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Inactivation of human immunodeficiency virus type 1 by nonoxynol-9, C31G, or an alkyl sulfate, sodium dodecyl sulfate

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Abstract

A highly desirable approach to prevention of human immunodeficiency virus type 1 (HIV-1) transmission during sexual intercourse is the development of nontoxic, topical, broad spectrum microbicides effective against transmission of cell-associated and cell-free virus. Toward this end, the HIV-1 inactivation potential of surface active agents C31G and an alkyl sulfate, sodium dodecyl sulfate (SDS) was assessed. Because of its extensive use as a microbicidal agent, nonoxynol-9 (N-9) was used as a reference against which C31G and SDS were compared. Viral inactivation was measured using HIV-1 LTR-β-galactosidase indicator cells (expressing CD4 or CD4/CCR5) derived from HeLa cells, a cell line of human cervical adenocarcinoma origin. In experiments which examined inactivation of cell-free HIV-1, C31G was generally more effective than N-9. Viral inactivation by SDS occurred at twice the concentration necessary to achieve similar levels of inactivation using either N-9 or C31G. Using HeLa and HeLa-derived cells in cytotoxicity studies, it was demonstrated that SDS is as much as 11 and five times less cytotoxic than N-9 or C31G, respectively, during 48 h of continuous exposure. SDS (unlike C31G and N-9) can inactivate non-enveloped viruses such as human papillomavirus (HPV) [Howett, M.K., Neely, E.B., Christensen, N.D., Wigdahl, B., Krebs, F.C., Malamud, D., Patrick, S.D., Pickel, M.D., Welsh, P.A., Reed, C.A., Ward, M.G., Budgeon, L.R., Kreider, J.W., 1999. A broad-spectrum microbicide with virucidal activity against sexually transmitted viruses. Antimicrob. Agents Chemother. 43(2), 314–321]. Since addition of SDS to C31G or N-9 may make the resulting microbicidal mixtures broadly effective against both enveloped and non-enveloped viruses, several surface active agent combinations were evaluated for their abilities to inactivate HIV-1. Addition of SDS to either C31G or N-9 resulted in mixtures that

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were only slightly less effective than equivalent concentrations of C31G or N-9 alone. To investigate inactivation of cell-associated infectivity, HIV-1 IIIB-infected SupT1 cells were treated with N-9, C31G, or SDS. Inactivation of cell-associated infectivity required higher microbicide concentrations than were needed for inactivation of cell-free virus. However, the relative activities of N-9, C31G, or SDS were similar to those seen in assays of inactivation using cell-free virus. These studies suggest that C31G and SDS may be attractive candidates for human trials as topical microbicides effective against HIV-1 transmission since both function at concentrations that provide effective viral inactivation with low levels of cytotoxicity. SDS microbicides (used alone or with other microbicides) may provide the added advantage of protection from HPV infection.

Keywords: Human immunodeficiency virus; N-9; C31G; SDS; Vaginal microbicide; MAGI assay

1. Introduction

The human immunodeficiency virus type 1 (HIV-1) has been identified as the causative agent of the acquired immune deficiency syndrome (AIDS) as well as a host of other degenerative disorders (Levy, 1993; Price, 1994; Tillmann et al., 1994; Krebs and Wigdahl, 1996; Pantaleo and Fauci, 1996; Kolson et al., 1998). During the course of the AIDS epidemic, the incidence of transmission during the perinatal period and heterosexual intercourse has increased dramatically (McCarthy et al., 1992; Kallings, 1993; Kesson and Sorrell, 1993), especially among populations of developing countries in Asia, Africa, and South America (UNAIDS/WHO, 1998). Despite promotion of condom usage in AIDS awareness education programs and the availability of the female condom (Bounds, 1997), the use of mechanical barriers to reduce the incidence of HIV-1 transmission has not gained universal acceptance (Campbell and Baldwin, 1991; Norr et al., 1996). The availability of effective, nontoxic, broad spectrum microbicides for intravaginal use would result in less frequent transmission of sexually transmitted disease (STD) pathogens such as HIV-1 and would provide women with an additional measure of protection independent of the male partner.

Design and development of microbicides with the potential to prevent HIV-1 transmission must take into consideration the form and tropism of the infectious inoculum. At present, the relative contributions of cell-free and cell-associated virus to infectivity within the vagina are unclear. Mononuclear cells isolated from semen of HIV-1-positive men can infect lymphocytes in vitro (Levy, 1988), and infection of primary human keratinocytes requires cocultivation with cell-associated HIV-1 (Ramarli et al., 1995), suggesting that HIV-1 transmission may occur, at least in part, via cell-associated virus. Strains of HIV-1 transmitted during heterosexual intercourse are likely to be macrophage tropic, as suggested by the abundance of monocytes over lymphocytes in seminal and vaginal lavage mononuclear cell populations (Wolff and Anderson, 1988; Anderson et al., 1998), increased expression of the coreceptor CCR5 over CXCR4 in cervical tissues (Patterson et al., 1998), and isolation of primarily macrophage tropic HIV-1 strains in the peripheral blood soon after infection (Zhu et al., 1993).

A number of active microbicidal agents are available for use in preventing transmission of HIV-1 and other pathogens responsible for STDs, including nonoxynol-9 (N-9), octoxynol-9, benzalkonium chloride, menfegol, and a number of other compounds (Rosenberg and Gollub, 1992; Pauwels and De Clercq, 1996). The majority of in vitro studies concerning the microbicidal efficacy of spermicides have focused on N-9, which has activity against HIV-1, herpes simplex virus type 2 (HSV-2), *Neisseria gonorrhoeae*, and, in some studies, against *Chlamydia trachomatis* (North, 1988). However, N-9 toxicity in the genital tract in conjunction with concurrent STDs has stimulated concern that N-9 could potentiate HIV-1 infection or coinfection with one or more STD pathogens (Kreiss et al., 1992; Kirkman and Chantler, 1993). In addition, a recent clinical study demonstrated that vaginal application of a film containing N-9 (used in conjunction with condoms) did not reduce the rates of new HIV-1, *N. gonorrhoeae*, or *C. trachomatis* infections, bringing the in vivo microbicidal efficacy of N-9 into question (Roddy et al., 1998).

The potential of two compounds [C31G and sodium dodecyl sulfate (SDS)] has been investigated as topical microbicides to reduce or prevent transmission of pathogens associated with common STDs. C31G used in the present study is an equimolar mixture of two amphoteric, surface active molecules: a C14 alkyl amine oxide and a C16 alkyl betaine. C31G has broad spectrum antibacterial, antiviral, and antifungal activity (Corner et al., 1988, 1990; Calis et al., 1992; Thompson et al., 1996; Wyrick et al., 1997). However, recent studies (Malamud et al., 1998; Howett et al., 1999) have demonstrated that C31G, like N-9 (Hermonat et al., 1992), cannot inactivate human papillomaviruses (HPV), sexually-transmitted viruses implicated in the genesis of cervical cancer (zur Hausen, 1996). To circumvent this shortcoming, alternative agents were sought that were effective against non-enveloped viruses such as HPV. SDS, a surface active, alkyl sulfate compound recognized for its widespread commercial uses in personal hygiene (e.g. toothpaste) and cleaning products, was selected because of its surfactant properties, its ability to effectively denature protein, its relatively low cytotoxicity, and its common availability (Reynolds et al., 1967; Tanford, 1968; Singer and Tjeerdema, 1993). Recent studies have demonstrated that SDS effectively inactivates HPV (at a concentration of 0.025%), Shope cottontail rabbit papillomavirus (CRPV; 0.05%), and bovine papillomavirus (BPV; 0.05–0.005%) as well as HIV-1 and HSV-2 (Howett et al., 1999). In the present study, these findings have been expanded upon and they have demonstrated the ability of C31G and SDS (alone or in combination) to inactivate HIV-1 infectivity in vitro. The results show that C31G has a greater capacity for inactivating both cell-free and cell-associated HIV-1 than N-9, while SDS is slightly less effective than N-9 or C31G. Both C31G and SDS may be attractive candidates for human trials as topical microbicides effective against HIV-1 transmission since both function at concentrations that provide effective viral inactivation with low levels of cytotoxicity.

