Broadly neutralizing monoclonal antibodies (nAbs) specific for HIV are being investigated for use in HIV prevention. Due to their ability to inhibit HIV attachment to and entry into target cells, nAbs may be suitable for use as topical HIV microbicides. As such, they would present an alternative intervention for individuals who may not benefit from using antiretroviral-based products for HIV prevention. We theorize that nAbs can inhibit viral transmission through mucosal tissue, thus reducing the incidence of HIV infection. The efficacy of the PG9, PG16, VRC01, and 4E10 antibodies was evaluated in an ex vivo human model of mucosal HIV transmission. nAbs reduced HIV transmission, causing 1.5- to 2-log_{10} reductions in HIV replication in ectocervical tissues and \approx 3\text{-log}_{10} reductions in HIV replication in colonic tissues over 21 days. These antibodies demonstrated greater potency in colonic tissues, with a 50-fold higher dose being required to reduce transmission in ectocervical tissues. Importantly, nAbs retained their potency and reduced viral transmission in the presence of whole semen. No changes in tissue viability or immune activation were observed in colonic or ectocervical tissue after nAb exposure. Our data suggest that topicaly applied nAbs are safe and effective against HIV infection of mucosal tissue and support further development of nAbs as a topical microbicide that could be used for anal as well as vaginal protection.

With circulating drug-resistant human immunodeficiency virus (HIV) on the rise in many communities where preexposure prophylactics (PrEP) will be used (1), the risk of transmitting virus with reduced susceptibility to antiretrovirals (ARVs) is possible. To circumvent this risk, several non-ARV microbicide candidates are being considered. Previous investigations of non-ARV microbicides used non-HIV-specific formulations of compounds, such as surfactants, polyanions, and buffering agents; however, all were ineffective at preventing HIV acquisition. Among them, the surfactant nonoyxynol-9 (N-9), which is commercially available as a spermicide, was shown to have anti-HIV activity in vitro (2). However, clinical evaluation of N-9 was stopped due to increased HIV incidence in women using an N-9 vaginal gel (3). Additionally, N-9 was shown to cause tight junction disruptions in epithelial cells in vitro (4) and to be harmful to beneficial vaginal flora (5). Other candidates, such as BufferGel (6), the carrageenan derivative Carraguard (7), and polyanionic gels (PRO 2000 [6] and cellulose sulfate [4]), were all unsuccessful as HIV microbicides. However, the new non-ARV microbicide candidates being investigated are HIV-specific agents and include broadly neutralizing monoclonal antibodies (nAbs).

While nAbs have been investigated extensively in the development of HIV vaccines, this increased focus has been placed on their development for HIV prevention. Originally isolated from chronically HIV-infected individuals, nAbs were shown to retain potent neutralizing activity across a broad range of HIV clades (8). These highly cross-reactive antibodies are found in only a small subset of HIV-infected individuals (9, 10), and they develop their broad cross-reactivity through a process of somatic hypermutation over 2 to 4 years (10, 11). They bind epitopes on key regions of the HIV envelope and directly inhibit the ability of virions to engage entry receptors on target cells, thereby reducing viral infection. nAbs such as VRC01, b12, and NIH 45-46 exert their HIV-inhibitory activity by binding to the CD4 binding site (12), while 4E10, 10E8, and 2F5 bind to the membrane-proximal external region (MPER) at the base of the envelope spike (12), and others, such as PG9 and PG16, recognize quaternary glycosylation motifs on the exposed variable loops of gp120 (12). nAbs have shown efficacy in reducing HIV transmission in vitro (8, 13) and in animal models of HIV transmission in vivo (14, 15). This ability to inhibit viral transmission is particularly important in the context of HIV prevention, as it is better to prevent HIV acquisition than to overcome the complications inherent to treating an established HIV infection. nAbs also bridge the gap between non-HIV-specific compounds and ARV drugs in the spectrum of HIV microbicide candidates. The antibodies are specific for HIV, but their activity has not been shown to affect viral sensitivity to HIV enzyme inhibitors. Conversely, viral neutralization by nAbs is not expected to be hampered by drug resistance mutations in traditional ARV drug targets—HIV reverse transcriptase, protease, and integrase. This is because HIV envelope proteins are not under the selective pressure of HIV enzyme inhibitors. Hence, nAbs are expected to retain efficacy against viruses that are ARV resistant.

