ACIDFORM Inactivates Herpes Simplex Virus and Prevents Genital Herpes in a Mouse Model: Optimal Candidate for Microbicide Combinations

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The acidic vaginal milieu is presumed to inactivate pathogens but is neutralized by semen. This notion fostered the development of acid-buffering products, such as ACIDFORM (developed by Program for Topical Prevention of Conception and Disease, Rush University, and licensed by Instead), as microbicides. However, the extent and mechanism of protective activity provided by buffering gels is not known. Exposure of herpes simplex virus (HSV) to pH 4.5 or lower irreversibly inactivated HSV and reduced HSV yields by at least 90%; exposure to pH 5.0 had little or no effect. Pretreatment of HSV-2 with pH 3.5–4.5 triggered proteolysis, disrupting the HSV particle and resulting in a reduction in binding and invasion. ACIDFORM protected 21 (81%) of 26 mice from genital herpes, compared with 3 (12%) of 25 mice who received a placebo gel. ACIDFORM retained significant activity if mice were challenged with HSV delivered in seminal fluid. These findings suggest that ACIDFORM offers considerable protection against HSV and may be an optimal candidate for developing combination microbicides.

Herpes simplex virus (HSV) infections are the most common cause of genital ulcer disease worldwide, accounting for >60% of cases in many sexually transmitted infection clinics in developing countries [1]. Forty-five million Americans are infected with HSV-2,

the serotype most commonly associated with genital herpes [1, 2]. The magnitude of the HSV-2 epidemic is amplified by the impact that it has on HIV [3]. Epidemiological studies demonstrate that being seropositive for HSV-2 increases the risk of HIV acquisition, and, likewise, the infectiousness of individuals coinfected with HIV-1 and HSV-2 increases during periods of HSV-2 reactivation [4–6]. Infectious HIV particles are found in genital herpes ulcers and in cervicovaginal secretions during HSV-2 reactivation, suggesting that coinfection may also increase HIV transmission [7, 8]. These observations prompted the initiation of acyclovir prophylaxis trials to determine the proportion of new HIV infections that could be prevented by suppression of HSV-2 alone. The increasing prevalence of genital herpes and its link to HIV transmission highlight the need for preventive strategies to combat both epidemics. One strategy is the development of vaginal microbicides that inactivate the viruses or prevent viral entry and replication [9]. Major classes of microbicides being advanced in clinical trials, which are active against HIV and HSV, include anionic polymers that inhibit binding

and entry and acid-buffering agents designed to maintain the genital tract pH.

The healthy human vagina is acidic, with a pH ranging from 3.5 to 4.5, primarily because of lactic acid and hydrogen peroxide produced by lactobacilli. This acidic environment is presumed to contribute to innate immunity, although this notion has not been extensively evaluated. Notably, when semen enters the vagina, the pH increases to ∼7.0 because of the buffering activity of the ejaculate (pH 7.2–8.2). Thus, women are exposed to microbes at an optimal pH for transmission. These observations provided the rationale for developing acid-buffering compounds as candidate topical microbicides. Yet, few studies have explored the extent or mechanism of activity provided by acidic pH [10–12]. Rosenthal found that entry of HSV into a human epithelial cell line, HEp-2, was reduced at pH 6.3, but this inhibition was completely reversed on neutralization of the medium [11]. In contrast, in studies conducted with Chinese hamster ovary (CHO) cells expressing an HSV coreceptor, in-

fection was reduced by 50% if HSV particles were incubated with buffers of pH 5.0–5.5 and completely inactivated at pH ≤4.7 [12]. However, HSV entry is mediated by endocytosis in CHO cells, which is distinct from human epithelial cells, in which entry is mediated primarily by pH-independent fusion [12]. Thus, it is unclear to what extent acid-buffering gels would inhibit HSV infection of cells in the female genital tract.

Two acid-buffering products, BufferGel (developed by Re-Protect) and ACIDFORM (developed by Program for Topical Prevention of Conception and Disease [TOPCAD], Rush University, and licensed by Instead) are currently in clinical trials. ACIDFORM itself has a pH of ∼3.5 and buffers twice its volume of semen to maintain a pH of 4.45 in vitro [13]. BufferGel is formulated at a pH of 3.9–4.0 and, when mixed 1:1 with semen, buffers to a pH of 4.5–5.0; when mixed 3:1, it achieves a pH of 5.3–5.7 [14].

