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The antimicrobial peptide Dermaseptin S4 inhibits HIV-1 infectivity in vitro

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Abstract

Most of HIV-1 infections are acquired through sexual contact. In the absence of a preventive vaccine, the development of topical microbicides that can block infection at the mucosal tissues is needed. Dermaseptin S4 (DS4) is an antimicrobial peptide derived from amphibian skin, which displays a broad spectrum of activity against bacteria, yeast, filamentous fungi, and herpes simplex virus type 1. We show here that DS4 inhibits cell-free and cell-associated HIV-1 infection of P4-CCR5 indicator cells and human primary T lymphocytes. The peptide is effective against R5 and X4 primary isolates and laboratory-adapted strains of HIV-1. Its activity is directed against HIV-1 particles by disrupting the virion integrity. Increasing the number of DS4-positive charges reduced cytotoxicity without affecting the antiviral activity. The modified DS4 inhibited HIV-1 capture by dendritic cells and subsequent transmission to $CD4^+$ T cells, as well as HIV-1 binding on HEC-1 endometrial cells and transcytosis through a tight epithelial monolayer.

Keywords: Dermaseptin S4; Microbicide; Herpes simplex virus type 1

Introduction

The predominant mode of human immunodeficiency virus type 1 (HIV-1) transmission worldwide is via heterosexual contact, with a higher rate of transmission to females than to males (UNAIDS, 2002). For sexual transmission to occur, infectious HIV-1 must cross the mucosal epithelium. Epithelial cells in genital and gastrointestinal tracts do not express CD4, but HIV-1 can bind to the cell membrane using a galactosyl-ceramide (Bomsel and Alfsen, 2003). HIV-1 could cross the pluristratified squamous epithelium of the vagina, exocervix or anus by physical breaches, or after capture or infection of dendritic cells (DCs). The transfer of HIV-1 across the monostratified epithelium of the endocervix, rectum, or gastrointestinal tract can occur by transcytosis, binding on Langerhans cells or infection of the intraepithelial lymphocytes (Bomsel, 1997; Bomsel and Alfsen, 2003; Pope and Haase, 2003; Stone, 2002). After crossing the epithelial barrier, HIV-1 can infect CCR5-expressing DCs, macrophages and T lymphocytes in the submucosa (Geijtenbeek et al., 2000; Greenhead et al., 2000; Spira et al., 1996), then spread to circulating $CD4^+$ T cells.

Since most new infections occur in developing countries, new methods of HIV prevention that can be controlled by women are urgently needed. These methods include the use of topical microbicides able to prevent HIV-1 entry through mucosa. Topical HIV-1 inhibitors may target the incoming virus at several steps of molecular events that drive viral entry (Eckert and Kim, 2001; Wyatt et al., 1998). They may also target specifically submucosal cells to prevent infection (Hu et al., 2004; O'Hara and Olson, 2002; Pierson and Doms, 2003; Reimann et al., 2002; Shattock and Moore, 2003).

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Inhibitors that target the envelope glycoprotein (Env) inhibit the binding of gp120 to CD4 or co-receptor (Bewley, 2001; Chang et al., 2003; Moulard et al., 2000; Poignard et al., 2001) or the fusion stage (Kilby et al., 1998). HIV-1 membrane is fragile and prone to disruption by many chemical agents. However, viral membrane is derived from the host cells from which it buds. Thus, the cytotoxicity of drugs that target viral membrane has to be evaluated on epithelial cells (Van Damme et al., 2002). Sexually transmitted diseases (STDs) have a marked effect on both viral shedding in the genital tract and the risk of acquiring HIV-1 infection, since they increase inflammation and create lesions in the vaginal or rectal tissues (Kovacs et al., 2001). Hence, an ideal microbicide should protect not only against HIV-1 but also against genital herpes and common bacterial infections.

Dermaseptin S4 (DS4) is a 28-residue antimicrobial peptide isolated from frog skin (Mor et al., 1991, 1994). This linear cationic peptide adopts an amphipathic α-helical conformation upon association with lipid bilayers, leading to membrane permeabilization and microbe death. Selective membrane recognition is related to the lipid composition of the target membrane and its electrical potential (Gaidukov et al., 2003; Kustanovich et al., 2002). DS4 displays a broad spectrum of activity affecting Gram-negative and Gram-positive bacteria, yeast, filamentous fungi, *Plasmodium falciparum*, and the enveloped herpes simplex virus type 1 (HSV-1) (Belaid et al., 2002; Efron et al., 2002; Mor et al., 1994). Increasing the positive charge and reducing the hydrophobicity of the native peptide have been shown to correlate with selective antimicrobial activity and reduced toxicity in mammalian cells (Kustanovich et al., 2002).

In this study, we investigated the antiviral effect of DS4 against HIV-1 in vitro. The ability of DS4 to reduce cell-free or cell-associated HIV-1 transmission was evaluated on different target cells: P4-CCR5 indicator cells, human primary T lymphocytes, and monocyte-derived dendritic cells (DCs). We also investigated the capacity of DS4 to impair HIV-1 attachment to human epithelial cells and DCs, as well as transcytosis through a tight epithelial barrier. The effect of the peptide on the viral particle was explored, and to pre-clinically test DS4 as a potential topical microbicide, we established the levels of efficacy versus toxicity.

Results

Dermaseptin S4 inhibits HIV-1 infection of P4-CCR5 indicator cells

The antiviral effect of DS4 was first evaluated using a single cycle virus infectivity assay on P4-CCR5 indicator cells (Lorin et al., 2004). These cells express the CD4, CCR5, and CXCR4 HIV-1 receptors and are stably transfected with *LacZ*, inducible by HIV Tat. Therefore, they are susceptible to HIV-1 isolates and express β-galactosidase upon infection. P4-CCR5 cells were infected with HIV-1_{LAI} in presence of increasing concentrations of DS4 (0.35–11.2 μM). Fig. 1 shows that DS4 inhibited HIV-1 infection of reporter target cells in a dose-dependent manner. More than 90% reduction of infection was observed in presence of 3.5 μM DS4 and 50% reduction in presence of 1.5 μM. P4-CCR5 cells viability was determined after 24-h exposure to increasing concentrations of DS4. Fig. 1 shows that cells remained viable in the presence of DS4 concentrations that inhibited 90% of HIV-1 infection. However, DS4 concentrations higher than 5 μM were significantly cytotoxic in this assay. For this reason, we modified DS4 peptide with the aim to reduce its toxicity without affecting its antiviral activity.

