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Enhancement of Human Immunodeficiency Virus Type 1 Replication Is Not Intrinsic to All Polyanion-Based Microbicides▽

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Polyanion-based microbicides have been developed to prevent the sexual transmission of human immunodeficiency virus (HIV). Recent data suggest that polyanions have the capacity to enhance HIV type 1 (HIV-1) replication at threshold antiviral concentrations. Evaluation of the microbicide candidates SPL7013 and PRO 2000 revealed no specific enhancement of two CCR5 HIV-1 strains in human peripheral blood mononuclear cells compared to enfuvirtide (Fuzeon). The enhancement effect is likely to be a function of the assay conditions and is not an intrinsic property of these polyanions.

SPL7013, the active component of VivaGel, is a dendrimer-based microbicide, which is currently undergoing clinical evaluation. SPL7013 has broad-spectrum activity against human immunodeficiency virus type 1 (HIV-1) and type 2 and herpes simplex virus by blocking the entry of these viruses into the host cell (3, 6, 14). SPL7013 inhibits HIV_{Ba-L} replication in human cervical cultures (5) and colorectal explant cultures (1). SPL7013 has demonstrated low toxicity in cervical and colorectal epithelial cell lines, peripheral blood mononuclear cells (PBMCs), and macrophages and does not impair the resistance of polarized epithelial cells (6). VivaGel has demonstrated favorable safety profiles when administered vaginally and rectally in macaques (16) and in clinical studies of healthy human female volunteers (T. McCarthy, J. Paull, G. Heery, S. Evans, S. Xia, J. O'Loughlin, H. McDonald, E. Ribic, Y, Lungershausen, and B. Trudinger, presented at the 8th Annual Meeting of the Alliance for Microbicide Development, Washington, DC, 2005) and male volunteers (4). It is effective in preventing the vaginal transmission of the CXCR4 SHIV89.6P strain in macaques and SHIV162P3 infection of macaque PBMCs (11).

SPL7013 belongs to a class of microbicides termed polyanions that also include the linear polymers PRO 2000, cellulose sulfate (CS) (Ushercell), and Carraguard (2, 7, 17). Members of this class differ chemically from each other, and this is likely to impart different pharmacological, toxicological, and antiviral properties (2). CS and Carraguard have failed to demonstrate efficacy in preventing HIV transmission in phase III clinical trials (18, 22), which has cast doubt regarding the potential in vivo efficacy of other members of the polyanion microbicide class (9, 19). A recent report claims that CS enhances the infection and replication of CCR5 strains of HIV-1 at threshold antiviral concentrations in human PBMCs (9, 19) and that this phenomenon has contributed to the increased trend in HIV acquisition observed in women using CS in a recent randomized, double-blind placebo-controlled phase III trial (22). The same study also states that PRO 2000 enhances HIV-1 replication in vitro and that this phenomenon is a property of all polyanions (19).

We conducted studies to determine whether SPL7013 enhances HIV-1 replication in human PBMCs in vitro. PRO 2000 was included as an example of a polyanion reported to cause enhancement of HIV-1 replication (19), and enfuvirtide (Fuzeon) was included as an example of a nonpolyanionic entry inhibitor (13). Phytohemagglutinin (PHA)-stimulated human PBMCs from one or two uninfected donors per experiment were prepared as described previously and maintained in Teflon pots to prevent depletion of monocytes by adherence (12). Stimulated PBMCs were transferred to medium containing 5% interleukin 2 and treated with different concentrations of SPL7013, PRO 2000, or enfuvirtide for 1 h at 37°C and 5%CO_2 prior to infection with either HIV_{Ba-L} (~1,700 50% tissue culture infective doses [$TCID_{50}$]/well) or a CCR5 clade C isolate (92BR025, ~300 $TCID_{50}$/well). Half medium changes were performed at days 3, 6, 10, and 13 postinfection, and clarified culture supernatants were stored for determination of virion-associated reverse transcriptase (RT) activity (23) and HIV p24 antigen using an enzyme-linked immunosorbent assay-based assay (Vironostika HIV-1 Antigen Micro-Elisa system p24 assay; Biomerieux).