The objective of the present study was to more extensively define the extent of anti-HSV activity provided by acidic pH
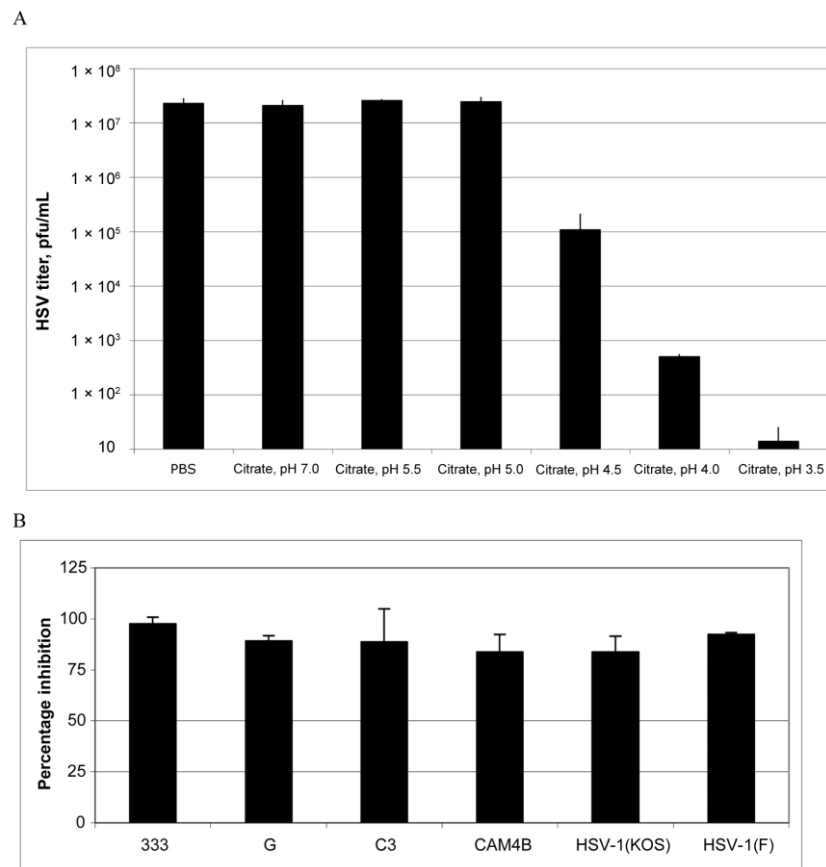

Figure 1. Impact of low pH buffer on herpes simplex virus (HSV) infection of CaSki cells. *A,* HSV-2(G), treated with citrate buffers at the indicated pH for 1 h, neutralized by diluting 1:5 in medium, and inoculated onto CaSki cells. Viral plaques were counted 48 h after infection, and the HSV titer was calculated. Results are means of 3 independent experiments conducted in duplicate. *B,* Different strains of HSV-1 and HSV-2 (∼250 pfu/well), exposed to citrate buffer, pH 4.5 and 7.0, for 1 h, neutralized by dilution in media, and plated onto cells for 1 h. Infectivity was obtained by counting plaques. Results are presented as plaque-forming units after treatment with citrate, pH 4.5, as a percentage of plaque-forming units formed after treatment with citrate, pH 7.0, and are means of 3 independent experiments conducted in duplicate.

buffers and the mechanism of antiviral activity and to evaluate the efficacy of ACIDFORM in a mouse genital herpes model.

MATERIALS AND METHODS

Cells and HSV. CaSki cells (human cervical epithelial cell line) were obtained from the American Type Culture Collection and maintained as described elsewhere [15]. The laboratory strains used were HSV-2(G), HSV-2(333), HSV-1(KOS), and HSV-1(F). The clinical isolate CAM4B (gift from P. Spear, Northwestern University) has been described elsewhere [16]. An additional clinical isolate, C3, was obtained from the clinical microbiology laboratory at the Mount Sinai Medical Center. Clinical isolates were passed no more than 3 times in Vero cells before study.

Reagents. ACIDFORM was obtained from TOPCAD; 4% PRO 2000 and its matched placebo gel were obtained from Indevus Pharmaceuticals. The hydroxyethylcellulose (HEC) placebo formulation has been developed for use in clinical trials as a "universal" placebo and was provided by CONRAD [17]. Citrate buffer (70 mmol/L sodium citrate and 4 mmol/L KCl) was adjusted to different pHs by adding NaOH. Measurements were obtained with a pH meter (model 215; Denver Instrument). During in vitro experiments, pH was measured using pH-indicator strips (ColorpHast; EMD).

Infection assays. Plaque assays were conducted to evaluate the antiviral activity of pH buffers. CaSki cells were plated in 12-well dishes and grown to near-confluence. Duplicate wells were exposed to HSV (range of serial dilutions, 0.005–5 pfu/cell) that had been pretreated for 1 h at 37°C with citrate buffer, pH 3.5–7.0, or PBS, pH 7.4. To determine whether the effects on HSV were irreversible, the HSV:buffer mixtures were diluted

1:5 with Dulbecco's MEM (DMEM) to yield a final pH of ∼7.0 before plating onto cells. After 1 h of incubation at 37°C, the cells were washed 3 times and overlaid with Medium 199 supplemented with 1% fetal bovine serum and 1% pooled human gamma globulin (199-O). Plaques were quantified 48 h after infection by immunostaining, using an anti-human IgG antibody peroxidase conjugate (Calbiochem) and with the HSV titer (plaque-forming units per milliliter) calculated. Only wells in which the number of plaques ranged between 25 and 250 were used to calculate the HSV titer.