Biochemical modifications reduce DS4 cytotoxicity

It has been previously shown that increasing the net-positive charge and reducing the hydrophobicity of DS4 resulted in reduced hemolytic activity and high antibacterial activity (Efron et al., 2002; Feder et al., 2000; Kustanovich et al., 2002). In the present study, DS4 analogs were synthesized by introducing deletions, substitutions, or both in the native 28-aminoacid sequence of DS4 (Table 1). Both antiviral activity and cytotoxicity of these analogs were evaluated using P4-CCR5 target cells. Peptide concentrations causing 50% inhibition of HIV-1 infectivity (IC_{50}) and concentrations causing 50% cytotoxicity (CC_{50}) after 24 h of exposure were measured. The selectivity index (SI), the ratio CC_{50}/IC_{50}, was calculated and results for all analogs are summarized in Table 1. The C-terminal amidation of native DS4 had no significant effect on the peptide toxicity for P4-CCR5 cells or on its anti-HIV-1 activity. Deletions of the C-terminal region dramatically affected the anti-HIV-1 activity: DS4-(1–16)a was 5-fold less active than the native peptide, and DS4-(1–12)a or DS4-(1–9)a were inefficient, even at a concentration of 100 μM. Likewise,

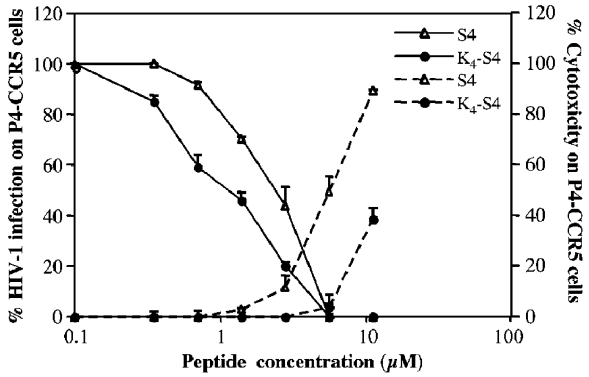

Fig. 1. Effect of dermaseptins S4 and K_4-S4 on HIV-1_{LAI} infection and cytotoxicity. P4-CCR5 cells were subjected to a 2-h infection with HIV-1_{LAI} in presence of the indicated concentration of the different forms of dermaseptin S4. β-Galactosidase production was quantified 48 h post-infection (solid lines). Cytotoxicity was quantified by trypan blue exclusion following a 24-h exposure on P4-CCR5 cells (dashed lines).

Table 1
Selectivity index of DS4 derivatives

Peptide	Sequence	IC_{50}[a]	CC_{50}[b]	SI[c]
S4	ALWMTLLKKVLKAAAKAALNAVLVGANA	2	4.5	2.25
S4a	————————————————————NH_2	2	5.6	2.8
K_4-S4	——K————————————————NH_2	1.4	16.8	12
S4-(1–16)a	————————————NH_2	>19.2	19.2	1
K_4-S4-(1–16)a	——K————————————NH_2	28	>100	>3.6
S4-(1–12)a	————————————NH_2	>100	>100	1
S4-(1–9)a	————————NH_2	>100	>100	1
S4-(6–28)	—————————————————————	>100	>100	1

[a] Peptide concentration (μM) that induces 50% infection inhibition on P4-CCR5 cells by HIV-1_{LAI}.
[b] Peptide concentration (μM) that causes 50% cytotoxicity on P4-CCR5 cells.
[c] Selectivity index: ratio CC_{50}/IC_{50}.

N-terminal deletion reduced the cytotoxicity of the peptide but weakened its anti-HIV-1 activity (DS4-(6–28) was 10-fold less active). On the contrary, a positive charge monosubstitution without shortening the length of the peptide (Met → Lys in position 4) reduced the cytotoxicity but did not affect the anti-HIV-1 activity. Dermaseptin K_4-S4 has the highest selectivity index among all analogs tested. Taken together, these results show that making shorter DS4 abolishes its cytotoxicity but reduces its anti-HIV-1 activity, and that a positive charge substitution in dermaseptin K_4-S4 reduces the cytotoxicity without affecting the anti-HIV-1 activity. All further experiments on P4-CCR5 cells were performed with dermaseptin K_4-S4 at non-toxic concentrations lower than 3.5 μM.

Dermaseptin K_4-S4 is active against X4 and R5 HIV-1

We examined the spectrum of activity of dermaseptin K_4-S4. Fig. 2A shows that dermaseptin K_4-S4 is active against a broad spectrum of HIV-1 viruses: the infectivity of the laboratory adapted LAI strain as well as several primary clade B or A and X4- or R5-tropic isolates (Bx08, US660, US714, HT 593, 3253, NDK, JR-CSF) were inhibited. This observation is consistent with a compound, which interacts rather with the plasma membrane phospholipids than with proteins (Strahilevitz et al., 1994). Fig. 2B shows that dermaseptin K_4-S4 also reduced the transmission of HIV-1_{LAI} from infected CEM cells to P4-CCR5 cells. This result indicates that the peptide is able to inhibit cell-associated virus infection as well. However, we cannot exclude that in this experiment dermaseptin inhibited also free particles newly produced by infected T cells.

Dermaseptin K_4-S4 is active before or at viral entry

To determine which part of HIV-1 life cycle is inhibited, we introduced the peptide at different stages in single cycle infection experiments. Infections were performed on P4-CCR5 cells, with constant doses of both HIV-1_{LAI} (1 ng p24) and dermaseptin K_4-S4 (3.5 μM). Fig. 3A shows that dermaseptin K_4-S4 was active only when added before or present at the time of infection. When the peptide was added 1-h post-infection, no activity was observed. This suggests that dermaseptin K_4-S4 acts either directly on viral particles or at the virus-cell interface before or at the moment of attachment and/or entry. Pre-treatment of P4-CCR5 cells with the peptide, followed by washing prior to infection with HIV-1_{LAI}, had no effect on infectivity (Fig. 3A). In contrast, cell-free incubation of HIV-1_{LAI} with 3.5 μM dermaseptin K_4-S4 before infection resulted in a strong reduction of infectivity (Fig. 3B).

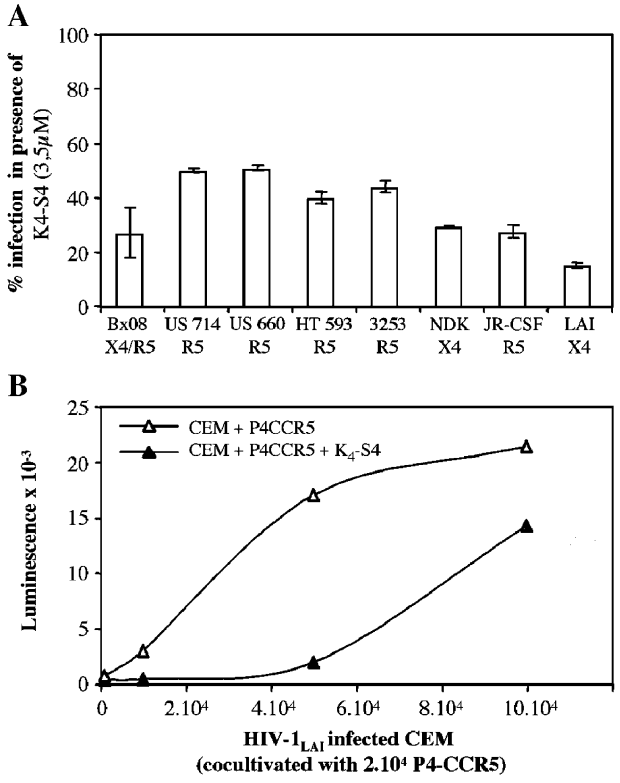

Fig. 2. Spectrum of anti-HIV activity of dermaseptin K_4-S4. (A) The percentage of infection of P4-CCR5 cells by HIV-1 primary isolates (Bx08, US714, US660, HT593, NDK, JR-CSF (clade B), 2553 (clade A)) or laboratory-adapted strain LAI in presence of 3.5 μM K_4-S4 is presented. The 100% of infection for each virus was determined in absence of K_4-S4. Cells were washed 24 h post-infection and β-galactosidase production was quantified 48 h post-infection (error bars indicate standard deviation). (B) CEM cells were infected with HIV-1_{LAI} and then co-cultivated with P4-CCR5 cells in absence or presence of K_4-S4 (3.5 μM). β-Galactosidase activity was measured in P4-CCR5 cells 48 h post-coculture.