In this study, the efficacy of nAbs—PG9, PG16, VRC01, and 4E10—was evaluated in human mucosal tissue ex vivo. While studies of nAb efficacy have been conducted using animal models, this study is the first to evaluate the efficacy of topically applied nAbs by using a relevant human mucosal model of HIV transmis-
controls were inoculated with 3,000 TCID50 HIV-1JR-CSF in 100 μl solutions (St. Louis, MO).

Whole human semen from pooled donors was purchased from Lee Bio-Solutions (St. Louis, MO).

Unless otherwise noted, culture media were purchased from Pittsburgh, PA) by using the Reed-Muench method (17).

vical explants were activated with 0.5

5-mm-diameter explants were mounted with the epithelium upward on gel foam inserts in 12-mm permeable transwell supports and sealed with Matrigel.

Colonic explants were activated with 0.5 μg/ml PHA-P and cultured in RPMI 1640 supplemented with 5% (vol/vol) human A/B serum, 1% Pen-Strep-l-Glut, 0.5 mg/ml piperacillin-tazobactam (Zosyn; Wyeth, Collegeville, PA), 100 U/ml Hl-L-2 (Roche Diagnostics, Indianapolis, IN), and 2.5 mM HEPES (HyClone, Logan, UT).

Efficacy evaluations in human tissue ex vivo. Paired polarized explants were treated with nAbs 24 h (colon) or 48 h (ectocervix) after setup. nAbs were applied at 2 times the final concentration to the apical surface of the appropriate explants in duplicate and cultured for 1 h at 37°C. Controls were treated similarly with either medium only or the HSV-8-N antibody. After 1 h, each explant was inoculated apically with 50,000 TCID50 (ectocervix) or 10,000 TCID50 (colon) HIV-1JR-CSF suspended in RPMI 1640 or 50% (vol/vol) pooled whole human semen (Lee Bio-Solutions, St. Louis, MO). Tissues were incubated for 24 h, after which the basolateral medium was collected and the explants were washed with 1× Dulbecco’s phosphate-buffered saline (DPBS; Mediatech, Inc., Manassas, VA). The basolateral medium was replenished with fresh medium supplemented with 100 U/ml Hl-L-2 and subsequently collected every 3 to 4 days for up to 21 days postinfection and stored at −80°C. Viral replication was monitored by measuring HIV p24 in the basolateral medium by using the Millipore HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA) kit (PerkinElmer, Waltham, MA). Individual ectocervical explants were fixed in formalin at 21 days postinfection and processed for immunohistochemical (IHC) analysis of intracellular HIV p24 antigen (19).

Evaluation of immune activation and tissue viability ex vivo. Nonactivated polarized ectocervical and colonic explants were treated on the apical surface with 1.5 μM and 0.03 μM nAbs, respectively. For controls, tissues were treated with medium only (negative control) or with 0.5 μg/ml PHA-P and 100 U/ml Hl-L-2 in the basolateral medium (positive control). All treatments were performed in duplicate, and tissues were cultured for 24 h at 37°C and 5% CO2. The basolateral medium was sampled 24 h after treatment and used for quantitative comparison of inflammatory cytokines (gamma interferon [IFN-γ], IL-10, IL-1β, IL-6, IL-8, tumor necrosis factor alpha [TNF-α], granulocyte-macrophage colony-stimulating factor [GM-CSF], IL-12, macrophage inflammatory protein 1β [MIP-1β], IL-18, and IL-15) by using a Milliplex human cytokine/chemokine magnetic bead panel (EMD Millipore Corp., Billerica, MA). For viability assays, nonactivated polarized explants were treated with nAbs as described above for 24 h. Control tissues were treated apically with medium only or with a 1:5 dilution of Options Gynol II Extra Strength gel (Caldwell Consumer Health LLC, Madison, WI), containing 3% N-9. After 24 h of treatment, explants were processed for histology and viability determination by MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay.