To determine the anti-HSV activity of a combination of pH 4.5 buffer and PRO 2000, CaSki cells were exposed in duplicate to serial dilutions of HSV-2(G) that had been pretreated for 1 h at 37°C with citrate buffer, pH 4.5 or 7.0, or PBS and PRO 2000 (formulated or unformulated PRO 2000, equivalent to 0, 0.1, 1, and 10 μg/mL concentrations of drug). The mixtures were diluted 1:4 with DMEM to yield a final pH of ∼7.0 before plating onto cells and infection was monitored as described above.

Time-course assay. To determine how quickly HSV is inactivated, the virus was preincubated with different pH buffers and neutralized with DMEM at different times after incubation before infecting CaSki cells.

Structural integrity of HSV exposed to acidic pH. Infected cell lysates were incubated for 30 min. at 37°C with DMEM adjusted to pH 3.5–7.0 by the addition of citrate buffer in the presence or absence of 1 mmol/L phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich). The treated lysates were layered onto a 10-mL continuous 30%–60% (wt/vol) sucrose gradient and centrifuged at 22,000 *g* for 1 h (SW41 rotor; Beckman Optima LE-80K) [18]. Four equal-volume fractions were col-
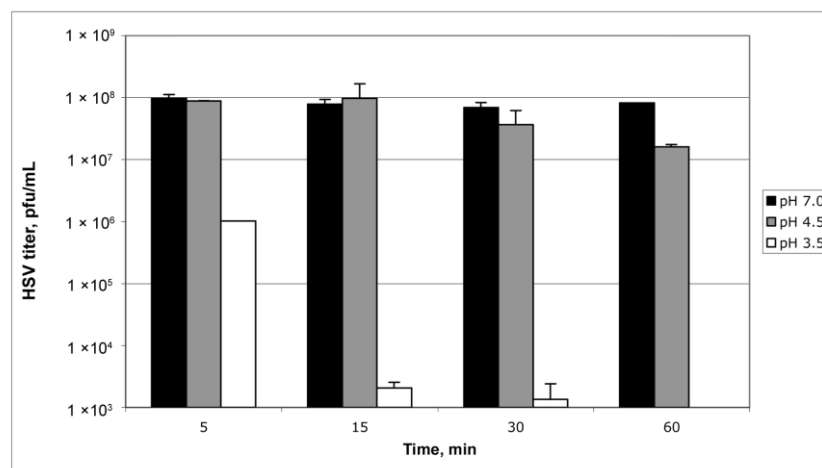

Figure 2. Kinetics of herpes simplex virus (HSV) inactivation. HSV-2 (333) was pretreated with citrate buffer, pH 3.5, 4.5, or 7.0, and neutralized at the indicated time points before inoculating cells for plaque assay. Results are HSV titer calculated after exposure to different pH buffers and are means of 3 independent experiments conducted in duplicate.

lected and analyzed by preparing Western blots and probing each fraction for the presence of the envelope glycoprotein B (mouse monoclonal antibody [MAb] 1123; Goodwin Institute) and the capsid protein VP5 (1:500) (mouse MAb 6F10; Santa Cruz Biotechnology). The HSV titer in each fraction was determined by plaque assay.

Binding assay. HSV was treated with citrate buffers or PBS for 1 h at 37°C and neutralized with serum-free media before inoculation of CaSki cells (1 pfu/cell) for 5 h at 4°C. Unbound HSV was removed by washing 3 times with cold PBS, and cell-bound HSV was analyzed by preparing Western blots of cell lysates and probing with anti-gD MAbs (MAb 1103; Goodwin Institute).

For all SDS-PAGE and Western blot experiments, the proteins were suspended in SDS sample buffer (100 mmol/L Tris-Cl [pH 6.8], 1% β-mercaptoethanol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) and boiled for 4 min. The proteins were separated on an 8.5% SDS-polyacrylamide gel

and transferred to a polyvinylidene difluorride membrane (Perkin Elmer). Membranes were incubated with the indicated primary antibodies, then rinsed extensively and incubated with horseradish peroxidase–conjugated goat anti-mouse IgG (1:1000) (Calbiochem) for 2 h and developed using Chemiluminescence Reagent Plus (Bio-Rad). Blots were scanned and analyzed using the CellDoc 2000 system (Bio-Rad Laboratories) [19, 20]. To control for protein loading, blots were stripped and probed for β-actin (MAb A5441; Sigma-Aldrich).

