Cyanovirin-N Inhibits AIDS Virus Infections in Vaginal Transmission Models

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ABSTRACT

The cyanobacterial protein cyanovirin-N (CV-N) potently inactivates diverse strains of HIV-1 and other lentiviruses due to irreversible binding of CV-N to the viral envelope glycoprotein gp120. In this study, we show that recombinant CV-N effectively blocks HIV-1Ba-L infection of human ectocervical explants. Furthermore, we demonstrate the in vivo efficacy of CV-N gel in a vaginal challenge model by exposing CV-N-treated female macaques (Macaca fascicularis) to a pathogenic chimeric SIV/HIV-1 virus, SHIV89.6P. All of the placebo-treated and untreated control macaques (8 of 8) became infected. In contrast, 15 of 18 CV-N-treated macaques showed no evidence of SHIV infection. Further, CV-N produced no cytotoxic or clinical adverse effects in either the in vitro or in vivo model systems. Together these studies suggest that CV-N is a good candidate for testing in humans as an anti-HIV topical microbicide.

INTRODUCTION

HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) is transmitted primarily by vaginal and rectal sexual intercourse. Although HIV initially was reported to be most prevalent among homosexual men, it is now clear that unprotected heterosexual intercourse is responsible for the majority of HIV infections worldwide.1,2 Young women (ages 15–24 years old) in the hardest-hit countries are now up to three times more likely to be infected than males of the same age. Although a prophylactic vaccine would provide an ideal strategy to prevent sexual transmission of HIV, no clinically effective anti-HIV vaccine is likely to be globally available for some time. While consistent and reliable condom use is effective in preventing sexual transmission of HIV,2–4 this largely male-controlled method is not widely accepted among many at-risk groups.4 Thus, additional preventive strategies are needed to combat this growing epidemic. An effective vaginal microbicide is one strategy that could significantly reduce the spread of HIV infection.

HIV infection of host cells is a stepwise process beginning with binding of the viral envelope glycoproteins to both the CD4 receptor and one or more chemokine receptors and ending with fusion of viral and cell membranes.5–7 In theory, a compound that could block one or more of these initial steps of HIV infection might serve as an effective topical microbicide to block mucosal HIV transmission. One such recently discovered compound that irreversibly inhibits HIV entry into host cells is cyanovirin-N (CV-N).8–11 The potent virucidal activity of CV-N occurs through a novel molecular mechanism involving multivalent interactions of CV-N with high mannose oligosaccharides comprising the HIV envelope glycoproteins.10,11 Thus, its antiviral properties8–11 make CV-N an ideal candidate for development as a topical microbicide to prevent sexual HIV transmission.

In this paper, we describe in vitro and in vivo efficacy studies of CV-N in vaginal transmission models. First, we explored the in vitro effect of CV-N on HIV infection of human ectocervical explants.12 Then we evaluated the efficacy of a gel-formulated CV-N as a topical vaginal microbicide against a chi-

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meric simian/HIV-1 virus, SHIV89.6P, in a macaque model. We found CV-N at doses tested showed no adverse effects and a highly effective infection-blocking agent in both model systems. In a companion paper we describe similar results of CV-N gel as a topical microbicide for the prevention of rectal transmission of SHIV89.6P.

METHODS AND MATERIALS

In vitro model: evaluation of CV-N efficacy against HIV-1 Ba-L infection of human ectocervical explants