Statistical analysis. All variables were inspected using descriptive statistics and graphical methods. For analyses, raw HIV p24 values were log transformed before their distribution was heavily skewed. Linear mixed models were used to test the effects of semen, nAb treatment, and different nAb doses on the trajectory of HIV p24 during the 21-day culture period. For all models, random effects for explants and tissue donors were included to adjust for any clustering effect of repeated measures within each explant and tissue donor. Separate models were used for ectocervical and colonic data.

The model used to evaluate the effects of different nAbs on HIV p24 included dummy variables for nAbs, day of culture, and the interaction between day of culture and individual nAbs as fixed effects. To evaluate the effects of semen on nAb potency, the model included the presence or absence of semen, day of culture, and the interaction between these factors as fixed effects. The effect of semen on HIV p24 was also investigated while adjusting for the effect of nAbs by using a model that included the pres-
ence or absence of semen and dummy variables for each nAb, day of culture, and the interactions between day of culture and semen or nAb treatment as fixed effects. The effects of individual nAbs at each dose were investigated with dose and day of culture as dummy variables for each nAb and with interactions between day of culture and dose or each nAb as fixed effects. A model using dose, day of culture, and their interaction as fixed effects was used to interrogate the effect of nAb dose on HIV p24. Finally, the effects of semen and nAb dose were investigated separately for each antibody, using a model that included the effects of semen, nAb dose, and their interaction separately for each antibody. All analyses were performed in R (R Foundation for Statistical Computing, Vienna, Austria).

Comparisons between treatments and viability outcomes from MTT viability experiments were made by one-way analysis of variance (ANOVA). The effects of nAb treatment on cytokine concentrations were compared using two-way ANOVA and the Holm-Sidak correction for multiple comparisons. Colon and ectocervical data were analyzed separately, and analyses were performed using GraphPad Prism, version 6.05 (GraphPad Software, La Jolla, CA).

RESULTS

Assessment of nAb efficacy in vitro. Using TZM-bl cells, the IC_{90}s of all nAbs against HIV-1_{R-C58} infection in vitro were derived. The potencies of nAbs VRC01-N and 4E10-N, produced in the transgenic N. benthamiana expression system, were also compared to those of the same nAbs produced in the traditional CHO cell system (VRC01 and 4E10) and were shown to have equivalent inhibitory activities (P > 0.05) (Table 1). The IC_{90} values indicated that nAb potencies had the following order: PG16 > PG9 > VRC01/N > 4E10/4E10-N. Due to their equivalance to CHO cell-produced nAbs and their greater availability, the Nicotiana-produced antibodies were used in the subsequent work.

nAbs reduce HIV transmission in human mucosal tissue ex vivo. To define nAb potencies in mucosal tissue, equivalent molar concentrations of IgG were used for all nAbs. Since the IC_{90} value of VRC01-N was intermediate in the range of those for the other nAbs used in this study (Table 1), the doses of all nAbs were standardized to the effective concentrations of VRC01-N. For ectocervical tissue, 1.5 μM and 0.3 μM IgG concentrations were used, which were the doses of IgG equivalent to 50× and 10× the IC_{90} of VRC01-N, respectively. Similarly, for colonic tissue, concentrations of 0.03 μM and 0.003 μM were used, which were equivalent to 1× and 0.1× the VRC01-N IC_{90}, respectively. The HSV-8-N isotope control antibody was used at a concentration of 0.3 μM in ectocervical tissues and 0.003 μM in colonic tissues.