2. Materials and methods

2.1. Cell culture

All inactivation and cytotoxicity measurements were performed using the following cell lines: HeLa (human cervical carcinoma; ATCC CCL-2.1); HeLa-CD4-LTR-β-gal (HCLB; AIDS Reagent Program #1470); P4-R5 (AIDS Reagent Program # 3580); and SupT1 (T lymphocyte; ATCC CRL-1942). The HeLa and HeLa-based cell lines were maintained in Dulbecco's modified Eagle's media (DMEM). HCLB and P4-R5 cells were propagated under selection using 0.2 mg/ml G418 and 0.1 mg/ml hygromycin B, or 1 μg/ml puromycin, respectively. SupT1 cells were maintained in RPMI 1640 media. All media were supplemented with 10% fetal bovine serum (FBS), L-glutamine (0.3 mg/ml), antibiotics (penicillin, streptomycin, and kanamycin at 0.04 mg/ml each), and 0.05% sodium bicarbonate.

2.2. Assessment of HIV-1 inactivation

The ability of N-9, C31G, or SDS to inactivate HIV-1 was assessed using in vitro assays that permitted rapid quantitation of remaining viral infectivity following exposure to selected microbicidal agents or combinations of agents. These assays used the HeLa-CD4-LTR-β-gal (Kimpton and Emerman, 1992) and P4-R5 (Charneau et al., 1994) cell lines, which stably express the cell surface protein CD4 (HCLB), or CD4 in conjunction with the chemokine receptor CCR5 (P4-R5). Both HeLa-derived cell lines also express β-galactosidase under the control of the HIV-1 LTR, which can be transactivated during HIV-1 infection. In assays using these cells, one β-gal-positive cell or multinuclear cell corresponds to one infectious virus particle. The HCLB and P4-R5 cell lines were used to quantitate infectious lymphocyte (T tropic) and monocyte tropic (M tropic) strains of HIV-1, respectively. Assays using these cell lines provide excellent means to quantitate infectious HIV-1 inactivation in that they resemble a plaque assay and do not depend on cocultivation, p24

ELISA, or reverse transcriptase assays to quantitate infectivity. Since HeLa cells are of human cervical epithelial origin, their use is also particularly relevant to our studies of cellular sensitivity to candidate vaginal microbicides.

HCLB or P4-R5 cells were plated at a density of 8×10^4 cells per well in 12 well culture plates 24 h prior to each experiment. Concentrated, cell-free viral preparations ($\sim 1.5 \times 10^6$ $TCID_{50}$/ml in RPMI with 10% FBS) of HIV-1 strains IIIB (T tropic), BaL (M tropic), and 89.6 (dual tropic) were obtained commercially (Advanced Biotechnologies, Columbia, MD). Experiments assessing inactivation of cell-associated infectivity were performed using SupT1 cells infected with HIV-1 strain IIIB 5 days prior to the experiment. Inactivation experiments were performed using 78 μl of cell-free virus or 8×10^4 cells from the infected SupT1 culture in a volume of 78 μl of RPMI 1640 (with 10% FBS). Cell-free virus or infected cells were mixed with 2 μl of N-9, C31G, or SDS diluted (in distilled water) from 1% aqueous stock solutions (obtained from Biosyn, Philadelphia, PA) to yield final concentrations of 0.0025–0.05% during the inactivation period. After incubation for 10 min at 37°C, the mixture was diluted 1:10 with RPMI 1640 (with 10% FBS) and added (300 μl/well in two wells) to the HeLa-derived indicator cells expressing the appropriate receptor/co-receptor combination. Following a 2 h adsorption period, new media (2 ml) was added to each well. After incubation at 37°C, 90% humidity, and 5% CO_2 for 48 h, cells were fixed and stained for β-galactosidase expression. β-gal-positive cells in duplicate wells were counted by light microscopy, averaged, and expressed as a percentage relative to the number of β-gal-positive cells in duplicate wells infected with virus incubated in the absence of any microbicidal compound. Statistical analyses were performed using Microsoft Excel.

2.3. Determination of agent cytotoxicity

Assessments of cytotoxicity used the SupT1 cell line as well as HeLa- and HeLa-derived cell lines. The choice of adherent cell lines used in these studies, dictated by the availability of indicator cell lines designed to detect infectious HIV-1, was relevant to the studies of compounds which may be developed as vaginal microbicides. The effect of each agent on cell viability was determined using an assay in which dehydrogenases in viable cells cleave the compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), yielding a purple product (formazan) which can be measured spectrophotometrically (Pauwels et al., 1988). Cells were seeded into 12 well plates at a density of 8×10^4 to 1×10^5 cells/well 24 h prior to introduction of the microbicides. Agents were filter-sterilized and diluted in sterile water for each experiment. Agents were added to triplicate wells resulting in final concentrations ranging from 0.00025 to 0.005%. Cells were incubated in the absence or presence of each agent at 37°C under 5% CO_2 and 90% humidity. At the conclusion of each experiment, 250 μl of MTT (5 mg/ml) was added to each well and incubated for 3 h at 37°C. Following removal of the media (by aspiration for adherent cells or centrifugation for suspension cells), intracellular formazan crystals were solubilzed for 5 min in 1 ml 10% Triton X-100 in acidified isopropanol (0.1 N). The resulting solutions were assayed spectrophotometrically at 570 nm and corrected for non-specific absorption at 690 nm. In this assay, measured absorbance is proportional to the viable cell number, and inversely proportional to the degree of cytotoxicity. Statistical analyses were performed using Microsoft Excel.

3. Results

3.1. SDS and C31G inactivate lymphocyte-, macrophage-, and dual-tropic strains of HIV-1

A microbicidal agent effective against HIV-1 must inactivate viruses of different cellular tropisms. To examine inactivation of a lymphocytic strain of HIV-1, high titer stocks of the T tropic HIV-1 strain IIIB were incubated with N-9, C31G, or SDS, and the remaining viral infectivity was assayed on HCLB indicator cells. Inactivation of HIV-1 strain IIIB (Fig. 1A) was concentration-dependent over the range of concentrations tested (0.0025–0.05%). Exposure to