Mouse model of genital herpes. Female BALB/c mice (weight, 18–21 g; age, 8–10 weeks) were pretreated with 2 mg/mL medroxyprogesterone acetate subcutaneously 5 days before HSV challenge. On the day of infection, mice were given a single dose of 40 μL/mouse of ACIDFORM gel, 4% PRO 2000 gel, or matched PRO 2000 vehicle. After 15 min, the mice were challenged intravaginally with 10^5 pfu/mouse of HSV-2 (C3) diluted in PBS or pooled human seminal fluid to a final volume of 20 μL/mouse. Mice were evaluated daily through day 14 after
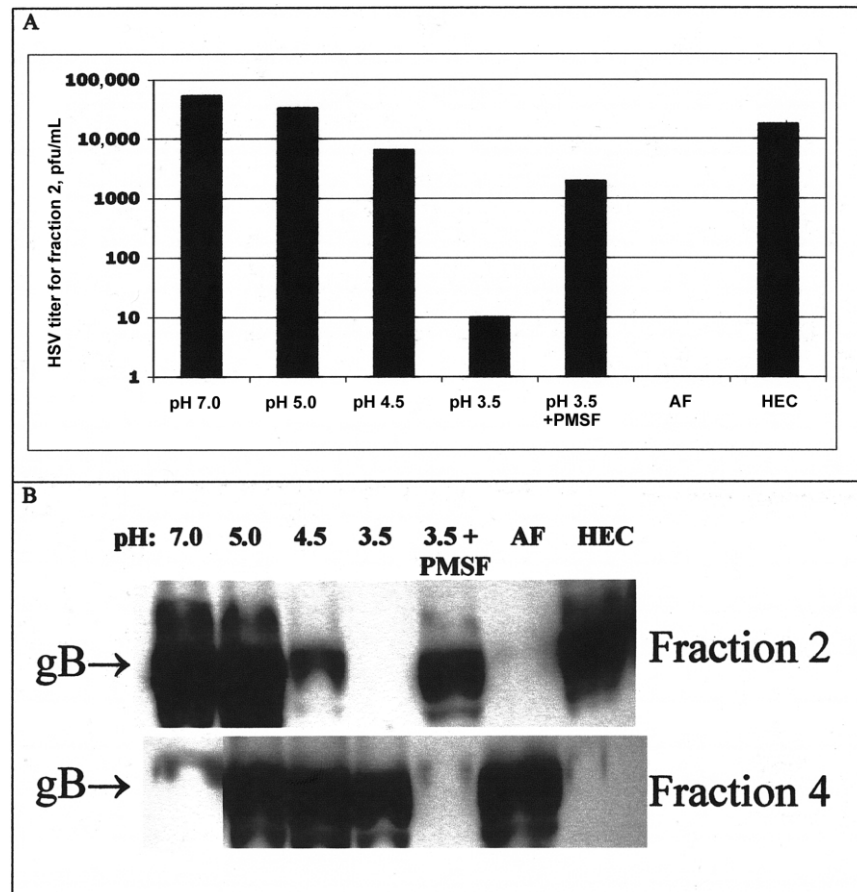

Figure 3. Acid treatment disruption of herpes simplex virus (HSV) integrity, leading to a loss in infection. Cell lysates were treated with the indicated pH citrate buffer or with ACIDFORM (AF) or hydroxyethylcellulose (HEC) gel in the absence or presence of 1 mmol/L phenylmethanesulfonyl fluoride (PMSF) and then applied to a continuous 30%–60% sucrose gradient. After centrifugation, 4 equal fractions were collected; the top fraction is fraction 1. *A,* HSV titer, determined by plaque assay on CaSki. Results are means of duplicates obtained in 2 independent experiments for fraction 2. *B,* Fractions, analyzed by SDS-PAGE and preparing Western blots and immunoblotting for HSV proteins. Representative gels probed for envelope glycoprotein B in fractions 2 and 4 are shown; similar results were obtained in 3 independent experiments.
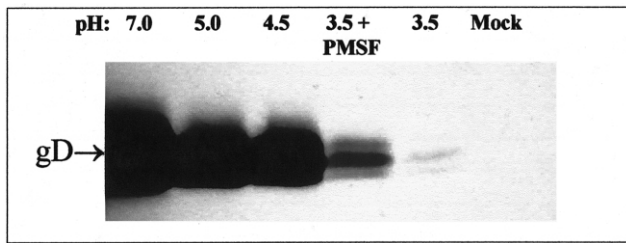
Figure 4. Binding reduction after treatment of herpes simplex virus (HSV) with low pH buffer. Stocks of HSV were treated with citrate buffers at the indicated pH for 1 h and neutralized by diluting in Dulbecco's MEM before infecting CaSki cells. For binding studies, precooled CaSki cells were exposed to the buffer-treated HSV stocks for 5 h at 4°C. Bound HSV was detected by preparing Western blots of cell lysates and probing with anti-gD monoclonal antibody. The gel is representative of results obtained in 3 independent experiments. Mock, mock infected; PMSF, phenylmethanesulfonyl fluoride.

washes were obtained on days 0, 1, and 6 after infection, by use of 100 μL of sterile saline solution; pooled (5 mice/group); and stored at −80°C after addition of antibiotics and protease inhibitors (Complete Protease Inhibitor Cocktail; Roche) for cytokine detection. Cytokines were quantified using multiplexed Luminex assays (Mouse Fluorokine MAP Base Kit [catalog no. LUM000]; R&D Systems) and analyzed by BioLuminex system (Applied Cytometry Systems). The vaginal pH was measured at baseline and at different times after application of gel alone or gel followed by inoculation with human seminal fluid (20 μL/mouse), by use of a 1.5-inch micro combination electrode (Accumet) attached to a portable pH meter (pHTester; Oakton Instruments).