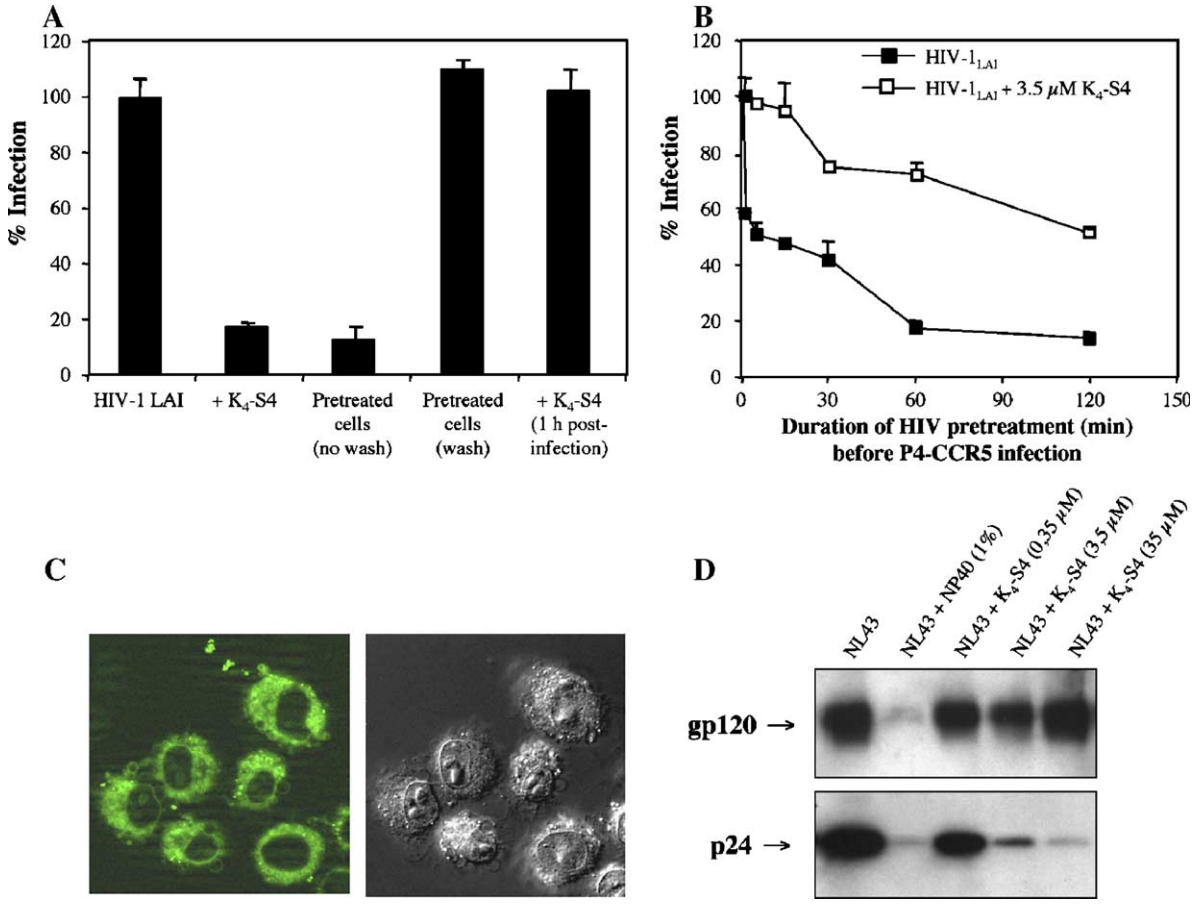

Fig. 3. Mechanism of anti-HIV activity of dermaseptin K_4-S4. (A) K_4-S4 (3.5 μM) was added at different time to P4-CCR5 cells infected with HIV-1_{LAI}. Cells were pre-incubated with the peptide 10 min at 37 °C, washed or not washed, and then submitted to 2-h infection. Alternatively, the peptide was added during viral entry or 1 h post-entry. Viral replication was monitored by measuring β-galactosidase in cells, 48 h post-infection. Results are means of two independent experiments performed in duplicate. (B) HIV-1_{LAI} was pre-incubated at 37 °C alone or in presence of K_4-S4 (3.5 μM) for different times and the remaining infectivity was evaluated on P4-CCR5. Cells were submitted to a 2-h infection by pre-treated virus and β-galactosidase was measured 48 h post-infection. Results are means of two independent experiments performed in duplicate (error bars indicate standard deviation). (C) P4-CCR5 cells were incubated for 10 min at 37 °C with fluoresceinated peptide (3.5 μM), washed, and observed under a confocal microscope (magnification x30). (D) Equal amounts of HIV-1_{NL43} (100 ng p24) treated with NP40 1% or K_4-S4 (0.35-35 μM) were ultra-centrifuged. Pelleted material was lysed and analyzed by Western blot using monoclonal anti-HIV gp120 and p24 antibodies.

Cellular localization of dermaseptin was examined by confocal microscopy using a fluoresceinated K_4-S4 peptide. P4-CCR5 cells treated with fluoresceinated K_4-S4 were labeled at their periphery and in the cytoplasm (Fig. 3C). It has been previously observed that DS4 was localized in erythrocytes cell membrane but not in cytoplasm (Feder et al., 2000). Here, we show that in nucleated cells the peptide is also localized in the cytoplasm. No difference was observed between 1 and 30 min of incubation, indicating that the uptake of the peptide takes less than 1 min. After washing, the peptide was not removed and cells remained labeled. When these cells were submitted to infection by HIV-1_{LAI}, no reduction of infection was observed (Fig. 3A). Taken together, these observations suggest that dermaseptin K_4-S4 affects primarily viral particles and inhibits HIV-1 infectivity only if the peptide in solution is able to bind directly to viral particles.