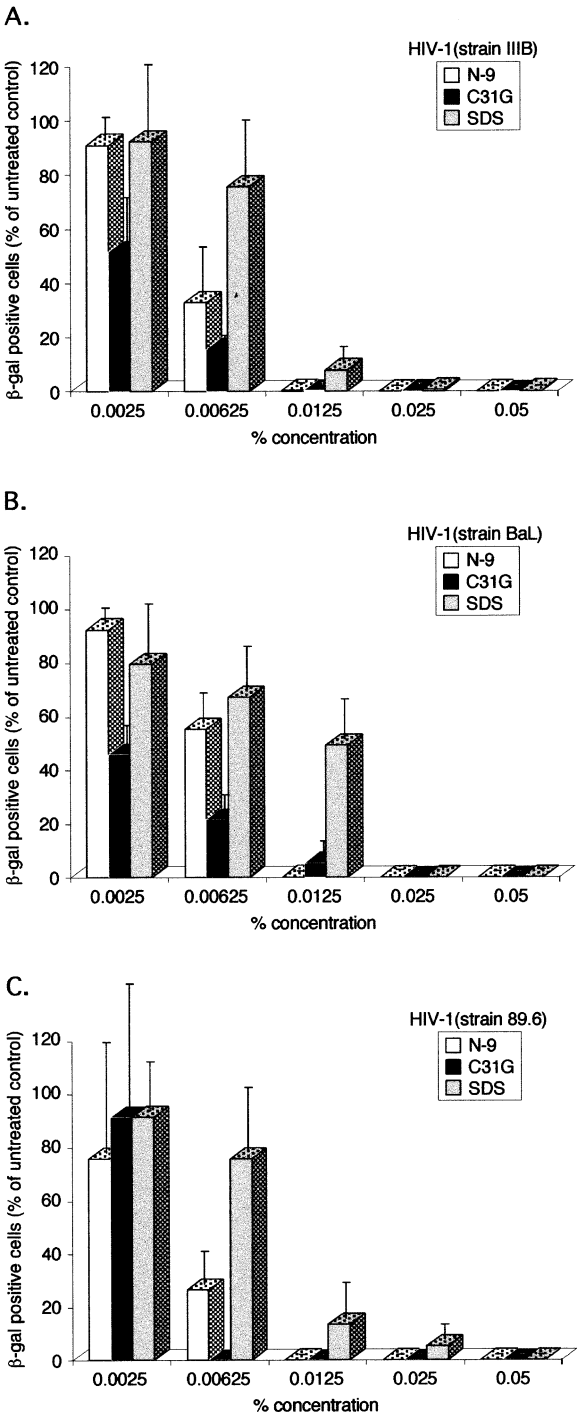

Fig. 1. (*Continued*)

concentrations of 0.00625% produced intermediate levels of inactivation (between 16 and 76% virus remaining after microbicide exposure). At this concentration, C31G was approximately twice as effective as N-9 and five times more effective than SDS. C31G inactivated HIV-1 IIIB more effectively than N-9 at concentrations below 0.0125%, and was equally effective at or above 0.0125%. At or below 0.0125%, SDS was equally or somewhat less effective than N-9. In general, SDS concentrations of approximately twice those of C31G achieved similar levels of inactivation. At concentrations at or above 0.025%, the infectivity of strain IIIB was reduced to less than 1% by each of the three agents.

In similar experimentation, inactivation of BaL (M tropic) and 89.6 (dual tropic) HIV-1 strains was assessed using the P4-R5 indicator cell line (Fig. 1B and C). HIV-1 strain BaL was effectively inactivated by all three agents at concentrations at or above 0.025% (Fig. 1B). As in experiments using HIV-1 IIIB, C31G and SDS were comparatively the most and least effective agents, respectively, used to inactivate HIV-1 BaL. At concentrations of 0.00625%, C31G was 2.5- and 3.1-fold more effective than either N-9 or SDS, respectively. In experiments using HIV-1 strain 89.6, exposure to 0.00625% C31G resulted in complete HIV-1 inactivation, while N-9 and SDS at the same concentrations reduced viral infectivity to 27 and 76%, respectively (Fig. 1C). At or above 0.0125%, HIV-1 strain 89.6 was completely inactivated by N-9 as well as C31G, while complete viral inactivation by SDS occurred at 0.05%.

Fig. 1. Lymphocyte-, macrophage- and dual-tropic strains of human immunodeficiency virus type 1 (HIV-1) are inactivated in the presence of nonoxynol-9 (N-9), C31G, or sodium dodecyl sulfate (SDS). Cell-free HIV-1 strain IIIB (A) was treated with N-9, C31G, or SDS, and used to infect HeLa-CD4-LTR-β-gal (HCLB) cells. Cell-free HIV-1 strains BaL (M tropic) (B) and 89.6 (dual tropic) (C) were treated with N-9, C31G or SDS, and used to infect P4-R5 cells. Assays were conducted as described in Section 2. Infectivity following exposure is expressed as a percentage relative to the number of β-gal-positive cells in duplicate wells infected with virus incubated in the absence of a microbicidal compound. Results shown are the average cell counts for a total of four wells per concentration in two independent experiments.

3.2. Short-term cytotoxicity of N-9, C31G, or SDS is time- and concentration-dependent

Since microbicidal effectiveness of surface active agents against enveloped viruses such as HIV-1 also implies a potentially disruptive effect on cellular membranes, experiments were carried out to evaluate the relative cytotoxicity of each microbicidal agent. Because the HeLa-derived indicator cells could themselves be subject to microbicidal agent toxicity during evaluations of virucidal activity, we proceeded to determine the impact of agent carryover on cellular function throughout the duration of the infectivity assays. In experiments in which HCLB and P4-R5 cells were used to quantitate viral infectivity (Fig. 1), the cells were exposed to each microbicide for 2 h at concentrations 10-fold lower than those used to inactivate HIV-1. Experiments were designed to measure the effect of short-term exposure to N-9,

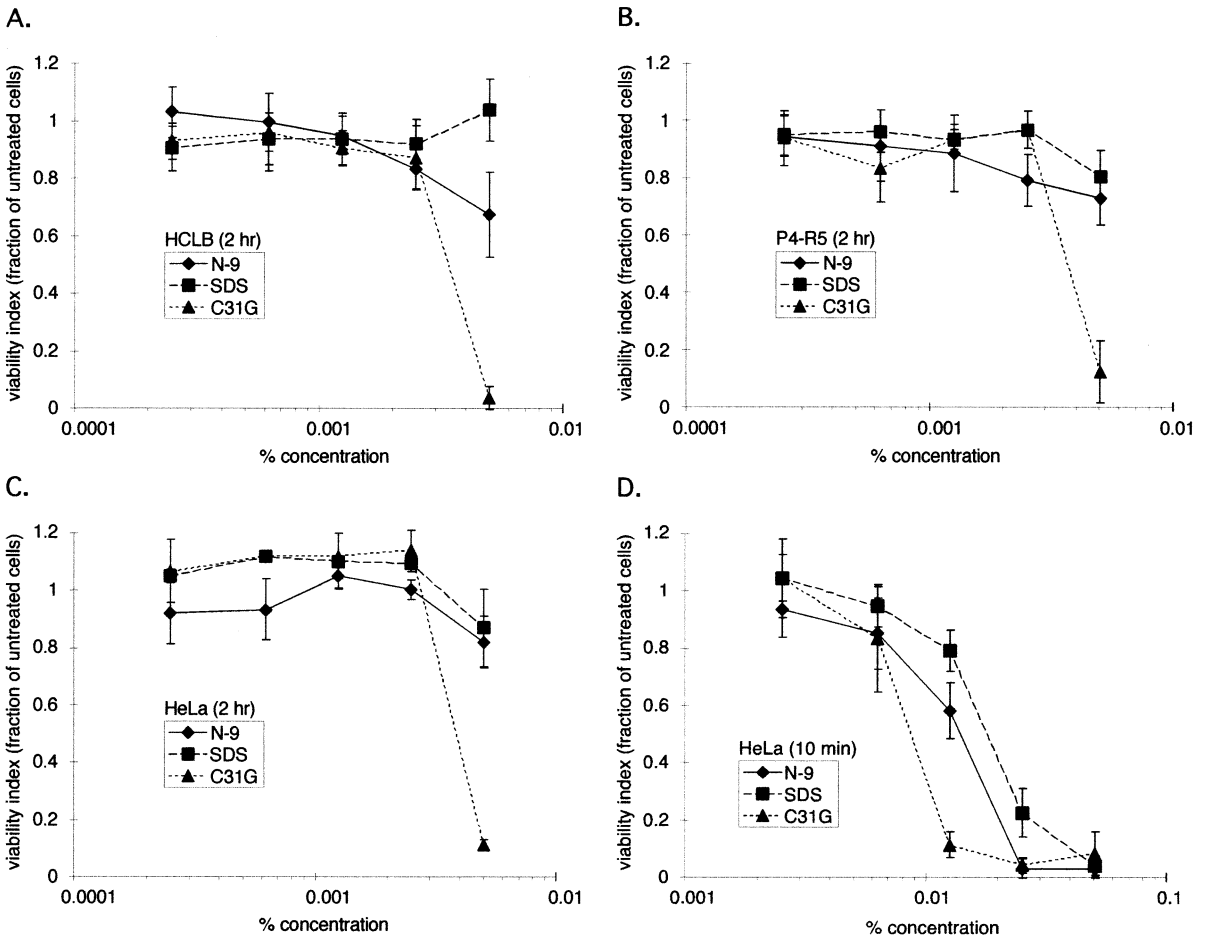

Fig. 2. Short-term cytotoxicity of nonoxynol-9 (N-9), C31G, or sodium dodecyl sulfate (SDS) is time- and concentration-dependent. Each microbicidal agent was added to (A) HeLa-CD4-LTR-β-gal (HCLB), (B) P4-R5, or (C) HeLa cells at the indicated concentrations in 2 ml of new media. Following a 2 h exposure period, media containing the microbicides was removed and replaced with new media. After a 24 h post-exposure incubation, viable cells were quantitated using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay. (D) HeLa cells were incubated with each microbicide at the indicated concentrations for 10 min at 37°C, and then assessed for viability as described in Section 2. Cell survival after treatment is expressed as the fraction of viable cells relative to the number of mock-exposed cells. Results shown are the average of two experiments in which triplicate wells for each concentration were assayed.