There was no enhancement of replication evident for either HIV strain at day 6 postinfection for all drugs tested (Fig. 1). In contrast, modest increases of not more than 60% above the level of replication for untreated controls were observed at days 10 and 13 postinfection between 1.0 and 3.2 μg/ml and 0.32 and 3.2 μg/ml for SPL7013 and PRO 2000, respectively (Fig. 1). The enhancement effect observed was greater for HIV_{Ba-L} (Fig. 1A, B, and C) than for the clade C strain (Fig. 1D, E, and F), irrespective of the drug tested, suggesting a strain-specific effect. Statistically significant increases in HIV_{Ba-L} replication compared to the infected untreated control were observed in the presence of 3.2 μg/ml (P = 0.014)

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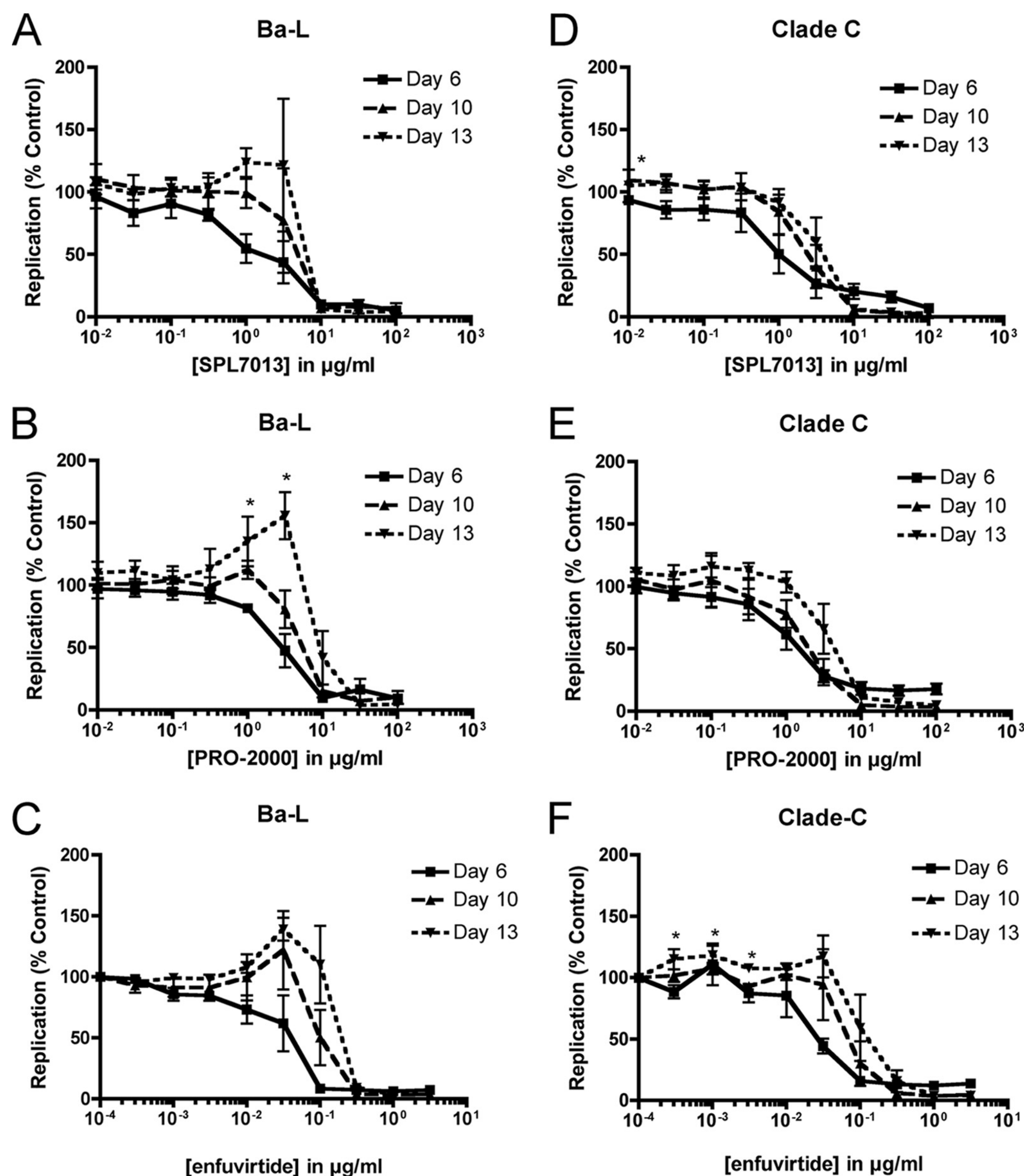