Dermaseptin K_4-S4 disrupts HIV-1 virion integrity

To examine the effect of K_4-S4 on the integrity of HIV-1 particles, HIV-1_{NL43} was treated with either K_4-S4 (0.35–35 μM) or 1%NP40 detergent, then concentrated by ultra-centrifugation. The composition of pelleted HIV-1 particles after treatment was analyzed by Western blot, using both anti-gp120 and anti-p24 antibodies. To be able to detect HIV-1 proteins by Western blot, we used high amounts of HIV-1_{NL43} (100 ng p24) in this experiment. Both HIV-1 gp120 envelope protein and the mature p24 Gag protein were removed from the HIV-1 particles after exposure to NP40 detergent (Fig. 3D). On the contrary, treatment with K_4-S4 removed only the p24 in a dose-dependent way. K_4-S4 concentrations higher than 3.5 μM depleted most of p24 Gag protein from particles, suggesting that the peptide destabilizes the HIV-1 core. HIV-1 envelope glycoprotein was still present in the pelleted material, even in presence of

high K_4-S4 concentration, suggesting that large viral membrane fragments were recovered by ultra-centrifugation (Fig. 3D). The activity of dermaseptins is mediated by interaction of their amphipathic N-terminal domain with membrane phospholipids (Kustanovich et al., 2002; Mor and Nicolas, 1994). Our observations suggest that K_4-S4 breaks the HIV-1 membrane and destabilizes the core without melting the lipid bilayer and subsequently reduces the attachment and/or entry of virus in target cells.

Dermaseptin K_4-S4 reduces HIV-1 capture and transcytosis by epithelial cells and HIV-1 capture and transfer by MO-DCs

The activity of dermaseptin K_4-S4 was then evaluated in HIV-1 capture and transfer experiments using epithelial endometrial cells or dendritic cells and primary T lymphocytes. Prior to perform these experiments, the cytotoxicity of dermaseptin K_4-S4 was evaluated on these cells. The viability of human epithelial HEC-1 cells, CD4 T cells (CEM), human PBL, and monocyte-derived dendritic cells (MO-DC) was evaluated and compared to that of P4-CCR5 cells after 3 h of exposure to increasing concentrations of K_4-S4 (Fig. 4). Interestingly, HEC-1 endometrial epithelial cells were less sensitive to K_4-S4 than the other cell lines tested. On the contrary, primary cells (PBL and MO-DC) were more susceptible than cell lines. For this reason, in all further experiments on primary cells, K_4-S4 was used only at low non-toxic doses (0.035–350 nM).

To assess the effect of dermaseptin K_4-S4 on HIV-1 attachment and transcytosis through a tight epithelial barrier, we used the human epithelial endometrial cell line HEC-1. HEC-1 cells were exposed to cell-free HIV-1_{NDK}, HIV-$1_{89.6}$, or HIV-$1_{JR\text{-}CSF}$ primary isolates in the presence of increasing non-toxic concentrations of dermaseptin K_4-S4. After elimination of unattached virus, HIV-1 attachment was evaluated by quantification of p24 antigen associated to the cells. Fig. 5A shows that 35 nM of dermaseptin K_4-S4 reduced about 50% of the amount of HIV-1 particles attached to HEC-1 cells. This inhibition was not dependent on the viral strain. The reduction of HIV-1 attachment to HEC-1 cells was probably the consequence of the destabilization of a part of HIV-1 particles by the peptide, and subsequent reduction the number of intact virions able to attach to cells. Given the mechanism of action of K_4-S4, this peptide is unlikely to inhibit specifically the interaction between HIV-1 gp120 and cell surface molecules. In this assay, dermaseptin K_4-S4 was active at a concentration 100-fold lower than the toxic concentration on the same cells, demonstrating that the peptide is still active at low doses.

To evaluate the capacity of K_4-S4 to inhibit HIV-1 transfer through a monostratified epithelium, we used a dual-chamber system. The apical chamber consisted of a confluent monolayer of HEC-1 cells, whereas the basal

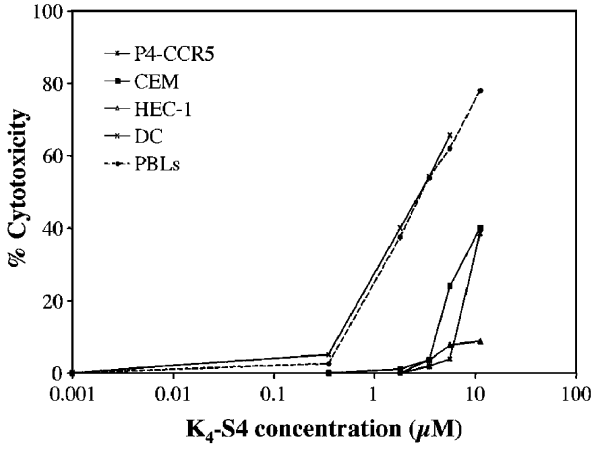

Fig. 4. Comparative cytotoxicity of K_4-S4 on different cells. Cytotoxicity of K_4-S4 was evaluated using the trypan blue exclusion method after 3 h exposure of cells to the peptide. Results are means of three independent experiments ± standard deviation.

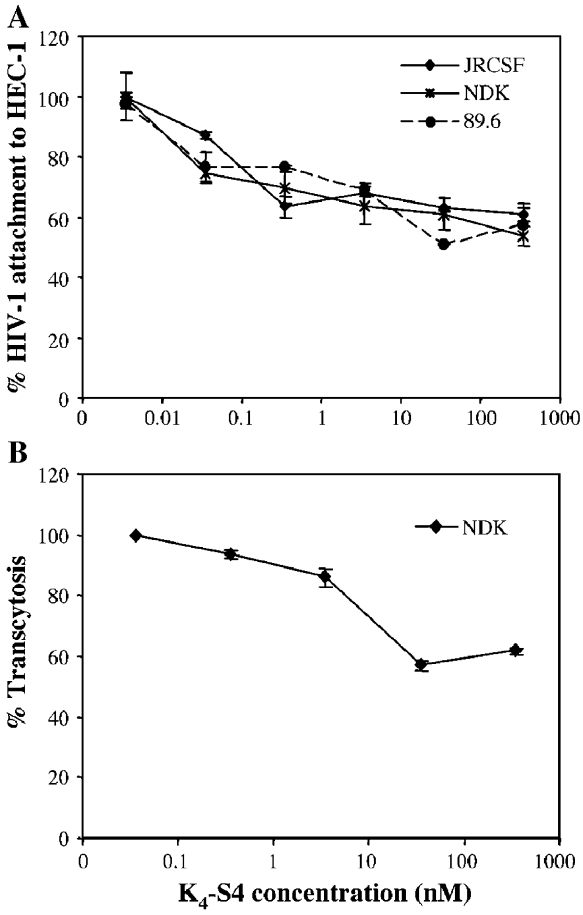

Fig. 5. Reduction of HIV-1 attachment to HEC-1 cells and transcytosis of cell-free HIV-1_{NDK} through a tight HEC-1 epithelial barrier. (A) HEC-1 cells were incubated with a dose range of K_4-S4 and 5 ng of virus for 1h30. Attached-virus was quantified by p24 capture ELISA. Results are shown as triplicate means ± standard deviation. (B) HIV-1_{NDK} (10 ng) was introduced with serial doses of K_4-S4 in the apical chamber of a transwell system for 24 h. Results of two independent experiments performed in duplicate are expressed as percentage of virus recovered in the basal chamber in the presence of K_4-S4 compared to the percentage of virus recovered in the absence of K_4-S4. Error bars represent standard deviations.

chamber contained medium. Cell-free HIV-1_{NDK} was added on the apical surface of HEC-1 cells together with increasing doses of dermaseptin K_4-S4. The transfer of HIV-1 through the artificial epithelium was assessed by quantification of p24 antigen present in the basal chamber after 24 h of incubation. Fig. 5B shows that transcytosis of cell-free HIV-1 was inhibited in a dose-dependent way correlating with the inhibition of attachment to the cells. To control that K_4-S4 did not alter the integrity of the epithelial barrier, the conductivity between apical and basal chambers was measured before and after peptide incubation. Conductivity remained constant (200 Ω/cm^2) during all the experiment.