C31G, or SDS on indicator cell viability. After a 2 h exposure to each agent followed by a media change and a 24 h incubation period, both N-9 and SDS had a minimal impact on HCLB (Fig. 2A) and P4-R5 (Fig. 2B) viability over the range of concentrations tested. Identical results were obtained in similar experiments in which MTT assays were performed immediately after the 2 h microbicide exposure, indicating that the post-exposure incubation had no effect on cell viability (data not shown). C31G also had minimal impact on cell viability over the range of 0.00025–0.0025%. Only at the highest concentration (0.005%) was C31G toxic, reducing cell viability to approximately 4–12%. Similar results were obtained using HeLa cells (Fig. 2C). Although C31G was toxic to HeLa-derived indicator cells at 0.005%, the impact of this cytotoxicity on our determinations of HIV-1 inactivation by C31G was minimal. Most (if not all) of the viral infectivity was inactivated by C31G at concentrations at or above 0.025% (subsequently diluted 1:10 to 0.0025% prior to incubation with the HeLa-derived indicator cells over the 2 h adsorption period). The results demonstrated that N-9, C31G, or SDS did not affect the viability of HeLa and HeLa-derived cells during short-term (2 h) exposure at concentrations at or below 0.0025%. Furthermore, these experiments indicated that the presence of these agents did not affect the outcome or interpretation of the assays of HIV-1 infectivity following microbicide exposure. Additionally, the similarities in concentration-dependent cytotoxicity between HeLa cells (Fig. 2C) and HeLa-derived cells (Fig. 2A and B) demonstrated that the genetic alterations and selection agents used to produce and maintain the HCLB and P4-R5 indicator cell lines did not affect cellular sensitivity to N-9, C31G, or SDS.

An efficacious vaginal microbicide must function within a range of therapeutic concentrations that maximize antimicrobial activity and minimize cytotoxicity in the surrounding tissues. To determine if such concentrations exist for N-9, C31G, or SDS, experiments were conducted in which microbicide-associated HeLa cell cytotoxicity was examined under conditions identical to those used to inactivate HIV-1 (10 min exposure to 0.0025–0.05% N-9, C31G, or SDS). Correlating the results of these experiments (Fig. 2D) to inactivation of HIV-1 IIIB (Fig. 1A), it has been demonstrated that exposure to 0.00625% N-9 or C31G reduced cell-free infectivity to 33 and 16%, respectively, while only decreasing cell viability to approximately 85%. Similarly, exposure to 0.0125% SDS resulted in 80% cell viability while reducing viral infectivity to 8%. These in vitro experiments demonstrated that all three agents could function at concentrations that provide effective viral inactivation and low levels of cytotoxicity.

3.3. SDS is less toxic than N-9 or C31G during extended exposure to each agent

Because the studies were directed toward identification of an effective anti-HIV-1 compound that is minimally toxic after prolonged in vivo exposure, the cytotoxicity of N-9, C31G, or SDS were also examined during chronic exposure to each agent. Cells assayed included both HeLa-derived indicator cell lines (HCLB, Fig. 3A; P4-R5, Fig. 3B) and the parental HeLa cell line (cervical adenocarcinoma; Fig. 3C). Cells were cultured in the presence of each agent (0.00025–0.005%) for 48 h prior to the MTT assay. SDS was consistently the least cytotoxic agent in all cell lines examined, causing less than 10% cell death at concentrations as high as 0.0025%. At the lowest concentration examined (0.00025%), N-9 was still considerably cytotoxic (54–76% cell viability following microbicide exposure), while C31G was less toxic (85–91% cell viability) and SDS had a negligible effect on viability. At a concentration (0.000625%) where the most toxic microbicide (N-9) reduced cell viability to no less than 50%, HeLa and HeLa-derived cell viability following exposure to SDS or C31G was 95 and 81%, respectively. A comparison of C31G and N-9 toxicity using the HeLa and HeLa-derived cell lines demonstrated that C31G was less toxic than N-9 at concentrations below 0.0025%, and equally or more toxic than N-9 at higher concentrations. As in the short-term (2 h) exposure experiments, the dependence of toxicity on N-9, C31G, or SDS concentration was very similar in