FIG. 1. Inhibitory effects of SPL7013, PRO 2000, and enfuvirtide on the replication of $HIV_{Ba\text{-}L}$ and the clade C 92BR025 strain in human PBMCs. PHA-stimulated PBMCs from at least one healthy donor were treated for 1 h with drug prior to infection with virus. Clarified culture supernatants collected at days 6, 10, and 13 postinfection were evaluated for virion-associated RT activity using a poly(rA)/oligo(dT) template/primer and $[^{33}P]$dTTP as the substrate. Data are expressed as virus replication of drug-treated cultures compared to the infected untreated control (representing 100% replication). Data were obtained from at least three independent assays using PBMCs from different individuals for each assay, with data from four assays used for SPL7013 and PRO 2000 tested against both viruses with the exception of $HIV_{Ba\text{-}L}$ on day 6 where data from three assays were used. For enfuvirtide, each data point represents data from three assays. The statistical significance of replication at greater than 100% of the infected, untreated control was determined using the nonparametric equality of medians Fisher's exact test where a P value of <0.05 was considered significant. As the alternative hypothesis was unidirectional ($>100\%$), a one-sided test was applied. Drug concentrations with statistically significant increases in virus replication above the infected, untreated control are denoted with an asterisk. Error bars indicate the standard errors of the means.

and 1 μg/ml ($P = 0.014$) of PRO 2000 at day 13 postinfection (Fig. 1B) and for cells infected with the clade C strain that were treated with 0.01 μg/ml ($P = 0.014$) of SPL7013 at day 10 postinfection (Fig. 1D). Notably, a similar replication pattern was observed for the clinically proven HIV-1 entry inhibitor, enfuvirtide (Fuzeon) (Fig. 1C and F) with statistically significant increases in HIV replication for the clade C virus in the presence of 0.31 to 3.1 ng/ml ($P = 0.014$) of peptide at day 13 postinfection (Fig. 1F). Measurement of HIV-1 p24 in supernatants collected at days 6 and 13 postinfection from $HIV_{Ba\text{-}L}$-infected cells treated with either SPL7013 or PRO 2000 demonstrated a similar replication pattern as observed for virion-associated RT activity with no enhancement at day 6 and increases of less than 50% of the untreated control at threshold antiviral concentrations (i.e., 1 to 3 μg/ml) of polyanion (Fig. 2). Taken together, these data demonstrate that the

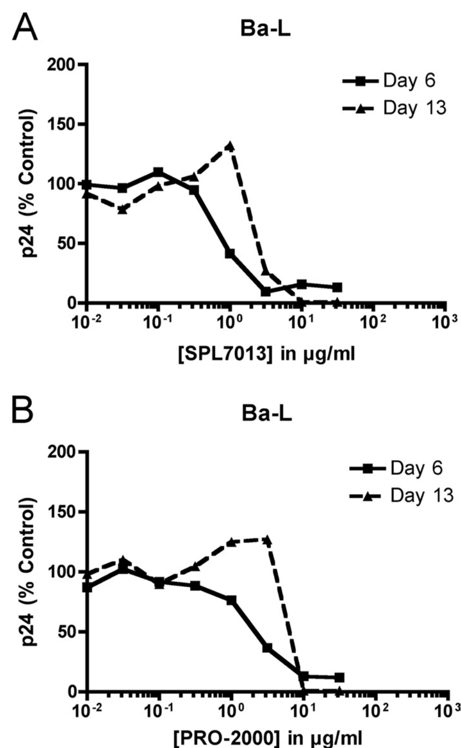

FIG. 2. Inhibitory effects of SPL7013 (A) and PRO 2000 (B) on the replication of HIV_{Ba-L} in human PBMCs using HIV p24 as the measure of virus replication. Experiments were performed as described in the legend to Fig. 1, except that clarified culture supernatants were subjected to a p24 enzyme-linked immunosorbent assay. Data are expressed as virus replication of drug-treated cultures compared to the infected untreated control and are derived from a representative assay.

enhancement effect seen at days 10 and 13 postinfection is not an intrinsic property of the polyanions tested. The effect may relate to specific conditions of the in vitro assay, such as the timing of target cell expansion, thus resulting in an increase in target cell numbers and therefore virion production compared to untreated infected cells, particularly at threshold antiviral concentrations. In this regard, we noticed that HIV_{Ba-L} replicated more robustly than the clade C strain in PBMCs, and this may have accounted for the greater "enhancement" of HIV_{Ba-L} replication observed in our assays. Assays that do not rely on measurement of virus replication following multiple rounds of replication such as single-round infections using a HIV reporter virus or reporter cell lines are likely to obviate the enhancement effect observed in our studies.