nAbs were applied to the apical surface of tissues for 1 h before inoculation with virus to simulate pericoital application of a topical microbicide preparation. Using this strategy, treatment of ectocervical tissues with 1.5 μM and 0.3 μM doses of VRC01-N, PG9, and PG16 caused significant reductions in HIV p24 over the 21-day culture period (P < 0.0001) compared to the levels in the untreated controls. Treatment with 1.5 μM VRC01-N, PG9, or PG16 caused a median reduction of 1.5, 1.9, or 1.8 log_{10} pg/ml HIV p24 at day 21 of culture, respectively, compared to the level in the untreated control (Fig. 1a; Table 2). Conversely, treatment with 4E10-N did not significantly reduce HIV p24 (P = 0.5797) (Fig. 1a; Table 2), as the concentrations used were below the in vitro IC_{90} of this nAb (Table 1). However, tissues pretreated with doses of 59.5 μM and 11.9 μM 4E10-N in ectocervical tissues and 1.19 μM and 0.119 μM in colonic tissues showed reductions in HIV transmission (see Fig. S1 in the supplemental material). These doses correspond to 50×, 10×, 1×, and 0.1× the IC_{90} of 4E10-N (Table 1), respectively, and also reflect 8- to 40-fold higher concentrations than the equivalent doses of nAbs used in this study. Treatment with the isotype control antibody, HSV-8-N, also did not affect HIV replication (P = 0.2830) (Table 2). There was also a greater reduction in HIV p24 with the use of 1.5 μM PG9 (P = 0.0171) and PG16 (P = 0.0302) at day 21 than with a 0.3 μM concentration of these nAbs (Table 2). While there was a trend, the high dose of VRC01-N was not significantly different from the low dose (P = 0.0810). This suggests that the neutralizing capacity of 0.3 and 1.5 μM doses of VRC01-N may have been saturated by the inoculum used in the ectocervical model. The dose effect observed with the PG9 and PG16 antibodies in the same model supports the observation that these nAbs are more potent and is discordant with the nAb hierarchy of potency observed in vitro (Table 1).

nAbs provide differential protection between human colonic and ectocervical tissues ex vivo. Using 0.03 or 0.003 μM nAbs (Fig. 1b), treatment with PG16 (P < 0.0001), PG9 (P < 0.0001), or VRC01-N (P < 0.0001) caused significant reductions in HIV p24 over the treatment period. Pretreatment of colonic tissues with 0.03 μM PG16, PG9, and VRC01-N yielded reductions of 3.0, 3.3, and 3.3 log_{10}, respectively, in the median HIV p24 level by day 21 of culture compared to the levels in untreated controls (Fig. 1b). A dose effect was observed in colonic tissues, as tissues treated with the low dose (0.003 μM) of VRC01-N, PG9, or PG16 had decreases in median HIV p24 of 1.2 log_{10} or less at day 21 (P < 0.0001) compared to tissues treated with 0.03 μM nAbs (Table 2). However, treatment with 4E10-N had no effect on HIV p24 at any of the concentrations used (P = 0.1994). The reductions of median HIV p24 of <0.5 log_{10} observed at day 21 for tissues treated with 4E10-N did not reach statistical significance and support the lack of potency observed with 4E10-N in this study. Infection curves for tissues treated with 0.003 μM PG9 or PG16 showed a delay in infection that was not observed with VRC01-N, with virus production expanding from 14 to 17 days postinfection (Fig. 1b). These results support the observation that PG9 and PG16 demonstrate superior potency in colonic tissue compared to ectocervical tissue (Fig. 1a) and are also discordant with the order of nAb potency described in our in vitro assays (Table 1). Higher doses of nAbs showed greater responses than lower doses of the same nAbs in colonic tissues, with the exception of 4E10-N.

nAbs retain potency in the presence of semen. During in vitro evaluations of some non-ARV microbicides, such as the polyanionic gel PRO 2000, semen was shown to abrogate anti-HIV activity (21). The counteractive effect of semen on microbicides has been attributed to an activity of seminal proteins on microbicide
moieties (21); the formation of amyloid fibrils that enhance HIV attachment to target cells (22); and the neutralizing effect of semen that lowers the vaginal pH, prolonging the survival of HIV virions (23–26). Hence, the potencies of nAbs in ectocervical (Fig. 2a) and colonic (Fig. 2b) tissues were compared in the presence and absence of whole human semen. Semen had no effect on HIV p24 concentrations ($P = 0.2382$) or on nAb potency in ectocervical tissue ($P = 0.0670$) compared to no-semen controls. In colonic tissues (Fig. 2b), the presence of semen had no effect on nAb potency during the 21-day culture period ($P = 0.0940$), and comparison of HIV p24 levels also showed similar levels of HIV replication in the presence or absence of semen ($P = 0.1177$). These data show no enhancement of HIV infection or effect on nAb activity in the presence of semen and collectively support the hy-