The protocols for explant cultures and efficacy assays were described previously. Briefly, ectocervical tissues were obtained from premenopausal women undergoing planned therapeutic hysterectomies at St. George’s Hospital, London. The CCR5-using HIV-1 Ba-L was used as a challenge virus in this study. Explants were treated with CV-N (0.02–1.0 μM) before (-5 or -60 min), simultaneously (0 min), or after (+60 min) exposure to HIV-1 Ba-L. For each viral exposure, explants were immersed in 1 ml cell-free virus (10⁵ TCID₅₀) for 2 hr at 37°C in the presence or absence of CV-N. For pretreated virus (PV), HIV-1 Ba-L was first incubated with CV-N for 1 hr at 37°C before being separated from CV-N by ultracentrifugation and then used to inoculate explants. For pretreated biopsy (PB), the explants were preincubated with CV-N for 1 hr at 37°C, washed to remove CV-N, and then inoculated with HIV-1 Ba-L. After incubation with virus, explants were washed five times in phosphate-buffered saline (PBS) and cultured in complete RPMI for 14 days. Infections of all explant cultures were monitored by p24 antigen enzyme-linked immunosorbent assay (ELISA). All samples showing greater than 95% inhibition by p24 ELISA were confirmed virus negative by HIV-1 DNA polymerase chain reaction (PCR) as described previously. The data (Fig. 1) represent the percent inhibition of p24 production at day 14 of culture, compared with control cultures... (PB), the explants were preincubated with CV-N for 1 hr at 37°C, washed to remove CV-N, and then inoculated with HIV-1 Ba-L. After incubation with virus, explants were washed five times in phosphate-buffered saline (PBS) and cultured in complete RPMI for 14 days. Infections of all explant cultures were monitored by p24 antigen enzyme-linked immunosorbent assay (ELISA). All samples showing greater than 95% inhibition by p24 ELISA were confirmed virus negative by HIV-1 DNA polymerase chain reaction (PCR) as described previously. The data (Fig. 1) represent the percent inhibition of p24 production at day 14 of culture, compared with control cultures exposed to virus in the absence of CV-N. Each data point is the mean of three independent experiments using explants from separate donors. The data of CV-N efficacy showed that infection of HIV-1 was completely prevented or inhibited during the timeframe of the assay.

In vivo efficacy studies of CV-N gels in SHIV-macaque model

Experimental design. Twenty-nine naive adult female cynomolgus macaques (Macaca fascicularis), virologically and serologically negative for type D retrovirus, SIV, and simian T-lymphotropic virus (STLV), were selected for this preclinical evaluation. Peripheral blood mononuclear cells (PBMC) from all the macaques demonstrated susceptibility to SHIV89.6P replication in vitro. Three of the macaques were used for testing the safety of CV-N gel in the vagina and 26 were used for preclinical evaluation of the efficacy of CV-N. For the safety evaluation, three macaques were given a single vaginal application of 2% CV-N gel into the cervicovaginal area. Other significant data about the safety of repeated applications of placebo and CV-N gels in the more sensitive anal and rectal areas are presented in our companion paper.

SHIV89.6P virus inoculum. The original SHIV89.6P was obtained from Dr. K. A. Reimann (Beth Israel Hospital, Boston, MA). For preparation of our virus stock, the original SHIV89.6P was propagated in phytohemagglutinin A-stimulated PBMC from naive pigtailed macaques. The infectious titer of this stock was approximately 10,000 TCID₅₀/ml in MT-2 cells. Virus stock was aliquoted into vials and frozen in liquid nitrogen until use. The intravaginal macaque infectious dose (1 MID₅₀) was determined to be 10 TCID₅₀. A dose of 100 TCID₅₀ (10 MID₅₀) resulted in a persistent 100% infection in methoxyprogesterone-pretreated macaques (C.-C. Tsai, unpublished data). Each macaque was inoculated intravaginally with 0.5 ml of inoculum containing 5000 TCID₅₀ (or 500 MID₅₀).
Cyanovirin-N (CV-N) gel as a topical microbicide. Purified recombinant CV-N (rCV-N) produced in Escherichia coli was used to prepare 0.5, 1, and 2% CV-N in aqueous gel with hydroxyethyl cellulose. The pH of the CV-N gel and placebo gel was adjusted to 5.8 with sodium hydroxide and/or hydrochloric acid. The placebo gel was CV-N free.

For gel application, 1 ml of the formulated CV-N gel or placebo gel was placed in a 3-ml syringe connected to a 14-gauge Teflon indwelling catheter. The catheter was inserted atranurally into the vagina of sedated macaques and the gel was delivered to the cervicovaginal area. The macaques were maintained in the knee-chest position for 20 min after gel application, and then were inoculated intravaginally with 0.5 ml virus (5000 TCID\textsubscript{50}). Virus control macaques were inoculated with virus only. To promote virus exposure to the mucosa, all macaques were kept in the same knee-chest position for 15 min after virus inoculation.