Seminal and cervicovaginal fluid. After Institutional Review Board approval at the Mount Sinai School of Medicine, informed consent was obtained before collection of cervicovaginal or semen samples from healthy female and male donors, respectively. An Instead SoftCup (Ultrafem) was inserted into the vagina for 1 h, in accordance with the manufacturer's instructions. The specimens were immediately transported on ice to the laboratory and diluted ∼1:1 with PBS before processing.
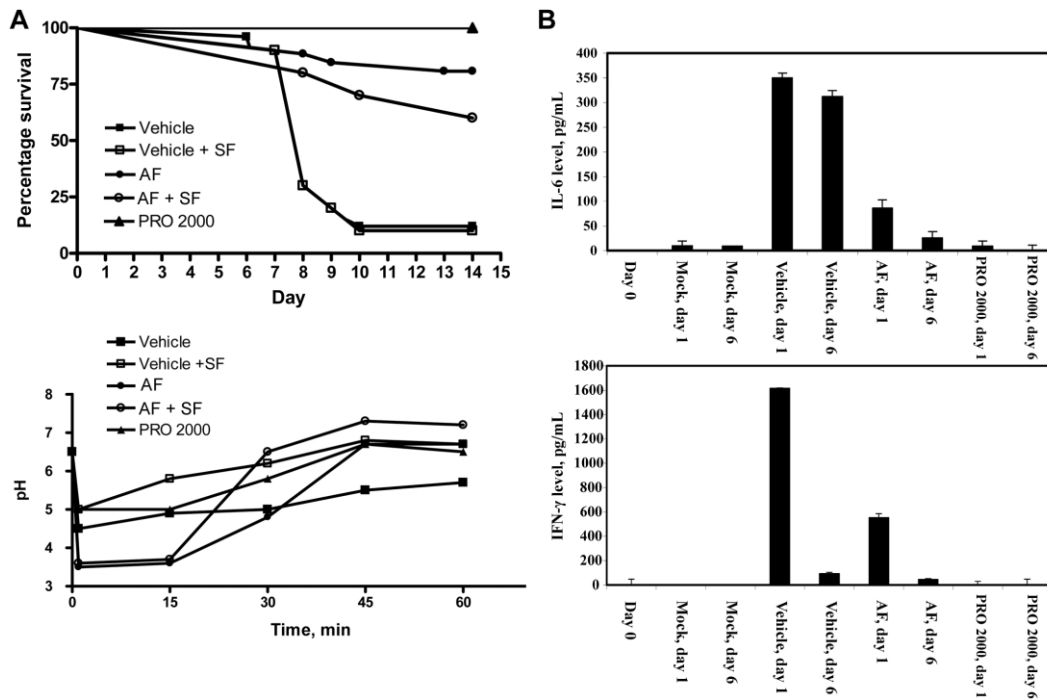
inoculation for evidence of infection, including erythema, edema, genital ulcers, hair loss around the perineum, and hind-limb paralysis. The mice were killed if symptoms of severe ulceration, hair loss, or hind-limb paralysis developed. Vaginal

Figure 5. ACIDFORM (AF) protection of mice from disease and prevention of the herpes simplex virus (HSV)–induced cytokine response. *A,* Female BALB/c mice, given a single dose of drug (AF, 4% PRO 2000, or its matched vehicle gel) intravaginally and challenged 15 min later with 10^5 pfu/mouse of HSV-2 (C3) diluted in either PBS or seminal fluid (SF) or mock infected. The mice were observed for any signs of disease during 14 days after infection and were killed if severe ulcerations or hind-limb paralysis was observed *(upper panel).* In parallel experiments, the intravaginal pH was measured at the indicated times, after giving the mice a single dose of the indicated formulation with or without subsequent exposure to human SF. Results are mean pH obtained from 3 independent experiments *(lower panel). B,* Vaginal lavages, obtained at baseline (day 0) and 1 and 6 days after infection and pooled (5 mice/group) for cytokine analysis. Results for interleukin (IL)–6 *(upper panel)* and interferon (IFN)–γ *(lower panel)* are mean ± SD from duplicate determinations.