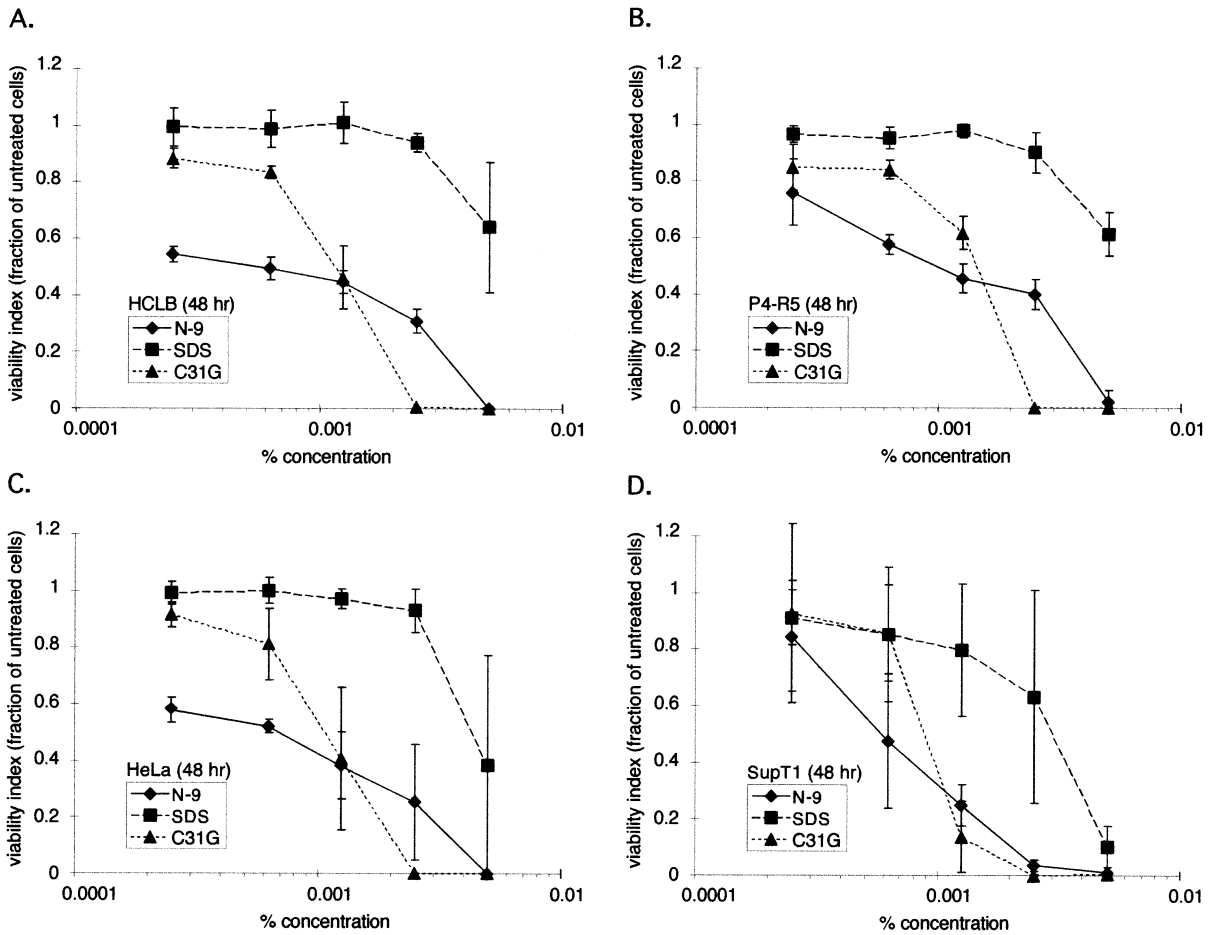

Fig. 3. Extended exposure to sodium dodecyl sulfate (SDS) results in less cytotoxicity compared to similar exposures to nonoxynol-9 (N-9) or C31G. Each microbicidal agent was added to (A) HeLa-CD4-LTR-β-gal (HCLB), (B) P4-R5, (C) HeLa, or (D) SupT1 cells at the indicated concentrations in 2 ml of new media. At 48 h after treatment, viable cells were quantitated using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell survival after treatment is expressed as the fraction of viable cells relative to the number of mock-exposed cells. Results shown are the average of two experiments in which triplicate wells for each concentration were assayed.

all HeLa and HeLa-derived cell lines tested, indicating that the procedures used to derive the HeLa-based HCLB and P4-R5 indicator cell lines did not change their sensitivity to longer duration (48 h) exposures to N-9, C31G, or SDS.

In anticipation of studies that examined inactivation of cell-associated infectivity (described below), the cytotoxicity of each microbicidal agent was examined using a cell line of T lymphocytic origin (SupT1; Fig. 3D). Compared to the HeLa and HeLa-derived cell lines, the SupT1 lymphocyte cell line was generally more sensitive to all three agents tested. For example, at 0.00125%, N-9, C31G, or SDS exposure reduced cell survival to 25, 14, or 80%, respectively. In contrast, HCLB cells were unaffected by exposure to the same concentration of SDS and were reduced in number to approximately 45% of the mock-exposed cells by N-9 or C31G. Although cell viability was affected very little by the lowest concentrations of SDS (91% cell survival) and C31G (93% cell survival), cytotoxicity increased

more rapidly with increasing microbicide concentration compared to that observed using the HeLa and HeLa-derived cells.

3.4. Cell lines of human cervical and immune system origin have a greater tolerance for SDS

To better illustrate the relative cytotoxicity of N-9, C31G, or SDS, concentrations at which the application of each agent resulted in a 50% reduction in cell viability (TC_{50}) were calculated from results of experiments depicted in Fig. 3. In all of the HeLa and HeLa-derived cell lines, the TC_{50} of C31G was somewhat greater than or equal to that of N-9 (Fig. 4), again indicating that these cells were less sensitive to C31G than to N-9. Similarly, SupT1 cells were slightly more viable in the presence of C31G than in the presence of N-9. In contrast, SDS was considerably less toxic than both N-9 and C31G in all cell lines used. For example, TC_{50} calculations using HCLB cells indicated that SDS was 10.6 times less toxic than N-9 and 5.2 times less toxic than C31G. In SupT1 cells, cell viability in the presence of SDS was 5.2- and 3.3-fold greater than the viability of cells exposed to either N-9 or C31G, respectively. These analyses more clearly illustrate the decreased cytotoxicity of C31G compared to N-9, and the even lesser impact that SDS has on the viability of both adherent and suspension cell lines.

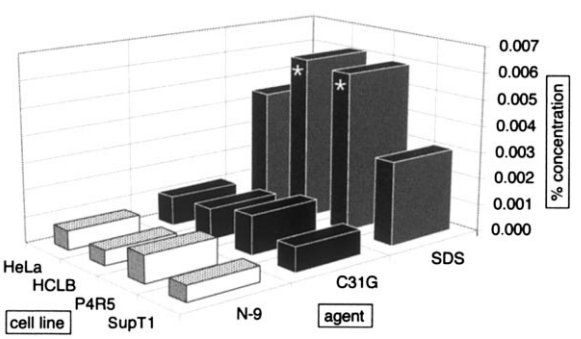

Fig. 4. Concentrations of sodium dodecyl sulfate (SDS) that result in 50% cytotoxicity are considerably higher than similar concentrations of nonoxynol-9 (N-9) or C31G. HeLa, HeLa-CD4-LTR-β-gal (HCLB), P4-R5, and SupT1 cell lines were used to assess agent cytotoxicity. The concentration at which each agent resulted in 50% cell death (TC_{50}) was calculated from experiments depicted in Fig. 3. An asterisk (*) indicates a value which was extrapolated above the highest concentration used in the experiment.

3.5. SDS does not appreciably affect the inactivation potential of C31G or N-9

Ideally, microbicides should prevent or reduce transmission of a broad spectrum of STD pathogens, including HIV-1, HSV-2, and HPV. However, N-9 and C31G are ineffective against non-enveloped viruses such as HPV (Hermonat et al., 1992; Howett et al., 1999). It has recently been shown that SDS inactivates HPV, CRPV, and BPV as well as HIV-1 and HSV-2 (Howett et al., 1999). Addition of SDS to preparations of N-9 or C31G may result in microbicidal mixtures that combine the efficacy of N-9 or C31G with the potential of SDS to inactivate non-enveloped viruses. To verify that mixed microbicides are not compromised to any great extent in their ability to inactivate HIV-1, experiments were carried out using combined aqueous solutions of C31G and SDS (Fig. 5A), or N-9 and SDS (Fig. 5B). C31G and SDS were compared to solutions composed predominantly of C31G (5:1 C31G:SDS) and predominantly of SDS (1:10 C31G:SDS) over a range of total agent concentration of 0.0025–0.05% (Fig. 5A). The ability of C31G supplemented with SDS (C31G/S) to inactivate HIV-1 was partially diminished compared to C31G alone. For example, at 0.00625%, C31G/S was ineffective, while C31G alone reduced viral infectivity to 67%. However, above 0.0125%, C31G/S and C31G were equal in their inactivation potential. In contrast, SDS supplemented with C31G (SDS/C) had virucidal activity similar to that of SDS alone. N-9 and SDS were also combined in the same ratios used for the C31G/SDS mixtures and assayed for inactivation potential (Fig. 5B). As observed in previous experiments (Fig. 1A), SDS was somewhat less effective than N-9 at concentrations of 0.00625 and 0.0125%. At a concentration of 0.00625%, the N-9/SDS mixture (N-9/S) resulted in an intermediate level of viral inactivation relative to N-9 alone. In contrast, inactivation of HIV-1 by SDS supplemented with