**Clinical observations and assessments.** All macaques were inspected for any signs of acute toxicity shortly after application of gels and virus inoculation. These signs included any expulsion or excretion, edema, erythema, or bleeding in the vagina. All macaques were inspected daily for general health condition. At predetermined time points, all macaques underwent a general physical examination. Other clinical assessments included periodic blood sampling for complete blood counts (CBC), serum biochemistry tests, and lymphocyte subset analysis. Blood samples for virological, immunological, and hematological evaluations were collected weekly during the first month postinoculation (PI), then every 2 weeks for 2 months, monthly for the next 2 months, and periodically for an additional 60 weeks (particularly CV-N gel-protected macaques).

**Laboratory measurements for virus detection.** Laboratory measurements included virus isolation, proviral DNA PCR, virus RNA PCR, and SHIV antibodies. Ethylenediaminetetraacetic acid (EDTA) blood samples obtained from the femoral veins were processed according to conventional methods. PBMC were separated from theuffy coat by centrifugation through lymphocyte separation media. Plasma was aliquoted and used for assays of SIV p27 antigenemia, SIV RNA PCR, and anti-SHIV antibodies. Peripheral lymph nodes from the macaques were collected for virus isolation and proviral DNA PCR.

**Virus isolation.** Virus levels in PBMC and lymph node mononuclear cells (LNMC) were estimated by quantitative co-cultures with MT-2 cells in 48-well tissue culture plates. The cell cultures and virus isolation were performed with the use of conventional methods. Except for CD4 T cell depletion induced by SHIV89.6P in later infection (8–10 weeks PI), CD8 cell-depleted and CD4-enriched PBMCs were used to coculture with MT-2 cells. Infection was determined on the basis of cytopathic syncytium effects and SIV p27 antigen production, measured by p27 ELISA (ZeptoMetrix Corp., Buffalo, NY).

**PCR for proviral DNA.** Nested PCR was carried out on genomic DNA from PBMC and LNMC with the use of SIV mac251-specific gag primers and conditions as described previously.\textsuperscript{19,20} The genomic DNA sample was amplified in a reaction mixture including Taq polymerase and AmpliTaq buffer (Perkin-Elmer Cetus, Norwalk, CT). After amplification, PCR products were analyzed by electrophoresis through a 1.5% agarose gel and visualized with ethidium bromide staining. The lower limit of detection of the viral DNA assay is 10 viral copies.

**SHIV RNA levels in plasma.** The real-time TaqMan PCR assay\textsuperscript{21} for SIV RNA was used to quantitatively measure SHIV89.6P RNA in macaque plasma. The gag gene of SIV-mac251 was used for the primers and the probe in the real-time TaqMan PCR (TaqMan, Applied Biosystems, Foster City, CA) was performed at Lucy Whittier Molecular Core Facility, UC-D, Davis, California as previously described.\textsuperscript{21} The lower limit of detection of the viral RNA is 30 copies/ml plasma.

**SHIV antibody assay.** Antibodies to SHIV89.6P were assessed and quantified with a commercial HIV-2 EIA kit (Sanofi-
Pasteur, Redmond, WA) as described previously. Plasma samples obtained at all time points were tested for the presence of SHIV antibodies according to the manufacturer’s protocol.

Lymphocyte subset analysis. Lymphocyte subsets were determined by using EDTA blood samples with mouse antihuman monoclonal antibodies that reacted with macaque lymphocytes. Specific CD4 cell numbers (Fig. 2) and CD4:CD8 cell ratio (data not shown) were calculated as described previously.

Plasma antigenemia. SHIV antibody-negative plasma samples taken during the first few weeks after challenge were analyzed for cell-free virus using a commercial SIVp27 antigen capture ELISA kit (ZeptoMetrix Corp., Buffalo, NY).

Virus detection in peripheral lymph nodes. Tissue samples of peripheral lymph nodes biopsied from CV-N gel-protected macaques, from alive control macaques at ≥10 months PI, and from control macaques at necropsy were used for virus isolation and PCR viral DNA. The cell suspensions were prepared from the biopsied lymphoid tissues and then used for virus isolation and PCR viral DNA with methods similar to those described above.