Successful transfer of HIV-1 across epithelial barriers would result in virus capture by dendritic cells and subsequent transmission to $CD4^+$ T lymphocytes. Therefore, we investigated the effect of dermaseptin K_4-S4 on HIV-1 capture by monocyte-derived DCs (MO-DCs) and on HIV-1 transmission from MO-DCs to autologous lymphocytes. MO-DCs were incubated with cell-free HIV-$1_{JR\text{-}CSF}$ or HIV-1_{NDK} in the presence of increasing non-toxic doses of K_4-S4. After removal of unattached virus, the amount of MO-DC-attached virus was evaluated by quantification of cell-associated p24 antigen. Fig. 6A shows that K_4-S4 reduced the amount of HIV-1 particles captured by MO-DCs in a dose-dependent way (concentrations ranging from 3.5–35 nM induced 50–60% inhibition). The active concentration of K_4-S4 in this assay was 10- to 100-fold lower than toxic concentration on MO-DCs. Therefore, the peptide is active on primary cells at low non-toxic concentrations. As in the case of HEC-1 cells, K_4-S4 is unlikely to inhibit specifically the interaction between gp120 and DC-SIGN but rather destroys viral particles.

We subsequently investigated the effect of K_4-S4 on HIV-1 transmission from MO-DCs to autologous lymphocytes. MO-DCs were incubated with HIV-1_{NDK} or HIV-$1_{JR\text{-}CSF}$ in the presence of increasing non-toxic doses of K_4-S4. After removal of free viral particles, autologous lymphocytes were added to the infected MO-DCs and co-cultivated for 6 days. HIV-1 production was assessed in co-cultures supernatants. Fig. 6B shows that K_4-S4 reduced the trans-

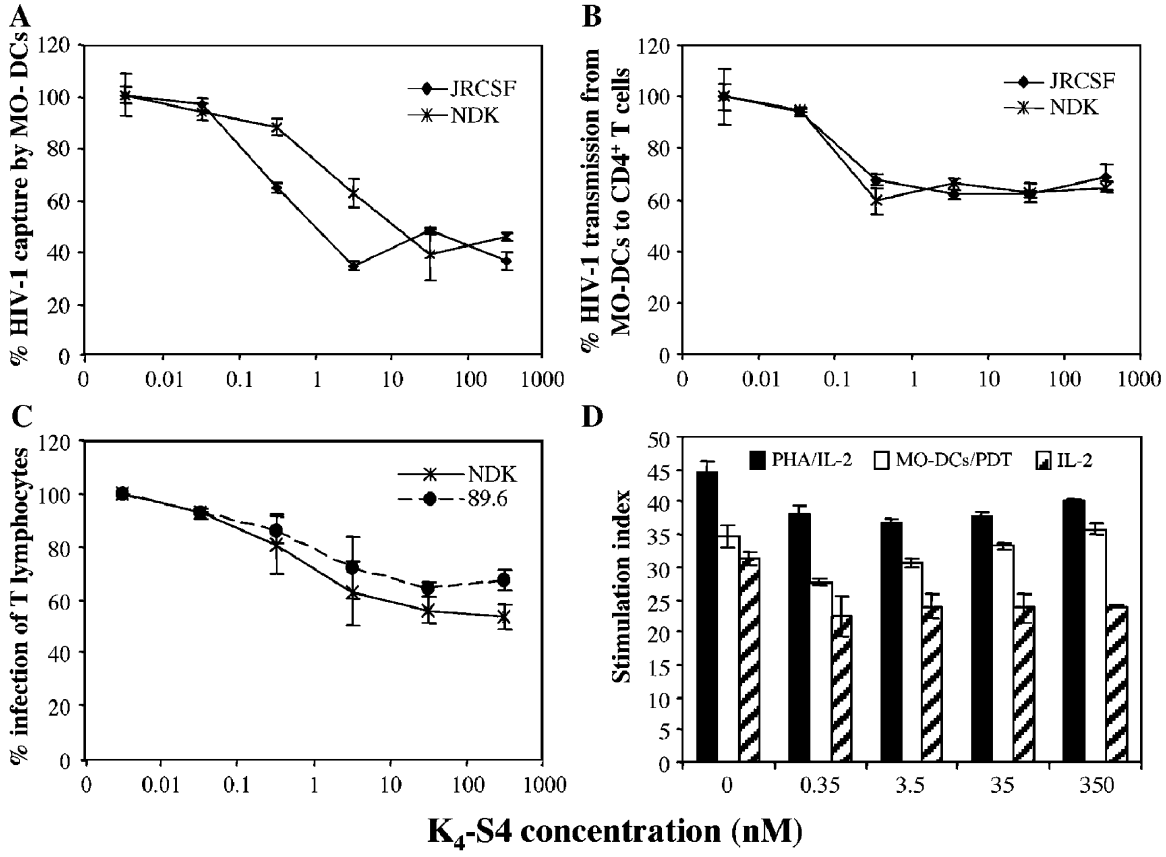

Fig. 6. Reduction of HIV-1 attachment to MO-DCs and MO-DC-mediated infection of autologous T cells. (A) MO-DCs were incubated with 20 ng/ml HIV-1 and increasing doses of K_4-S4 for 3 h at 37 °C. HIV-1 capture was measured in MO-DCs by p24 capture ELISA. (B) MO-DCs were incubated with 0.5 ng HIV-1 and a dose range of K_4-S4 for 3 h. Cells were washed and co-cultivated with autologous stimulated T cells (DC–T cell ratio 1:5). The viral production by T lymphocytes was evaluated on day 6 of co-culture by p24 capture ELISA in supernatants. The data are expressed as percentage of virus produced by T cells co cultivated with MO-DC/HIV-1/K_4-S4 compared to virus produced by T cells co cultivated with MO-DC/HIV-1. (C) Primary T lymphocytes were infected by HIV-1_{NDK} or HIV-$1_{89.6}$ in presence or absence of K_4-S4 and p24 was quantified on day 6. All results are presented as mean of triplicate ± standard deviation. (D) Proliferation capacity of T cells pre-treated with K_4-S4 (1 h 30, 0–350 nM). T cells were stimulated with IL2, PHA/IL2, or MO-DCs in presence of PDT (5 μg/ml) for 5 days. The results are expressed as stimulation index defined as mean cpm of stimulated samples/mean cpm of unstimulated samples (mean of 2 different donors ± standard deviation).

mission of HIV-1 infection from MO-DCs to autologous lymphocytes in a concentration-dependent way. This inhibition was not dependent on the viral strain. This inhibition is likely due to the reduction of HIV-1 particles captured by MO-DCs. Lastly, we investigated the effect of dermaseptin K_4-S4 on cell-free HIV-1 infection of PBLs. At the same concentration reducing HIV-1 attachment to epithelial cells and MO-DCs, K_4-S4 reduced similarly T lymphocytes infection by HIV-1 (Fig. 6C).