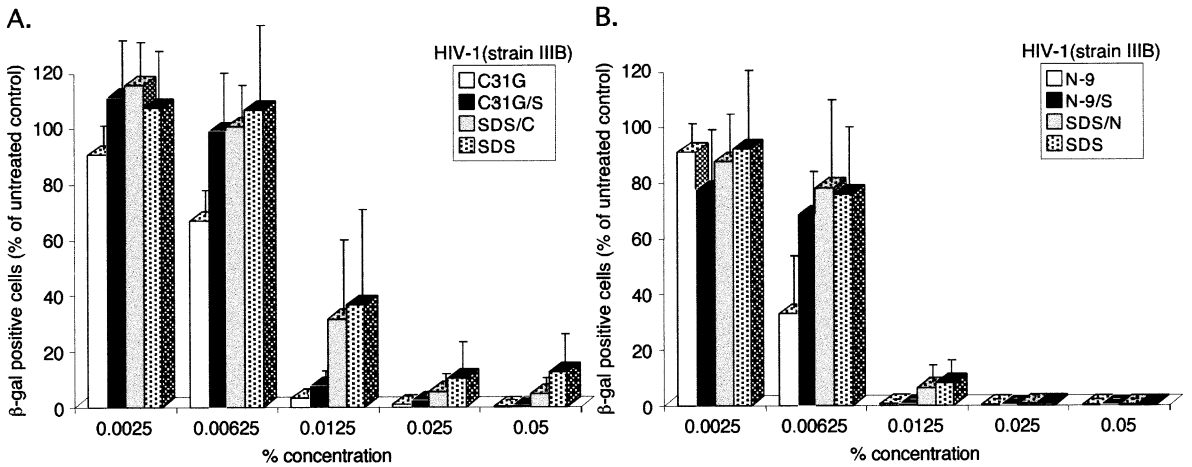

Fig. 5. Sodium dodecyl sulfate (SDS) does not appreciably affect the inactivation potential of C31G or nonoxynol-9 (N-9). HIV-1 strain IIIB was treated with selected concentrations of single or mixed solutions of (A) C31G and SDS, or (B) N-9 and SDS. Microbicide-treated virus was used to infect HeLa-CD4-LTR-β-gal (HCLB) cells. C31G supplemented with SDS (C31G/S) and N-9 supplemented with SDS (N-9/S) were 5:1 mixtures of C31G:SDS or N-9:SDS, respectively. SDS supplemented with C31G (SDS/C) and SDS supplemented with N-9 (SDS/N) were 1:10 mixtures of C31G:SDS and N-9:SDS. Assays were conducted as described in Section 2. Infectivity following exposure is expressed as a percentage relative to the number of β-gal-positive cells in duplicate wells infected with virus incubated in the absence of a microbicidal compound. Results shown are the average cell counts for a total of four wells per concentration in two independent experiments.

N-9 (SDS/N) was unchanged compared to SDS alone. In summary, addition of SDS to either N-9 or C31G (at the ratios examined) does not appreciably compromise the ability of the predominant component to inactivate HIV-1. In related experiments, it has been demonstrated that microbicide mixtures that contain SDS also inactivate papillomavirus (data not shown), verifying that SDS is active against non-enveloped viruses in the presence of other microbicides. These combined results are indicative of the potential of mixed microbicides as broad-spectrum agents with activity against both enveloped and non-enveloped viruses.

3.6. Addition of SDS to microbicide mixtures containing predominantly N-9 or C31G does not appreciably affect cytotoxicity

The cytotoxicity of mixed solutions of C31G/SDS and N-9/SDS were also evaluated in parallel with HIV-1 inactivation studies shown in Fig. 5. Since HeLa and HeLa-derived cell lines exhibited similar sensitivity to each microbicide in both short-term (2 h) and long-term (48 h) studies (Figs. 2 and 3), the cytotoxicity of combined agents in HeLa cells only were examined. HeLa cells were treated for 48 h with microbicidal agents alone or in combination and assayed using the MTT protocol (Fig. 6). Both C31G and SDS (Fig. 6A) demonstrated cytotoxicity over the concentration range tested comparable to that observed in previously described experiments (Fig. 3). The addition of a small amount of SDS to C31G (C31G/S) had no appreciable effect on the toxicity of C31G. However, SDS supplemented with C31G (SDS/C) was slightly more toxic than SDS alone at 0.005%. In experiments examining the toxicity of N-9/SDS combinations, N-9 supplemented with SDS (N-9/S) was less toxic than N-9 alone (Fig. 6B) only at the highest concentration tested. Addition of N-9 to SDS (SDS/N) resulted in a notable decrease in cell viability at all concentrations compared to SDS alone.

3.7. N-9, C31G, and SDS also decrease cell-associated HIV-1 infectivity

Since transmission of HIV-1 within the vaginal tract is likely mediated, in part, by HIV-1-infected

cells as well as cell-free virus, studies were initiated to examine the effectiveness of N-9, C31G, or SDS against cell-associated HIV-1 infectivity. SupT1 cells infected with HIV-1 (strain IIIB) were treated with selected microbicide concentrations and co-cultivated with HCLB cells to assess infectivity following treatment. Although HIV-1 is likely transmitted predominantly by HIV-1-infected macrophages during sexual intercourse, HIV-1-infected T lymphocytes were selected as the initial target for inactivation because SupT1 cells are readily infected and provide a high-titer viral inoculum as a challenging target for microbicidal agents. Like inactivation of cell-free virus (Fig. 1A), inactivation of SupT1-associated HIV-1 infectivity by each agent was concentration-dependent over the range of concentrations examined (Fig. 7A). The results using cell-associated virus demonstrated that, at or below 0.00625%, N-9, C31G, or SDS inactivated cell-associated virus roughly equally. Higher concentrations revealed differences in inactivation potential. At 0.025%, C31G reduced cell-associated infectivity to less than 3% and was approximately seven to nine times more effective than either SDS or N-9. At 0.05%, SDS was unable to completely abolish the infectivity. These data contrast those obtained using cell-free virus (Fig. 1A), in which less than 1% of the total infectivity remained following exposure to SDS at half the concentration (0.025%). The results demonstrated that N-9, C31G, or SDS inactivation of cell-associated HIV-1 infectivity required approximately twice the microbicidal concentration to achieve levels of inactivation similar to those seen using cell-free virus.

Microbicidal-dependent reductions in cell-associated infectivity may be the consequence of not only inactivation of progeny virus and interference with cell-to-cell viral transfer, but also increased cell mortality during microbicide exposure. To investigate the contribution of microbicide toxicity to the results, cytotoxicity assays were performed using uninfected SupT1 cells and short duration (10 min) exposures to the same microbicide concentrations used to inactivate SupT1-associated infectivity (0.0025–0.05%). These concentrations were 10-fold higher than microbicide concentrations used in cytotoxicity experiments described above (Figs. 2–4 and 6).

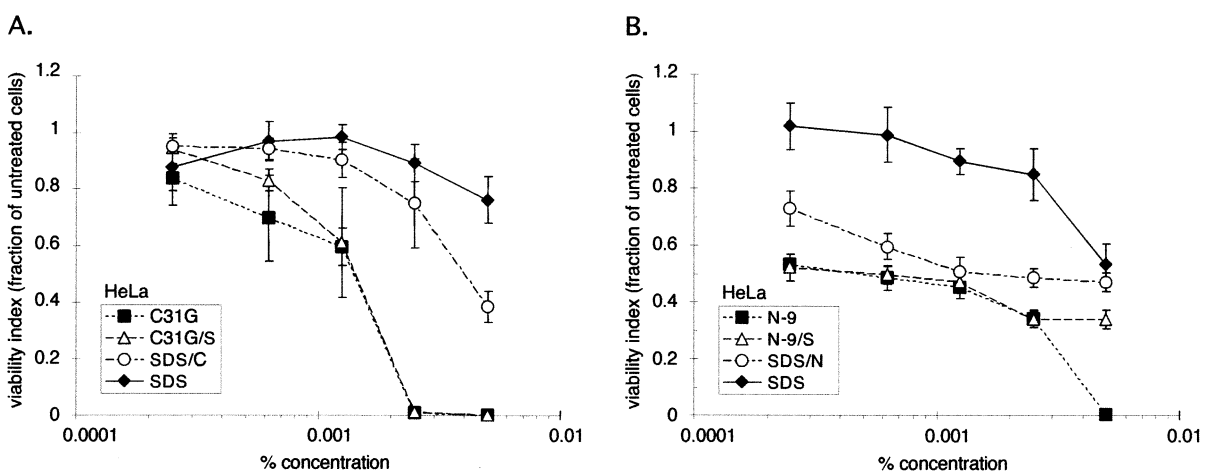

Fig. 6. Addition of sodium dodecyl sulfate (SDS) to microbicide mixtures containing predominantly nonoxynol-9 (N-9) or C31G does not appreciably affect cytotoxicity. HeLa cells were exposed to selected concentrations of single or mixed solutions of (A) C31G and SDS, or (B) N-9 and SDS. Each microbicidal agent or agent combination was added to HeLa cells at the indicated concentrations in 2 ml of new media. At 48 h after treatment, viable cells were quantitated using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell survival after treatment is expressed as the fraction of viable cells relative to the number of mock-exposed cells. Results shown are the average of two experiments in which triplicate wells for each concentration were assayed.