FIG 1 nAb efficacies in human ectocervical (a) and colonic (b) tissues ex vivo. Viral replication was monitored by p24 antigen ELISA on basolateral culture supernatants collected at 4, 7, 11, 14, 17, and 21 days postinfection. Data points represent the medians and interquartile ranges for ≥5 tissues from individual donors.
TABLE 2 Effects of nAbs on HIV p24 at day 21 postinfection in human tissues ex vivo

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cervix</th>
<th>Colon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dose (µM)</td>
<td>Log_{10} ΔHIV p24</td>
</tr>
<tr>
<td>VRC01-N</td>
<td>1.5</td>
<td>−1.523</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>−0.898</td>
</tr>
<tr>
<td>4E10-N</td>
<td>1.5</td>
<td>+0.067</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>−0.192</td>
</tr>
<tr>
<td>PG9</td>
<td>1.5</td>
<td>−1.924</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>−1.516</td>
</tr>
<tr>
<td>PG16</td>
<td>1.5</td>
<td>−1.752</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>−1.547</td>
</tr>
<tr>
<td>HSV-8-N</td>
<td>0.3</td>
<td>−0.005</td>
</tr>
</tbody>
</table>

^a P values represent comparisons of changes in p24 over time in tissues treated with each nAb, regardless of dose, relative to the levels in untreated tissue. Each P value, except those for HSV-8-N, corresponds to the values for each set of 2 rows.

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Early microbicide development was focused on vaginal products. However, recent efforts have expanded to optimize products to safely prevent HIV transmission during receptive anal intercourse as well. While these new products are directed at reducing the incidence of HIV among men who have sex with men (MSM), it has also been acknowledged that heterosexual anal intercourse may be underreported due to social taboos and other factors (27–29). This issue has shifted the paradigm of microbicide development toward a new generation of dual-compartment microbicides that are safe for both vaginal and rectal use (30, 31). Hence, in this study, we used models of rectal and vaginal mucosal transmission to evaluate the potential of topically applied nAbs. This study presents the first comprehensive preclinical evaluation of nAbs as a topical microbicide by use of a human ex vivo model of sexual HIV transmission. The data show that nAbs are potent and effective at reducing mucosal HIV transmission in human ectocervical and colonic tissues ex vivo. These results are concordant with animal studies where nAb preparations applied to the vaginal lumen before vaginal inoculation with simian-human immunodeficiency virus (SHIV) were protective (14, 32). Importantly, nAb potency was not affected by the presence of whole semen. In addition, this study shows that nAbs have a good safety profile, with no loss of tissue viability or immune activation that could preclude their use as an effective topical HIV microbicide.

The effective nAb dose for ectocervical tissue (1.5 µM) was 50 times higher than the effective dose for colonic tissue (0.03 µM) (Fig. 1). It is currently unclear why there are different effects of nAbs between colonic and ectocervical tissues. Pharmacokinetic studies of intravenously, intramuscularly, and orally administered ARVs have shown a differential deposition of drugs in female genital and rectal compartments. Higher drug levels are typically found in the rectal tissue than in the female genital tract (33, 34), and topically applied drugs are metabolized differently in the vaginal and rectal mucosae (35). However, in the context of a topically applied preparation of nAbs, where pharmacokinetic coverage is limited to the lumen, those observations do not explain why higher concentrations of topically applied nAbs are required to prevent viral transmission in cervical tissue than in colonic tissue. The greater potency of nAbs observed in this model of rectal mucosal transmission is not easily explained by differences in the size of inoculum used and merits further exploration. nAb potencies in the order PG16 > PG9 > VRC01-N >> 4E10-N were resolved in vitro. However, in the ex vivo model, the distinction between the potencies of PG9 and PG16 was not well defined. Lower doses of both of these nAbs caused a delay in infection...
Further attesting to the greater potency of these nAbs than that of VRC01-N or 4E10-N. This may suggest that further dilutions of PG9 and PG16 may have been needed in both tissue models to better define the comparative potencies of these antibodies.