K_4-S4 could have an immunosuppressive effect on T lymphocytes or MO-DCs, which could not be detected by the trypan blue exclusion method. Thus, we evaluated the proliferative capacity of these cells in presence of increasing doses of K_4-S4 (0–350 nM). Fig. 6D shows that $CD3^+$ T lymphocytes proliferation after specific stimulation by autologous MO-DCs of after non-specific stimulation by PHA and/or IL-2 was not significantly affected by K_4-S4.

Discussion

These experiments show that dermaseptin K_4-S4 inhibits the infectivity of primary and laboratory-adapted strains of HIV-1 and CCR5- or CXCR4-mediated infection. This peptide reduces partly HIV-1 attachment to human endometrial cells (HEC-1) and HIV-1 transcytosis through a tight HEC-1 monolayer. Similarly, HIV-1 attachment to MO-DCs and subsequent MO-DC-mediated infection of autologous T lymphocytes are reduced. HIV-1 infection is inhibited when dermaseptin K_4-S4 is present during virus-cell contact, but no reduction is observed when it is added after, suggesting that the antimicrobial peptide targets HIV-1 before entry. These experiments also show that K_4-S4 exerts a selective activity on viral particles and disturbs their organization by breaking the viral membrane, leading to the exposure of HIV-1 core and its dissociation. This "virucide" activity, which is non-specific of any type of HIV-1 and independent of host cells, results in a rapid loss of infectivity of HIV-1 before virus-cell contact.

When used at high concentrations, this natural antimicrobial peptide is cytotoxic in vitro for host cells. However, modifications can be achieved on the native sequence to reduce its toxicity without altering activity. To increase the selectivity index of DS4 for HIV-1, we tested for deletions and substitutions, based on previous studies performed with *Escherichia coli* and human red blood cells (Feder et al., 2000). Our results show that a positive charge substitution leads to a lower cytotoxicity of the peptide without altering its anti-HIV-1 activity. The derived peptide K_4-S4 appears to be the best candidate in these assays. The selective activity of antimicrobial dermaseptins depends on the membrane lipid composition of the microbe versus the host cell and its electrical potential (Diaz-Achirica et al., 1998). For example, although the lipid compositions of host cell and *P. falciparum* membrane are similar, the potential of the parasite membrane is higher than that of the host cell membrane, leading to a discriminating effect of DS4 (Efron et al., 2002). We observed that primary cells were more sensitive to the toxic effect of K_4-S4 than cell lines. However, the peptide was able to reduce 50% of HIV-1 infection on primary $CD4^+$ T cells at a concentration (35 nM) 10- to 100-fold lower than the toxic concentration on these cells. The discrepancy that we observed between the active doses of K_4-S4 against HIV-1 infectivity on P4-CCR5 cells and primary $CD4^+$ T cells could be explained by the higher susceptibility of P4-CCR5 cells to HIV-1 infection.

For sexual transmission to occur, HIV-1 must first cross the mucosal epithelium (Shattock and Moore, 2003). We used an in vitro model mimicking the penetration of HIV-1 through unstratified epithelium by using a polarized monolayer of endometrial epithelial cells (HEC-1). We observed that 35 nM of dermaseptin K_4-S4 inhibit about 50% of both HIV-1 attachment on HEC-1 and transcytosis through the tight epithelial barrier. This K_4-S4 concentration was 100-fold lower than the toxic concentration on HEC-1 cells. Interestingly, human endometrial epithelial cells are less sensitive to the toxic effect of K_4-S4 than other cells. This is an important point because these cells would be the major cell population in contact with a topical anti-HIV-1 microbicide. After passage across the epithelium, HIV-1 particles are captured by migratory dendritic cells (mediated by DC-SIGN or another mannose C-type lectin receptor) or infect susceptible cells (DCs, T cells, macrophages) (Pope and Haase, 2003; Shattock and Moore, 2003). HIV-1-carrying DCs can disseminate infectious virions to draining lymph nodes, where massive infection of $CD4^+$ T cells takes place (Geijtenbeek et al., 2000; Masurier et al., 1998). Blocking HIV-1 uptake by DCs and subsequent transmission to $CD4^+$ T cells is crucial for the prevention of HIV-1 sexual transmission. We observed that dermaseptin K_4-S4 reduces MO-DC-mediated viral transmission to autologous T cells by reducing the number of HIV-1 virions able to attach to MO-DCs, at concentrations (3.5–35 nM) that are 10- to 100-fold lower than the toxic concentration on these primary cells. As a control for toxicity, we show that these active doses do not inhibit antigen-presentation by DCs and do not alter activation of T lymphocytes. However, these non-toxic concentrations of K_4-S4 prevent only 50% of HIV-1 capture by MO-DCs and subsequent infection of primary $CD4^+$ T cells. To reach 90–100% reduction, a further modified derivative of dermaseptin with lower toxicity for host cells could be used at higher concentrations. Low non-toxic doses of dermaseptin K_4-S4 might be also tested in combination with other anti-HIV entry inhibitors or further modified to reduce its toxicity.

The cytotoxicity for host primary cells of a non-specific anti-HIV-1 compound is an important issue. Indeed, the nonoxynol-9, a non-specific surfactant, which destroys HIV-1 particles in vitro (Polsky et al., 1988), caused lesions in the vaginal epithelium in vivo and increased the probability of being infected with HIV-1 (Van Damme et al., 2002). Our observations show that dermaseptin K_4-S4 does not behave as a detergent since viral membrane structures are preserved.

Dermaseptins act as ionophore and create transient pores in membranes, which may enable the passage of low molecular weight molecules leading to the membrane lysis (Shai, 1999). The in vivo toxicity of dermaseptin K_4-S4 has to be studied to determine if this peptide could disrupt the epithelial barrier at low doses. Macaque challenge experiments with other microbicides in development predict that doses 1000 times higher than that active in vitro are required to provide protection in vivo (Lederman et al., 2004). The mucosal physiological conditions (pH, mucus viscosity) will influence the stability, toxicity, and activity of biologically active peptides. The antibacterial activity of dermaseptin K_4-S4 has been shown to be maintained a low pH conditions, suggesting that the peptide could be active in genital tract (Yaron et al., 2003). Whether the in vivo active versus toxic doses of dermaseptin K_4-S4 will remain similar to that observed in vitro has to be determined. Although dermaseptin K_4S4 has an improved toxicity profile, further perfection is desirable to reach the safety of an acceptable microbicide. One of the derivative evaluated in this study, the K_4S4(1–16)a analog, inhibited 50% of HIV-1 infection on P4-CCR5 cells with a concentration of 28 μM. This analog was less active than K_4-S4 but did not cause cell toxicity with concentrations up to 100 μM. The K_4S4(1–16)a derivative has proved antibacterial activity in vivo with no toxicity associated in mice which received a systemic injection of 250 μg (11 mg/kg) (Navon-Venezia et al., 2002). Such a shorter derivative with lower activity but lower toxicity should be evaluated in vivo.