Safety evaluation. Three healthy adult female cynomolgus macaques were given a single 1-ml application of 2% CV-N gel, which was delivered to the cervicovaginal area as described for the preclinical evaluation study. The macaques were maintained in the knee-chest position for 20 min after gel delivery and then rested in a housing cage, where they were carefully inspected for any signs of acute irritation immediately after gel application. This immediate inspection was followed by clinical observations for any adverse effects at 3 and 12 hr. Additional safety evaluations of 1% CV-N gel and placebo gel are described in our companion paper. Briefly, four macaques received seven daily doses (2 ml) of placebo gel and two macaques received four daily doses (2 ml) of 1% CV-N gel by intrarectal (IR) application. Their clinical health status and any signs of adverse effects were carefully inspected immediately after each daily IR application. About 14–16 hr after the final applications, the macaques were euthanized and examined immediately for any gross lesions. Tissue specimens from the anus and rectum were examined microscopically.

RESULTS

CV-N inhibits CCR5-using HIV-1Ba-L infection of human ectocervical explants

The CV-N inhibition of HIV-1Ba-L exposed explants was monitored by p24 ELISA. Virus (p24)-negative samples were confirmed DNA PCR as described previously (data not shown). The capacity of CV-N to inhibit the virus was dependent on both dose (0.2–1.0 μM) and time of treatment (~60 to +60 min relative to viral exposure). CV-N treatment of explant cells before virus exposure was more efficacious than CV-N treatment after virus exposure. Furthermore, pretreatment of virus with CV-N was sufficient to render the virus noninfectious. Thus, CV-N proved a potent inhibitor of virus entry and appeared to be a suitable anti-HIV agent for preclinical evaluation and development as a topical microbicide for preventing vaginal HIV transmission.

Effect of CV-N gel on vaginal transmission of SHIV89.6P in macaques

Preclinical evaluation of CV-N gels to prevent vaginal transmission of SHIV89.6P was conducted in 26 female cynomolgus macaques that were pretreated with a single intramuscular dose of methoxyprogesterone to synchronize the physiological condition of their lower genital tracts. The efficacy of CV-N gels was determined by laboratory measurements and clinical assessments. Following viral challenge, five of the six macaques in each of the three CV-N gel (0.5, 1, and 2% of CV-N) groups resisted virus challenge and remained uninfected as determined by virus isolation and viral DNA PCR (Table 1). In contrast, all virus control and gel control macaques became infected. Moreover, none of the uninfected macaques in the three CV-N gel groups developed virus-specific antibodies (detailed data are not shown but results are summarized in Table 2). The three CV-N gel-treated macaques that became infected, as well as most of the virus control and placebo gel macaques, developed virus-specific antibodies by 1 month after challenge and

![FIG. 2](image_url)

FIG. 2. CD4⁺ T cell counts in peripheral blood of macaques after vaginal inoculation with SHIV89.6P. Five of six macaques in each of the three CV-N gel groups were protected from infection and showed normal variable ranges of CD4⁺ T cell populations (A–C). Two of the three infected macaques in CV-N gel groups had a moderate CD4⁺ cell depletion and the third one (B) showed a severe CD4⁺ cell depletion. All placebo gel control and virus control macaques showed a typical, virus-induced CD4⁺ cell depletion (D–E) except for macaque 01014 (E), which had high CD4⁺ cell counts.
remained seropositive for the duration of study (data not shown). One infected macaque in the placebo gel group failed to seroconvert and was euthanized after 4 weeks due to rapid onset of SHIV-induced AIDS-like disease.

CD4^+ T cell levels in CV-N gel-protected macaques remained within normal ranges (Fig. 2A–C). Of the three gel-treated macaques that became infected, one, macaque A01044 (Fig. 2A), had a normal level of CD4^+ T cells while two others, macaques A01033 (Fig. 2B) and A01032 (Fig. 2C), showed a typical pattern of CD4^+ cell depletion. All CV-N gel-protected macaques maintained normal CD4^+:CD8^+ cell ratios (data not shown), but the three CV-N gel-treated and infected macaques displayed low CD4^+:CD8^+ cell ratios. All but one of the placebo gel and virus control macaques showed rapid CD4^+ cell depletion typical of SHIV infections (Fig. 2D and E), resulting in low CD4^+:CD8^+ cell ratios (data not shown). Macaque 01014 (Fig. 2E) had high levels of both CD4^+ and CD8^+ cells but maintained normal CD4^+:CD8^+ cell ratios (data not shown). Additionally, five of the six macaques in each of the three CV-N gel groups showed no viral RNA in plasma, whereas the single infected macaque in each CV-N gel group became chronically viremic (Fig. 3A–C). All placebo gel and virus control macaques had high plasma virus loads during their acute infections, developing either long-lasting or recurrent plasma virus loads with time (Fig. 3D and E).

