1998. Inhibition of HIV type 1 BaL replication by MIP-1alpha, MIP-1beta, and RANTES in macrophages. AIDS Res. Hum. Retroviruses **14**:233–240.
45. **Cocchi F, et al.** 1995. Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by $CD8^+$ T cells. Science **270**:1811–1815.
46. **Coffey MJ, Woffendin C, Phare SM, Strieter RM, Markovitz DM.** 1997. RANTES inhibits HIV-1 replication in human peripheral blood monocytes and alveolar macrophages. Am. J. Physiol. **272**:L1025–L1029.
47. **Lehner T.** 2002. The role of CCR5 chemokine ligands and antibodies to CCR5 coreceptors in preventing HIV infection. Trends Immunol. **23**: 347–351.
48. **Moriuchi H, Moriuchi M, Fauci AS.** 1997. Nuclear factor-kappa B potently up-regulates the promoter activity of RANTES, a chemokine that blocks HIV infection. J. Immunol. **158**:3483–3491.
49. **Vangelista L, Secchi M, Lusso P.** 2008. Rational design of novel HIV-1 entry inhibitors by RANTES engineering. Vaccine **26**:3008–3015.
50. **Iqbal SM, et al.** 2005. Elevated T cell counts and RANTES expression in the genital mucosa of HIV-1-resistant Kenyan commercial sex workers. J. Infect. Dis. **192**:728–738.
51. **Rebbapragada A, et al.** 2008. Bacterial vaginosis in HIV-infected women induces reversible alterations in the cervical immune environment. J. Acquir. Immune Defic. Syndr. **49**:520–522.
52. **Verhelst R, et al.** 2005. Comparison between Gram stain and culture for the characterization of vaginal microflora: definition of a distinct grade that resembles grade I microflora and revised categorization of grade I microflora. BMC Microbiol. **5**:61.
53. **Levin J, Bang FB.** 1968. Clottable protein in Limulus; its localization and kinetics of its coagulation by endotoxin. Thromb. Diath. Haemorrh. **19**: 186–197.
54. **Fichorova RN, et al.** 2004. Interleukin (IL)-1, IL-6, and IL-8 predict mucosal toxicity of vaginal microbicidal contraceptives. Biol. Reprod. **71**: 761–769.
55. **Fichorova RN, Rheinwald JG, Anderson DJ.** 1997. Generation of papillomavirus-immortalized cell lines from normal human ectocervical, endocervical, and vaginal epithelium that maintain expression of tissue-specific differentiation proteins. Biol. Reprod. **57**:847–855.
56. **Fichorova RN, et al.** 2005. Anti-human immunodeficiency virus type 1 microbicide cellulose acetate 1,2-benzenedicarboxylate in a human *in vitro* model of vaginal inflammation. Antimicrob. Agents Chemother. **49**: 323–335.
57. **Canny GO, Trifonova RT, Kindelberger DW, Colgan SP, Fichorova RN.** 2006. Expression and function of bactericidal/permeability-increasing protein in human genital tract epithelial cells. J. Infect. Dis. **194**:498–502.
58. **Trifonova RT, Pasicznyk JM, Fichorova RN.** 2006. Biocompatibility of solid-dosage forms of anti-human immunodeficiency virus type 1 microbicides with the human cervicovaginal mucosa modeled ex vivo. Antimicrob. Agents Chemother. **50**:4005–4010.
59. **Onderdonk AB, et al.** 1987. Qualitative assessment of vaginal microflora during use of tampons of various compositions. Appl. Envir Microbiol. **53**:2779–2784.
60. **Klein G, Pack A, Bonaparte C, Reuter G.** 1998. Taxonomy and physiology of probiotic lactic acid bacteria. Int. J. Food Microbiol. **41**:103–125.

61. **Senok AC, Verstraelen H, Temmerman M, Botta GA.** 2009. Probiotics for the treatment of bacterial vaginosis. Cochrane Database Syst. Rev. CD006289. http://www2.cochrane.org/reviews/en/ab006289.html.
62. **Antonio MA, Hawes SE, Hillier SL.** 1999. The identification of vaginal Lactobacillus species and the demographic and microbiologic characteristics of women colonized by these species. J. Infect. Dis. **180**:1950–1956.
63. **Spear GT, et al.** 2008. Comparison of the diversity of the vaginal microbiota in HIV-infected and HIV-uninfected women with or without bacterial vaginosis. J. Infect. Dis. **198**:1131–1140.
64. **Murray PR (ed).** 1995. Manual of clinical microbiology. ASM Press, Washington, DC.
65. **Delaney ML, Onderdonk AB.** 2001. Nugent score related to vaginal culture in pregnant women. Obstet. Gynecol. **98**:79–84.
66. **Onderdonk AB, Lee ML, Lieberman E, Delaney ML, Tuomala RE.** 2003. Quantitative microbiologic models for preterm delivery. J. Clin. Microbiol. **41**:1073–1079.
67. **Fichorova RN, Ross RA.** 2002. Vaginal microbiota regulates epithelial proinflammatory pathways. Keystone Symposia. NF-kappaB: Bench to Bedside. Keystone Symposia for Molecular and Cellular Biology, Keystone, Colorado.
68. **Li J, Yuan J.** 2008. Caspases in apoptosis and beyond. Oncogene **27**: 6194–6206.