Sexually transmitted diseases (STDs) that cause ulcers and epithelial inflammation greatly increase the efficiency of HIV-1 transmission by increasing both the infectiousness of HIV-1 and the susceptibility to HIV-1 infection (Galvin and Cohen, 2004). Ideally, topical microbicides should be inexpensive, easy to use, and stable under low pH condition, non-irritating to genital mucosal tissues, and have a broad spectrum of activity against a variety of sexually transmitted microbes within genital secretions and against HIV-1. Dermaseptin S4 is active against a large spectrum of bacteria, parasites, HSV-1, and in our study, against HIV-1 at non-toxic doses in vitro. Its activity could be evaluated against other STDs (HSV-2, *Trichomonas vaginalis*, *Tepanema pallidum*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Candida albicans*, *Gardnerella*, *Mobiluncus*, *Mycoplasma*, *Ureaplasma*, etc). Other compounds are active against both HIV-1 and HSV-2 (Bourne et al., 1999; Gyotoku et al., 1999; Herold et al., 2002; Zacharopoulos and Phillips, 1997). These compounds might reduce HIV-1 infection both directly and indirectly by reducing other STDs.

A number of potential microbicides are under development (Golding et al., 2003; O'Hara and Olson, 2002; Shattock and Moore, 2003; Stone, 2002; Wang et al., 2003). Some of them have been suggested to interact directly with gp120 or gp41 of HIV-1, such as polianions (dextran sulfate, PRO-2000, carageenan), T-20, Cyanovirin, Retrocyclin, mAb b12, or mAb 2F5. Others target CD4 or co-receptors, such as anti-CD4 mAbs, anti-CCR5 mAbs, PSC-RANTES, AMD3100, or anti-CCR5 peptides. These specific blocking molecules would subject the virus to selective pressure and might lead to the emergence of resistant strains (Moore and Doms, 2003). Using in combination molecules that target several steps in HIV-1 transmission could prevent drug resistance (Stone, 2002). Dermaseptin K_4-S4 prevents HIV-1 infection by disrupting HIV-1 particles without specific interactions with HIV-1 proteins or receptors. Thus, resistance to this peptide should not occur. Therefore, dermaseptin K_4-S4 deserves to be further evaluated in animal models to test its toxicity in mucosal tissues and its ability to block HIV-1 sexual transmission alone or in combination with other potential compounds.

Methods

Synthesis of peptides

Peptides were synthesized by the solid- phase method the f-moc (9-fluorenylmethyloxycarbonyl) active ester chemistry on a fully automated, programmable Pioneer Peptide Synthesis System (Applied Biosystems). Preloaded PAC-PEG-PS-resin and PAL-PEG-PS-resin were used to obtain free carboxyl and amidated peptide, respectively. Side chain protections were *tert*-butylcarboxyl for lysine and tryptophan, o-*tert*-butyl ether threonine, and trityl for asparagine. Cleavage of the peptidyl resins and side chain deprotection were carried out in trifluoroacetic acid (TFA), triethylsilane (TIS), and water (95:2.5:2.5 v/v/v) mixture (10 ml/g of peptidyl resin) for 2 h at room temperature. After filtration of the resin, the peptide-TFA filtrate was precipitated in ice-cold diethyl ether, then dissolved in 0.08% TFA and lyophilized. The crude peptide was purified to chromatographic homogeneity by reverse-phase high performance liquid chromatography (HPLC). HPLC runs were performed on preparative C_{18} columns with a linear gradient of acetonitrile in water (1%/min); both solvents contained 0.08% TFA. To confirm their composition, the purified peptides were subjected to amino acid analysis and mass spectrometry. Peptides were stored as lyophilized powder at −20 °C. Prior to experimentation, fresh solutions were prepared in water and diluted in the appropriate medium. For fluorescein-labeled peptides, fluorescein was introduced at the N-terminus of the peptide using fluorescein *N*-hydroxysuccinimide ester prior to TFA treatment.

Cells lines and virus strains

P4-CCR5 indicator cells (Hela-CD4-CXCR4-CCR5-HIVLTR-LacZ) express the CD4, CXCR4, and CCR5 HIV-1 receptors and have been stably transfected with *lacZ* under transcriptional control of the HIV-1 long terminal repeat (LTR) (Charneau et al., 1994). P4-CCR5 and CEM cells were maintained in Dulbecco's modified Eagle's medium

(DMEM) supplemented with 10% fetal calf serum and 1 mg/ml G418. Epithelial cell line HEC-1 was maintained in RPMI 1640 containing 10% FCS and antibiotics.

Different HIV-1 isolates were used: the laboratory adapted strain X4-tropic LAI, NL43, clade B R5/X4-tropic primary isolates Bx08 (C. Moog, INSERM, Strasbourg, France) and 89.6 (F. Barré-Sinoussi, Institut Pasteur, Paris, France), clade B R5-tropic primary isolates 92US660, 92US714, 92HT593, JR-CSF (NIH-AIDS Research and Reference Reagent Program), clade B X4-tropic primary isolate NDK (F. Barré-Sinoussi, Institut Pasteur, Paris, France), and a clade A R5-tropic primary isolate 3253 (G. Pancino, Institut Pasteur, Paris). These viruses were propagated on PBL from healthy donors stimulated with phytohemagglutinin (PHA) and interleukin-2 (IL-2) and quantified in cell culture supernatants by HIV-p24 ELISA (HIV-1 core profile ELISA, DuPont de Nemours, France). Primary R5-tropic HIV-$1_{JR\text{-}CSF}$ was amplified in monocyte-derived macrophages from healthy donors.

T lymphocytes and MO-DCs preparation

Human dendritic cells (DCs) were generated from monocytes. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized buffy coats of healthy adult donors by Ficoll density gradient centrifugation (MSL, Eurobio, France). PBMC were resuspended in RPMI 1640 medium supplemented with glutamine, penicillin (100 IU/ml), and streptomycin (100 μg/ml). Cells were seeded into 24-well plates (1×10^6 adherent cells/ml) and incubated at 37 °C for 45 min. Non-adherent cells were removed by four washes. Adherent monocytes were incubated in RPMI medium with 10% fetal calf serum (FCS), glutamine, and antibiotics in the presence of 10 ng/ml rhIL-4 and 10 ng/ml rhGM-CSF. Half the medium was replaced every 2 days. After 6 days of culture, non-adherent cells corresponding to the DC-enriched fraction were harvested, washed, and used for subsequent experiments. Flow cytometry analysis using forward scatter and side scatter properties (Becton Dickinson) demonstrated that the DCs were more than 90% pure. T cells were prepared from the monocyte-depleted cell fraction. The negatively selected T cells were collected and more than 98% expressed CD3 by flow cytometry analysis. Peripheral blood lymphocytes (PBLs) were cultured for 48 h in fresh medium supplemented with PHA (2.5 μg/ml) and IL-2 (1 μg/ml). PBLs were then washed and cultured in growth medium containing IL-2 (1 μg/ml) for 24 h.