Our data differ from the data of the study of Tao et al. (19), since we failed to observe enhancement of HIV-1 replication at day 6 postinfection (Fig. 1 and 2). Additionally, at no stage did we observe enhancement approaching that found by this group for either a laboratory-adapted clade B strain (~200%) or a primary clade C isolate (~800%). Only at later time points was modest (≤60%) enhanced replication observed at threshold antiviral concentrations, which was not polyanion specific. Our results are consistent with published data for PRO 2000, which show enhancement of HIV_{Ba-L} replication at day 10 postinfection in the presence of 0.6 to 9 μg/ml of polyanion (8, 19). While the target cell used in this study was a T-cell line, the similarity with our data suggests that this effect may also be a function of the assay conditions. The concentrations at which enhancement was observed for SPL7013 and PRO 2000 are orders of magnitude lower than the concentrations formulated for in vivo use, which are 30 mg/ml and 5 mg/ml, respectively (7, 17), although dilution after vaginal application may lead to low microgram levels of these polyanions in the genital tract.

We chose to evaluate whether SPL7013 and PRO 2000 specifically enhance replication of CCR5 strains of HIV-1 in PHA-stimulated PBMCs and evaluate virus replication at 6, 10, and 13 days postinfection using virion-associated RT activity for all assays and HIV p24 for a select number of assays to be able to compare our data to a previous study (19). In contrast to the Tao et al. study (19), we did not observe enhancement of HIV-1 replication at threshold antiviral concentrations at day 6 postinfection (Fig. 1 and 2), and we did not see a plateau in p24 levels at later time points, which has been suggested to represent maximum accumulation of virus in the culture supernatant (19). The reason for this is unclear, although it may be that p24 was more labile in our system. Nevertheless, the assay system we used was not unique and very similar to that used in the Tao et al. study (19).

Our findings are consistent with a previous study demonstrating that the polyanion dextran sulfate does not enhance HIV-1 replication in T lymphocytes (15). In contrast, dextran sulfate enhances HIV_{Ba-L} replication in monocyte-derived macrophages (10, 15). While the mechanism of this enhancement is unknown (15), one study reported that the enhancement by high-molecular-weight dextran sulfate is associated with conformational changes in the gp120 V2 region and increased interaction between gp120 and CCR5 (10). While we have demonstrated that SPL7013 and PRO 2000 are not specific enhancers of HIV replication, we cannot exclude the possibility that CS behaves differently than SPL7013 and PRO 2000 (19). Furthermore, we cannot exclude the possibility that SPL7013 and PRO 2000 are able to enhance replication of HIV-1 isolates in primary human macrophages, as reported for dextran sulfate (15).

Even if the apparent enhancement effect previously described for CS and PRO 2000 (19) were reproducible, its in vivo significance would remain unclear. In this regard, a recent study found no correlation between the in vitro enhancement of replication of simian immunodeficiency virus SIVmac239 bearing HIV RT in the presence of Carraguard and increased vaginal transmission of this virus in macaques (21). In addition, there was no evidence of increased HIV-1 transmission in the Carraguard phase III clinical trial, where imperfect compliance could have led to suboptimal carrageenan concentrations in the genital tract during a significant number of high-risk sex acts, thus representing a worst-case scenario with respect to enhancement of HIV transmission (18). Furthermore, recent data from the HPTN 035 trial which evaluated the safety and effectiveness of 0.5% PRO 2000 gel for the prevention of HIV infections in women revealed a 30% decrease in HIV transmission in women using PRO 2000 compared to placebo gel or no treatment (S. A. Karim, A. Coletti, B. Richardson, G. Ramjee, I. Hoffman, M. Chirenje, T. Taha, M. Kapina, L. Maslankowski, and L. Soto-Torres, presented at the 16th Conference on Retroviruses and Opportunistic Infections, Montreal, Canada, 2009, oral abstr. 48LB). While this study did not

reach statistical significance ($P = 0.1$), it is noteworthy that there was no evidence of an increase in HIV transmission by PRO 2000.

Of greater relevance with respect to the potential in vivo efficacy of a microbicide is the adherence of study participants and its antiviral potency relative to the extent to which the antiviral agent and/or vehicle compromise the integrity of the genital tract and increase inflammation and/or attenuate innate immune function, factors which may have contributed to the failure of CS in clinical trials (P. Mesquita and B. Herold, presented at Microbicides 2008, New Delhi, India, 2008) (20). Each polyanion has different properties, and the reported enhancement effect in PBMCs (9, 19) is not applicable to SPL7013, not reproducible in regard to PRO 2000, and has questionable relevance in vivo.

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The results and conclusion of this report are those of the authors and do not necessarily represent the views of NIH.

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