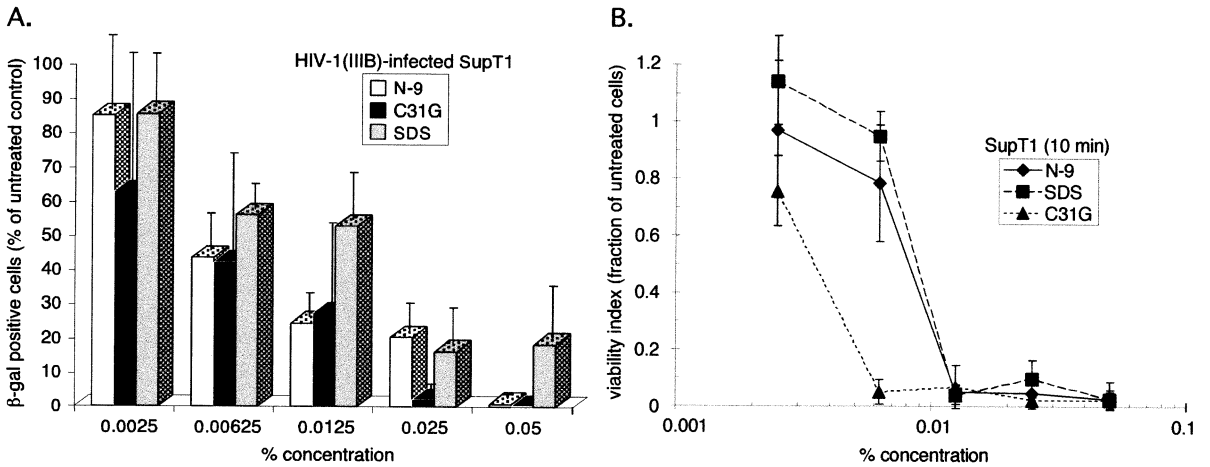

Fig. 7. Nonoxynol-9 (N-9), C31G, and sodium dodecyl sulfate (SDS) can reduce the cell-associated infectivity of HIV-1-infected SupT1 cells. (A) SupT1 T lymphocytes (8×10^4) infected 5 days prior with HIV-1 strain IIIB were pelleted and resuspended in new media to remove cell-free virus. After exposure to selected concentrations of each microbicide for 10 min at 37°C, the cells were diluted 1:10 and co-cultured with HeLa-CD4-LTR-β-gal (HCLB) cells for 2 h. Following one wash with PBS to remove the infected lymphocytes, the indicator cells were cultured and assayed as described in Section 2. Infectivity following exposure is expressed as a percentage relative to the number of β-gal-positive cells in duplicate wells infected with HIV-1-infected SupT1 cells incubated in the absence of a microbicidal compound. Results shown are the average cell counts for a total of four wells per concentration in two independent experiments. (B) SupT1 T lymphocytes (8×10^4) were incubated with selected concentrations of each microbicide for 10 min at 37°C, and then diluted 1:10 with new media. Following a 2 h incubation period, the cells were assessed for viability as described in Section 2. Cell survival after treatment is expressed as the fraction of viable cells relative to the number of mock-exposed cells. Results shown are the average of two experiments in which triplicate wells for each concentration were assayed.

SupT1 cells were incubated with N-9, C31G, or SDS for 10 min at 37°C. Following a 1:10 dilution in RPMI media and a 2 h incubation period, cell viability was assessed using the MTT assay (Fig. 7B). At the lowest concentration (0.0025%), both SDS and N-9 had negligible effects on SupT1 viability, while exposure to C31G decreased cell viability to 75% compared to mock-exposed cells. At the next highest concentration (0.00625%), exposure to SDS again resulted in a minimal decrease in cell viability, while the presence of N-9 reduced cell viability to 80%. In contrast, the presence of C31G at the same concentration decreased the number of viable cells to 5% of the mock-exposed cells. At or above 0.0125%, cell viability was decreased to less than 10% after exposure to N-9, C31G, or SDS. These results demonstrated that SDS is also comparatively less toxic than N-9 or C31G after short duration exposure to higher microbicide concentrations. Collectively, results presented in Fig. 7 demonstrate that microbicide concentrations that result in complete cell mortality do not necessarily lead to total elimination of viral infectivity.

4. Discussion

It has been demonstrated that cell-free preparations of HIV-1 or infectivity associated with HIV-1-infected T lymphocytes can be inactivated in vitro by N-9, C31G, or SDS. Although each agent effectively eliminated all of the infectivity at high concentrations (0.05% and above), lower concentrations revealed differences in inactivation potential as well as cellular sensitivity to short-term (10 min and 2 h) and extended exposure (48 h) to each microbicide. It has also been demonstrated that mixtures of N-9 and SDS or C31G and SDS could inactivate HIV-1, opening the possibility that microbicide mixtures could be used in place of single agents that are more limited in their activity and potency against a spectrum of STD pathogens.

There were clearly microbicide-dependent differences in HIV-1 sensitivity to each of the compounds examined. Using three HIV-1 strains of differing tropism, it has been demonstrated that C31G is generally more potent against HIV-1 than either N-9 or SDS (Fig. 1). At a concentration that resulted in intermediate levels of inactivation of HIV-1 strains IIIB and BaL (0.00625%), C31G was at least two and three times more effective than N-9 and SDS respectively, reducing viral infectivity to less than 22%. At that same concentration, C31G completely eliminated the infectivity of HIV-1 strain 89.6. In contrast, SDS was comparatively less effective than either N-9 or C31G. While the infectivity of strains IIIB, BaL, and 89.6 was greatly reduced (less than 6% remaining infectivity) by N-9 and C31G concentrations at or above 0.0125%, similar levels of inactivation by SDS occurred at or above a concentration of 0.025%. Despite the somewhat lower activity compared to N-9 or C31G, SDS remains a promising candidate microbicide since equivalent levels of inactivation using SDS required only a 2-fold increase in concentration compared to either N-9 or C31G.

These experiments also suggested the possibility that inactivation of HIV-1 is dependent on HIV-1 strain-related factors. For example, exposure to 0.00625% C31G reduced HIV-1 strains IIIB and BaL infectivity to approximately 16–22%. In contrast, HIV-1 strain 89.6 was completely inactivated by C31G at the same concentration. Additionally, SDS at 0.0125% was four to six times less effective against HIV-1 strain BaL compared to strains IIIB and 89.6. We hypothesized that at least two factors play roles in determining the susceptibility of each strain to surface active agents like C31G and SDS. First, strain-specific variations in the amino acid sequence of viral proteins gp120 and gp41 may alter the conformation of each protein and subsequently make them more or less refractile to denaturation by agents such as SDS. Proteins which differ in their primary amino acid sequence are differentially denatured by SDS (Wu et al., 1981). Less extensive differences in amino acid sequence, as found in proteins of different strains of HIV-1, may contribute to more subtle differences in denaturation and viral inactivation. Second, the source of the virus may affect its inactivation. Each of the strains used in the experiments was grown in a different host cell type (IIIB in the H9 human T lymphocyte cell line, BaL in primary human macrophages, and 89.6 in human CEMx174 cells). Because the viral envelope of HIV-1 is derived from the host cell membrane, the envelope of the progeny virus carries with it lipids from the infected cell. The process of viral budding, which involves sequestration of select lipids from the host cell membrane lipid pool (Aloia et al., 1988), results in the release of HIV-1 particles which differ in lipid content across strains. These differences may reflect variations in the host cell membranes, differences in growth conditions, or strain-dependent recruitment of lipids into the envelope (Aloia et al., 1988). Differences in phospholipid content may differentially alter the envelope integrity and impact inactivation by C31G or SDS. The significance of these findings is that vaginal microbicides must be optimized to inactivate viruses with cellular tropisms likely involved in HIV-1 transmission. Viral and biochemical factors involved in strain-dependent differences in microbicidal susceptibility are not clearly understood and warrant further investigation.