Table 1. Acid-buffering capacity of ACIDFORM (AF) mixed with human cervicovaginal secretions (CSs) and seminal fluid (SF) in vitro.

Mixture ratio, CSs:AF:SF	Final pH
1:1:1	3.5
1:1:2	5.0
1:1:4	6.5

NOTE. CSs (pH 4.2) were mixed with an equal volume of AF gel (pH 3.5) and with increasing amounts of SF (pH 8.0), and the final pH was determined. Results are representative of 2 independent experiments.

The supernatants were separated from the cells by centrifugation (1000 g for 10 min at 4°C), and antibiotics and amphotericin were added. The supernatants were aliquoted and stored at -80°C. Seminal fluid from 2–3 donors was pooled for mouse experiments. To evaluate the acid-buffering capacity of ACIDFORM gel in vitro, the gel was first mixed with an equal volume of cervicovaginal secretions, and then increasing amounts of seminal fluid were added. The resulting pH was measured using pH-indicator strips (ColorpHast; EMD).

RESULTS

HSV inactivation at pH 4.5. To determine the extent of irreversible inactivation provided by acidic pH, serial dilutions of HSV-2(G) were treated for 1 h with different citrate pH buffers and neutralized by diluting with media, and HSV infectivity was monitored by plaque assay. At pH 4.5, HSV infection was reduced ~100-fold, and significantly greater inactivation was observed if HSV was exposed to pH 3.5 (figure 1*A*). However, little or no antiviral activity was observed at pH 5.0. Although there was variability with respect to the extent of antiviral activity, a pH of 4.5 inhibited infection by all the laboratory-adapted and clinical isolates of HSV-1 and HSV-2 tested (figure 1*B*). Time-course studies were conducted to examine how quickly acidic pH inactivates HSV. Treatment with pH 3.5 rapidly inactivates HSV; in contrast, inactivation at pH 4.5 was substantially slower (figure 2). Notably, an inoculum effect was observed for pH 4.5 buffer, which inactivated only 1 log if the titer of inoculating HSV was 10^8 pfu/mL (figure 2), compared with a 2-log inhibition when the titer was 10^7 pfu/mL (figure 1*A*). In contrast, the pH 3.5 buffer produced 4–6 log inhibition of HSV, independent of the inoculating HSV titer. The kinetics and extent of inactivation were similar for HSV-2(G) and C3 (data not shown).

Proteolysis triggered by acid and disrupts HSV particles to inhibit binding and entry. The irreversible inactivation of

HSV after exposure to acidic pH buffers suggests that acid may trigger changes in the structural integrity of HSV particles to prevent infection. One hypothesis is that acidic pH disrupts HSV particles by triggering proteolysis. To test this notion, HSV-infected cell lysates were treated with different pH buffers or with ACIDFORM or HEC placebo gel and then applied to a sucrose gradient. After centrifugation, 4 equal-volume fractions were collected from the top and analyzed for the presence of HSV proteins and infectivity. A HSV band was easily visible in fraction 2 from gradients containing HSV treated with HEC, pH 5.0 or 7.0 buffers, but not with ACIDFORM, pH 4.5 or 3.5 buffers. Moreover, there was complete loss of HSV infectivity in fraction 2 from the gradients treated with ACIDFORM and a 4-log reduction in the titer of pH 3.5–treated lysates (figure 3*A*). No HSV proteins could be identified in fraction 2 after treatment with pH 3.5 or ACIDFORM gel. Instead, almost all the proteins were detected at the bottom of the gradient in fraction 4 (figure 3*B*). The HSV integrity and infectivity were partially restored if cell lysates were treated with pH 3.5 in the presence of 1 mmol/L PMSF, which inhibits serine and cysteine proteases (figure 3).

To explore the impact that acid has on HSV binding, the HSV-infected cell lysates were treated with buffers of varying pH for 1 h, neutralized by diluting in DMEM, and then allowed to bind to CaSki cells for 5 h at 4°C. Binding was reduced after treatment with buffer of pH 4.5 and essentially undetectable after treatment with buffer of pH 3.5 (figure 4). The binding was partially restored if acid treatment was conducted in the presence of PMSF. Acid treatment with buffers of pH 4.5 or lower also resulted in a marked reduction in the transport of the tegument protein VP16 to the nucleus, a surrogate marker for HSV entry, and in expression of the HSV immediate early gene product, ICP4 (data not shown). These findings support the hypothesis that exposure to acidic pH (4.5 or lower) triggers proteolysis of HSV particles, physically disrupting HSV to impair binding and entry.