Antiviral activity assays on P4-CCR5 cells

P4-CCR5 cells were seeded in 96-well plates (20,000 cells per well) and incubated at 37 °C in DMEM, 10% FCS for 24 h. Infections were performed by replacing the medium by 50 μl DMEM, 10% FCS, DEAE dextran (10 μg/ml) containing HIV-1 virus (1 ng p24) and increasing DS4 concentrations. Cells were infected for 2 h at 37 °C with HIV-1_{LAI} and 24 h with primary isolates. After washing to remove the unattached virus and the peptide, cells were incubated at 37 °C. After 2 days, the β-galactosidase activity was measured using a Chemiluminescence Reporter Gene Assay (Roche, USA). To examine whether K_4-S4 interacts primarily with HIV-1 or cells, the peptide (3.5 μM) was pre-incubated at 37 °C with cell-free HIV-1_{LAI}. The remaining viral infectivity was then assessed over time on P4-CCR5 cells. Alternatively, DS4 (or DMEM 10% FCS as control) was pre-incubated with cells for 1 h at 37 °C and cells were washed prior to infection with HIV-1_{LAI}.

Cytotoxicity assays

The cytotoxicity of dermaseptin was evaluated on P4-CCR5, HEC-1, PBLs, and monocytes-derived dendritic cells (MO-DCs) by quantifying cell viability using the trypan blue-exclusion method following 3–24 h exposure to increasing concentrations of DS4 or K_4-S4 derivative. More sensitive assays were also used to evaluate K_4-S4 cell toxicity: the immune functions of MO-DCs and T lymphocytes were evaluated using proliferation assays after treatment with K_4-S4. Bulk $CD3^+$ T cells from healthy donors were incubated for 1 h 30 with K_4-S4 increasing concentrations in 96-well plates (10^5 cells per well, in RPMI 1640, 10% FCS). Cells were then stimulated with PHA, IL-2, or MO-DCs in presence of purified derivative of tuberculin (PDT) (5 μg/ml) for 5 days in 5% CO2 at 37 °C. All assays were performed in triplicate. Cells were pulsed with 0.5 μCi/well (Saphire et al., 2001) thymidine 18 h before the end of incubation then harvested using an automatic collector (EG and G Wallc, Turku, Finland). Isotope incorporation was measured with a Microbeta Liquid Scintillation Counter (EG and G Wallc, Turku, Finland) and stimulation index was determined as the ratio between mean cpm in stimulated samples and mean cpm in control samples.

Cellular localization of fluorescent peptide by microscopy

P4-CCR5 cells were incubated in presence of the fluoresceinated peptide K_4-S4 at 1–10 μM. After 1–30 min incubation, cells were washed and analyzed (non-fixed cells). Microscope images were taken using a Zeiss axioplan 2 imaging microscope with Apotom system.

HIV-1 virion isolation and immunoblotting

HIV-1_{NL43} (100 ng p24) was incubated alone or in presence of increasing concentrations of K_4-S4 (0.35–35 μM) at 37 °C for10 min. The same amount of virus was incubated with NP40 detergent (1%) at 4 °C for 30 min. Samples were then ultra-centrifuged at 30,000 rpm through 5-ml PBS at 4 °C for 1 h 30. Pelleted material was resuspended in 50 μl of 150 mM NaCl, 50 mM Tris, pH 8, 1% NP40, 0.5 mM PMSF, and 0.2 mg/ml Pefabloc (Interbiotech, France). Proteins were denatured by treatment

with urea and β-mercaptoethanol for 5 min at 65 °C, and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (4–12% NuPage, Invitrogen). After transfer to cellulose membrane (Amersham Pharmacia Biotech), blots were probed with a mouse monoclonal anti-HIV p24 antibody (23A5G4) or with mix of mouse monoclonal anti-HIV gp160 and gp120 antibodies (110H, 160A, O. Schwartz, Institut Pasteur, France). A goat anti-mouse IgG horseradish peroxidase (HRP) conjugate (Amersham) was used as a secondary antibody. Peroxidase activity was visualized with an enhanced chemiluminescence detection Kit (ECL+, Pierce).

HIV-1 attachment on epithelial cells and transcytosis

Human epithelial endometrial cells (HEC-1 cell line) were incubated in 48-well plates with HIV-1 (5 ng p24) and increasing doses of dermaseptin K_4-S4. Each sample was performed in triplicate. After 1 h 30 min of incubation, unattached virus was removed (4 washes) and cells were lysed (1% Triton X-100 for 45 min at 37 °C). Cell lysates were harvested and centrifuged at 1800 rpm for 5 min. The amount of HIV-1 p24 associated to cell lysates was evaluated using HIV-p24 ELISA (HIV-1 core profile ELISA, DuPont de Nemours, France). To analyze HIV-1-free particles transcytosis through an epithelial monostratified barrier, HEC-1 cells were grown as a tight polarized monolayer on a permeable polycarbonate support (0.4-μm pore-diameter, Transwell, Costar, Cambridge, MA) as previously described (Bomsel et al., 1998; Hocini et al., 2001). The tightness of the monolayer of HEC-1 cells was monitored by controlling that resistivity remained above 200 Ω/cm^2 in presence of K_4-S4. HIV-1 (10 ng p24) was added on the apical pole of the HEC-1 monolayer together with increasing doses of dermaseptin K_4-S4 and cells were incubated for 24 h at 37 °C. HIV-1 transcytosis was assessed by detecting the presence of p24 antigen in the basolateral chamber of the transwell. The inhibition of transcytosis was expressed as the percentage of p24 antigen recovered in the basolateral chamber in the presence of dermaseptin K_4-S4 by comparison to that recovered without peptide.

HIV-1 attachment on human dendritic cells and DC-mediated infection of autologous T cells

After 6 days of differentiation, human DCs were washed three times and seeded into 96-well culture plates (5×10^5 cells/well). HIV-1 (20 ng p24) together with increasing doses of dermaseptin K_4-S4 were added on cells and incubated for 3 h at 37 °C. Each sample was performed in triplicate. After four washes to remove the unattached virus, cells were lysed by incubation for 45 min at 37 °C with 1% Triton X-100. Cell lysates were harvested and centrifuged at 1800 rpm for 5 min. The amount of cell-associated HIV-1 was evaluated using HIV-1 p24 ELISA (HIV-1 core profile ELISA, DuPont de Nemours, France). To assess the transmission of HIV-1 from dendritic cells to autologous T cells, DCs were incubated into 96-well culture plates (5×10^5 cells/well) and infected with HIV-1 (0.5 ng p24) in the presence of increasing doses of dermaseptin K_4-S4 for 3 h at 37 °C in a 5% CO_2 atmosphere. Cells were washed four times and autologous stimulated T cells were added onto infected DCs at a DC–T-cell ratio of 1:5. Each sample was performed in triplicate. Culture supernatants were harvested every 3 days and fresh medium was added. Supernatants were inactivated with 1% Triton X-100 and frozen at −20 °C. The viral production by T lymphocytes was evaluated after 6 days of culture by measurement of p24 in supernatants using capture ELISA.

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