It was also shown that the viability of cells cultured in vitro was dependent on the choice of microbicide, microbicide concentration, and exposure duration. During short-term exposure (2 h), both N-9 and SDS were minimally toxic at the concentrations tested while C31G was considerably toxic only at the highest concentration (0.05%). During long-term (48 h) exposure, all three agents caused increased cell mortality with increased concentration. SDS was by far the least cytotoxic of the three compounds after long-term exposure. This was also apparent in comparisons of HeLa TC_{50} values calculated after either 10 min (Fig. 2D) or 48 h exposure (Fig. 4). After 48 h, the TC_{50} for SDS decreased by a factor of 4 compared to the TC_{50} calculated after 10 min exposure. The TC_{50} for C31G decreased by a factor of 8, indicating that it was twice as cytotoxic as SDS under the in vitro conditions used. In contrast, the TC_{50} value for N-9 decreased by

a factor of 20, indicating that N-9 is considerably more toxic than either SDS or C31G after extended exposure. Concentration-dependent differences in long-term toxicity between N-9 and C31G, as suggested by the lower toxicity of C31G below 0.0025%, may be related to mechanistic differences in membrane disruption (Thompson et al., 1996). Dissimilar cytotoxicity during long-term exposure may also be relevant to adverse effects associated with repeated use of vaginal microbicides. In vivo studies have indicated a direct correlation between repetitive use of N-9 and toxicity. Daily vaginal application of N-9 over 1 week in clinical trials resulted in vaginal irritation as well as inflammation and associated recruitment of CD8-positive lymphocytes and macrophages (Stafford et al., 1998). The decreased cytotoxicity of C31G and SDS may offer significant advantages over N-9 when repeated and more extended exposures are considered.

The microbicidal efficacy of N-9, C31G, or SDS is closely related to the ability of each to disrupt lipid and protein interactions within the membrane. This ability is, in turn, related to the property of self-association, as measured by the critical micelle concentration (CMC) of each agent. The CMCs of N-9, C31G, and SDS are 0.073, 0.041, and 0.5 mM, respectively (McConlogue, 1998). At a concentration which resulted in partial HIV-1 inactivation (0.00625%), the concentration of SDS ($\sim$0.2 mM) was less than half the CMC for SDS. In contrast, N-9 and C31G ($\sim$0.1 mM) were both present at concentrations which exceeded their CMCs. Differences in CMCs may account for decreased HIV-1 inactivation by SDS at low concentrations. Furthermore, the 10-fold higher CMC of SDS (compared to N-9 or C31G) may correlate with the much lower cytotoxicity of SDS.

An extension of the inactivation experiments using each agent singly was to combine two compounds to examine the effectiveness of mixed microbicidal agents. Mixed agents may combine the inactivation potential of each component and exhibit broad activity against HIV-1 and other pathogens. It has been demonstrated that a mixed microbicidal agent composed predominantly of C31G supplemented with SDS was only slightly less effective (at lower concentrations) against HIV-1 (strain IIIB) and no more cytotoxic than C31G. These results suggest the possibility that a C31G/SDS mixture may be effective against a broad spectrum of enveloped viruses and bacterial agents due to the activity of C31G (Corner et al., 1988, 1990; Calis et al., 1992; Thompson et al., 1996; Wyrick et al., 1997), as well as non-enveloped viruses like HPV due to the presence of SDS (Malamud et al., 1998; Howett et al., 1999). The slightly reduced efficacies of C31G or N-9 supplemented with SDS suggest the need for careful evaluation of microbicide mixtures for adverse interactions between mixture constituents. Interactions between constituents of a mixed microbicide may adversely impact the microbicidal effectiveness of marginally compatible mixtures. The experiments described here, which were performed to verify that mixed microbicides are not compromised to any great extent in their ability to inactivate HIV-1, are only the prelude to more extensive studies. Investigations will now advance toward a more thorough examination of the properties of a wide array of microbicide combinations. These studies will culminate in the identification of one or more combinations that exhibit maximal microbicidal activity, minimal cytotoxicity, and component compatibility.

The inactivation of cell-associated HIV-1 infectivity was also examined. It was concluded from the results that, compared to inactivation of cell-free virus, higher microbicidal agent concentrations are required to interfere with the transfer of infectivity from HIV-1-infected SupT1 cells to the HCLB indicator cells. Higher amounts of N-9, C31G, or SDS are likely to be needed to offset microbicide association with and sequestration by cellular lipids and proteins. In addition, a comparison of concentration-dependent viral inactivation and cytotoxicity (Fig. 7A and B) demonstrated that viral infectivity could be readily detected at and above microbicide concentrations of 0.0125%, despite the high level of cell mortality. These results indicate that decreased cell-free and cell-associated HIV-1 infectivity is not closely associated with microbicide-dependent cell mortality, and that infectious virus may still be present at microbicide concentrations that re-

sult in total cell mortality. Finally, our results suggest that the relationship between in vitro cell viability and microbicidal effectiveness is dependent on the choice of microbicide. For example, exposure to 0.00625% C31G decreased infectivity to just less than 50% and SupT1 viability to 5% (Fig. 7). In contrast, exposure to SDS or N-9 resulted in similar decreases in infectivity but much smaller reductions in cell viability. These data indicate that SDS (and N-9) may have an advantage over C31G at lower concentrations when the balance between microbicide effectiveness and cytotoxicity is considered.

The efficacy of N-9, C31G, and SDS will also be affected by the vaginal environment. Although the in vitro experiments provide a comparison of the relative activity and toxicity of each microbicidal agent, their results almost certainly do not accurately reflect the characteristics of these microbicides under in vivo conditions. The in vivo cytotoxicity (as well as the microbicidal effectiveness) of each of these agents will undoubtedly be affected by the morphological structures of keratinized vaginal and cervical tissues and factors associated with the vaginal milieu, including pH and the presence of cervical mucus or semen. These factors will likely decrease the in vivo sensitivity of cells within the vaginal and cervical epithelia to microbicide exposure and allow use of higher microbicide concentrations that are effective against HIV-1 (and other sexually transmitted disease pathogens). In vivo toxicity can be estimated using the rabbit vaginal irritation test, and indeed, the surfactants tested here (N-9, C31G, and SDS) have all been judged to be safe at concentrations of 1–5% in this in vivo model (data not shown). In related experiments, it has also been demonstrated that HeLa cells are approximately half as sensitive to N-9, C31G, or SDS exposure (10 min at 0.0025–0.05%) compared to SupT1 cells, as measured by TC_{50} (compare Fig. 2D and Fig. 7B). Such differential sensitivity between cell types could allow the use of higher microbicide concentrations that are effective against HIV-1-infected immune cells and cell-free virus yet less cytotoxic to cells of the vaginal and cervical epithelia. Experiments already in progress are addressing the impact of vaginal and cervical environmental factors on each microbicide.

These investigations have demonstrated the inactivation of both cell-free and cell-associated forms of HIV-1 by C31G and SDS alone or in combination. Formulations which incorporate SDS in combination with either N-9 or C31G may serve as broad spectrum barriers not only to HIV-1 transmission, but also to non-enveloped viruses such as HPV, which are more refractile to inactivation by single agents such as N-9 or C31G. These studies highlight the potential of these compounds as components of vaginal microbicide formulations and lay the foundation for future human clinical trials of these agents.

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