ACIDFORM protection of mice from genital herpes. A mouse genital herpes model was adopted to determine whether the antiviral properties of acidic pH translated to protection in vivo. Mice were treated with ACIDFORM or, for comparison, 4% PRO 2000 or its matched vehicle gel and 15 min later challenged intravaginally with 10^5 pfu/mouse of HSV-2(C3). ACIDFORM protected 21 (81%) of 26 mice from genital herpes, whereas only 3 (12%) of 25 mice pretreated with the vehicle gel were protected ($P = .0001$, log-rank test). 4% PRO 2000 gel protected 10 (100%) of 10 mice, which was not significantly different than the protection observed for ACIDFORM (figure 5*A*, *upper panel*). Moreover, ACIDFORM retained significant protective activity if HSV was introduced diluted in seminal fluid rather than in PBS ($P = .0064$, log-rank test). Not only did ACIDFORM protect against disease, but it also inhibited
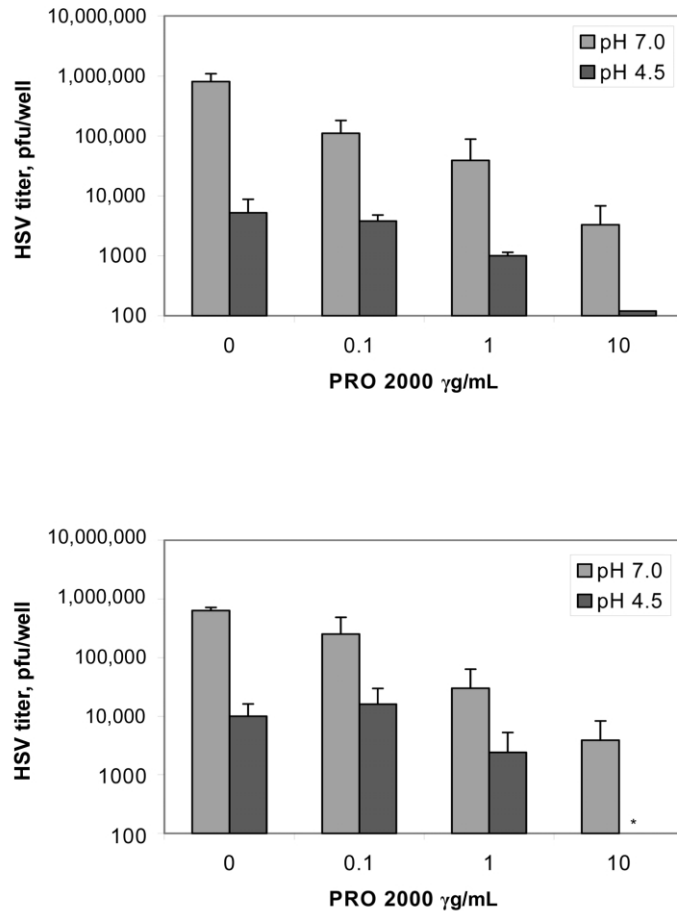
Figure 6. Additive protection against herpes simplex virus (HSV) in vitro provided by combining acidic pH buffer with PRO 2000. CaSki cells were exposed to HSV-2 (G) (range of serial dilutions, 0.005–1 pfu/cell) that had been pretreated for 1 h at 37°C with citrate buffer, pH 4.5 or 7.0, or PBS buffer, pH 7.4, combined with PRO 2000 (unformulated drug *[upper panel]* or formulated gel *[lower panel]*, equivalent to 0, 0.1, 1, and 10 µg/mL of drug). The mixtures were diluted 1:4 with Dulbecco's MEM to yield a final pH of ~7.0 before plating onto cells, and infection was monitored by immunostaining plaques 48 h after infection. Results are mean ± SD from 2 independent experiments conducted in triplicate; the asterisk indicates that no HSV plaques were detected in the wells inoculated with 1 pfu/cell.

the HSV-induced cytokine response. Vaginal washes were collected from mice at baseline and 1 and 6 days after HSV inoculation. As shown in figure 5*B*, pretreatment with ACID-FORM or PRO 2000, but not with the vehicle gel, blocked the HSV-induced increase in the levels of proinflammatory cytokines interleukin-6 and interferon-γ. Vaginal application of gel in the absence of HSV challenge did not induce any increase in cytokine levels (data not shown).

The vaginal pH in the mice was monitored after gel application alone or followed by challenge with human seminal fluid (figure 5*A*, *lower panel*). The baseline pH in mice (6.5) is higher than that observed in humans. Immediately after application of ACIDFORM, the vaginal pH dropped to ~3.5, but, in the presence of seminal fluid, the pH returned to baseline by 30 min. In contrast, PRO 2000 and its matched vehicle gels, although designed to have some acid-buffering capacity, had only

transient effects on vaginal pH, and by 15 min after application the pH was 5, a pH that would not be anticipated to provide protection.