The delay in infection observed with PG9 and PG16 treatment (Fig. 1) was likely due to a small portion of the inoculum not being neutralized by nAb treatment due to insufficiency of the antibody dose used. This low-level infection would have taken some time to expand enough to generate p24 concentrations that were above the ELISA limit of detection, hence the observed outgrowth. It is unlikely that this outgrowth of virus was due to the emergence of escape mutants, as exposure to a single dose of nAbs would not have provided sufficient selective pressure in this model. However, it must be acknowledged that infection by viruses with natural polymorphisms in envelope glycoproteins that make them resistant to neutralization by a single antibody is possible and may be prevented more effectively by using combinations of anti-HIV antibodies that simultaneously target multiple key envelope epitopes.

The relative lack of potency demonstrated by 4E10-N was not surprising, as this antibody, although broadly neutralizing, has
been characterized as being only moderately potent (36). 4E10 exerts its main HIV-inhibitory activity by MPER binding; however, this antibody is also polyreactive, engaging in relatively short-lived, low-avidity interactions with other hydrophobic moieties (37). These may contribute to the decreased potency of 4E10 in HIV neutralization. The observed lack of potency may also be attributed to slower neutralization kinetics seen for MPER antibodies, such as 4E10 and 2F5. These antibodies require an epitope conformation that is thought to only be realized post-receptor engagement in more neutralization-resistant HIV-1 isolates, such as HIV-1JR-CSF (38).

Seminal plasma contains factors that enhance HIV transmission (22, 39) and others that may interfere with microbicide activity (21). Previous microbicide evaluations showed that PRO 2000 gel protected mice from HSV-2 transmission. However, in the presence of semen, the product showed decreased efficacy in vitro (21). It was postulated that this effect may have been due to seminal protein interactions with the polyanion that blocked binding to HSV-2. This effect of semen essentially precludes the use of PRO 2000 for prevention of mucosal HIV transmission, where the putative inoculum is HIV-infected semen. In contrast, evaluation of nAb potency in the presence...
of semen showed that nAb activity in this model was not affected.

Preliminary safety data on the use of topically applied nAbs (Fig. 3) suggest that they are suitable for use as a topical on-demand prophylactic. In experiments to determine if there was immune activation due to nAb treatment, nAbs generally caused no changes in concentrations of inflammatory mediators, suggesting their suitability as topical microbicides. Additional safety evaluations may be necessary to determine the safety of other nAb applications. Previous nonantiretroviral microbicide candidates were shown to cause immune activation. Increased expression of IP-10 in the female genital tract has been associated with increased HIV acquisition (40) and is indicative of an inflammatory milieu. Treatment of ectocervical tissue with VRC01 was associated with decreased expression of IP-10 (Fig. 3c), suggesting that these nAbs do not increase production of soluble proinflammatory mediators.

Regarding a nAb microbicide, antibodies are highly specific for their cognate HIV epitopes and are native to the human body, hence nAbs applied lumina are expected to have negligible adverse effects that would preclude a therapeutic benefit. Traditional pharmacoeutic antibody production uses transgenic mouse or human cell systems, both of which can be cost prohibitive to producing an affordable antibody-based topical microbicide. The cost of producing a pharmacoeutic-grade supply of antibodies by using mammalian cell or animal systems was estimated to be $5 to 6 million over 18 months. In comparison, the use of transgenic plant production systems has made large-scale production of antibodies feasible and relatively cheap. Production of a pharmacoeutic-grade supply of antibodies in transgenic plant production systems was estimated to cost $0.5 to 0.8 million over 12 months (16). These time and cost savings are important factors for making a nAb microbicide accessible for use in resource-limited settings. The data reported here show that nAbs are effective in modes of both rectal and vaginal transmission and may be considered for formulation as a dual-compartment microbicide product. Hence, an antibody-based microbicide could possibly expand microbicide application beyond vaginal use in high-risk women to rectal application and use in other vulnerable populations. A nAb microbicide may also be recommended for use by heterosexual couples engaging in both vaginal and anal sex. nAb efficacy is not expected to be affected by HIV mutations selected as a result of suboptimal ARV use, hence a nAb microbicide could be used by HIV-serodiscordant couples. In addition, nAbs may provide a non-ARV microbicide option for individuals who choose to avoid the side effects of using an HIV drug. Our findings obtained using unformulated nAbs suggest that these antibodies can prevent mucosal transmission of HIV when applied pericoitally and would likely be safe and effective as topical vaginal or rectal microbicides.

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