In an attempt to predict the acid-buffering capacity of ACID-FORM gel in vivo, we combined human cervicovaginal secretions, ACIDFORM gel, and seminal fluid together and measured pH (table 1). Presuming that the volume of cervicovaginal secretions is ~2 mL and that a single applicator of gel is 2 mL, ACIDFORM would buffer an equal volume (2 mL) of seminal fluid to pH 3.5. However, as the volume of seminal fluid increases, the acid-buffering properties are overcome. Thus, ACIDFORM may not be sufficient alone but could provide an excellent vehicle for formulation of candidate drugs. Combining the inactivating activity of acidic pH with the antiviral activity of an entry inhibitor, for example, may provide greater protection than afforded by either drug alone. To test this no-

tion, we compared the antiviral activity of unformulated (figure 6, *upper panel*) or formulated (figure 6, *lower panel*) PRO 2000 diluted in pH 7.0 or 4.5 buffer. Results indicate that the anti-HSV effects of PRO 2000 combined with acidic pH are additive.

DISCUSSION

These studies demonstrate that the acidic environment of the healthy female genital tract (pH 3.5–4.5) provides innate protection against HSV. However, when semen enters the vagina, the pH increases because of the buffering activity of the ejaculate. The acid-buffering properties of ACIDFORM could restore the acidic environment, thus providing protection against HSV. Mechanistic studies indicate that exposure of HSV to pH 3.5–4.5 physically disrupts HSV particles, leading to a reduction in binding and entry. The effects are partially reversed in the presence of PMSF, suggesting that acid may activate cellular proteases.

Viral inactivation is rapid and substantial at pH 3.5 but is less effective at pH 4.5, which results in 1–2 log inhibition of infection and requires exposure to the acidic environment for ∼30–60 min. These in vitro observations are supported by results of mouse studies. ACIDFORM reduced the vaginal pH to 3.5 for 15 min in mice, even after challenge with human seminal fluid, and provided significant protection against HSV disease. However, the acid-buffering capacity is short-lived in the mouse model, suggesting that ACIDFORM alone would not be sufficient as a microbicide if the kinetics of acid buffering are similar in humans. The buffering capacity is also overcome in the presence of increasing volumes of seminal fluid in vitro. This suggests that the extent of antiviral activity will also depend on the relative volume of gel and seminal fluid. Clinical trials are necessary to determine the kinetics and extent of acid buffering in humans, in whom the pH of the genital tract is substantially lower than in mice (pH 3.5–4.5 vs. 6.5) and in whom the volume and surface area of the genital tract differ.

The observation that PRO 2000 and its matched vehicle gel had less of an acid-buffering effect led us to test a combination of acid pH and PRO 2000 (formulated and unformulated) in vitro. We found that the anti-HSV effects of PRO 2000 when combined with pH 4.5 buffer were additive. Thus, combining an acid-stable microbicide with ACIDFORM or a comparable bioadhesive acid-buffering agent may provide greater protection than afforded by either product alone.

Both ACIDFORM and PRO 2000 prevent the HSV-induced proinflammatory cytokine response associated with genital HSV challenge. This observation may have important implications with respect to coinfection because proinflammatory cytokines may increase HIV replication or recruit HIV target cells. Thus, microbicides that block the HSV-induced proinflammatory response in the genital tract may indirectly reduce HIV infection.

Additional studies suggest that acid-buffering gels may pro-

vide some protection against other sexually transmitted infections, including *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, and HIV infection [21–26]. Notably, the reported effect of acid pH on HIV is somewhat variable. O'Connor et al. found HIV-1 to be sufficiently acid stable to remain infective after exposure to phosphate/citrate buffer, pH 4.5, for 60 min, although incubation of HIV with pH 3.5 buffer for 60 min completely inhibited the cytopathic effect [27]. Acid sensitivity of gonococcal clinical isolates was reported to be strain and growth-phase dependent [24], and BufferGel and ACIDFORM provided some protection in a mouse model of *N. gonorrhoeae* genital tract infection [27]. ACIDFORM protected mice against *C. trachomatis* [28], whereas BufferGel failed to protect macaques [23]. The differences in results may reflect the lower pH achieved by ACIDFORM. In the macaque study, the vaginal pH 30 min after application of BufferGel ranged from 4.8 to 5.3 [23]. Given our in vitro findings, this pH range is unlikely to prevent HSV infection. Notably, BufferGel did protect mice from vaginal HSV challenge in one study in which infection was monitored primarily by assessing HSV titers in vaginal washes [29]. In another study, BufferGel failed to protect mice if they were inoculated with 10^5 pfu, but it did protect ∼60% of the mice challenged with a lower inoculum, 10^4 pfu [30].

These studies demonstrate that acidic pH irreversibly inactivates HSV by disrupting HSV particles, leading to a reduction in binding, entry, and infection. ACIDFORM protected mice from HSV and prevented the HSV-induced cytokine response. Extrapolation of these findings to humans is difficult because of differences in the mouse and human vaginal pH and surface-to-volume ratio and because it will depend on the volume and buffering capacity of semen. These studies support the further clinical advancement of ACIDFORM or comparable acid-buffering gels, preferably combined with other microbicides, because combination therapy for systemic treatment of infections has been shown to provide clear benefits.

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