**Virus detection in peripheral lymph nodes**

To determine whether the virus had localized in lymphoid tissues, we examined peripheral lymph nodes biopsied from CV-N-treated and control macaques after 10 months, and from the other control macaques at necropsy, for virus isolation and viral DNA PCR. All lymph nodes from the five protected macaques in each of the three CV-N gel groups were negative for virus infection. In contrast, lymph nodes from all control macaques and the three CV-N gel-treated but infected macaques were positive for virus (data not shown).

**Safety evaluation of the topical application of CV-N and placebo gels in macaques**

The three macaques that received a single application of 2% CV-N gel in the cervicovaginal area showed no adverse effects for up to 12 h after gel application. Additional significant safety data were evaluated in the most sensitive rectum as described in our companion paper. Briefly, four macaques that received seven daily applications of placebo gel in the rectum showed

![FIG. 3. Plasma viral RNA levels in macaques treated with CV-N gels or placebo gel after vaginal exposure to SHIV89.6P. Five of six macaques in each of the three CV-N gel groups were protected from infection and showed no viral RNA in plasma as measured by the SIV TaqMan PCR assay (A–C); one of the six macaques in each gel group was infected and showed a long-lasting virus load in plasma. Three macaques in each placebo gel control and virus control group had high virus loads at acute infection that at setpoint became recurrent low-level viremias (D–E); one macaque in each of the two control groups had a long-lasting high virus load. One macaque in placebo gel control was euthanized due to severe virus-induced disease.](image-url)
no abnormal effects or clinical adverse events. The two macaques that received four daily rectal applications of 1% gel had no irritation or demonstrable histopathological lesions in the rectum.

Conclusions of CV-N gel as topical microbicide against vaginal transmission of SHIV89.6P in macaques

The overall effectiveness of CV-N gels against vaginal transmission of SHIV89.6P in macaques is summarized in Table 2. All of the control macaques expressed detectable virus within 2 weeks postchallenge and showed high plasma viremia, cell-associated viremia, and CD4+ T cell depletion within 4 weeks of challenge. In contrast, five of the six macaques in each CV-N gel group showed no evidence of SHIV89.6P infection, although one macaque in each CV-N gel group did become infected. Peripheral lymph nodes biopsied from protected macaques after 10 months showed no evidence of virus infection. These results indicate that 0.5, 1, and 2% CV-N gels are effective in blocking vaginal transmission of cell-free SHIV89.6P in macaques. Importantly, in the follow-up study, all CV-N gel-protected macaques remained virus free 15 months after virus exposure.

DISCUSSION

Macaques of Asian origin are useful models for studies of vaginal transmission of HIV because they are very similar to women in terms of the gross and histological anatomy of the vagina and ectocervix. These features make Asian-origin macaques a particularly valuable resource for evaluating the efficacy and safety of candidate topical microbicides.

In this study, 15 of 18 CV-N gel-treated macaques were protected against vaginal SHIV challenge. Of these 15 uninfected CV-N-treated macaques, all needed only a single intravaginal insertion of a Teflon catheter for delivery of CV-N gel or virus. Curiously, the remaining three CV-N-treated macaques that became infected were from each of the three drug concentration groups. While we cannot rule out mere coincidence, we did observe during CV-N gel delivery that these macaques required multiple attempts to vaginally insert the drug-delivery catheter. The multiple attempts were due to resistance encountered during the insertion. This resistance slowly abated with the delivery of small amounts of the gel followed by renewed insertion attempts. The gel may have acted as a lubricant eventually allowing penetration to the cervicovaginal junction where the bulk of the gel was deposited. There was no visible evidence of trauma and no difficulties delivering the virus challenge to any of the macaques including these three. We were very careful to avoid trauma but cannot rule out that multiple insertion attempts of the Teflon catheters might have abraded the methoxyprogesterone-thinned mucosa thereby exposing the lamina propria and submucosa and facilitating virus entry.

It is important to note that all the macaques in this study were rendered more susceptible to SHIV challenge by treatment with progesterone prior to the study. Methoxyprogesterone treatment has been associated with an increase in the susceptibility to SIV infection and a marked thinning of the vaginal epithelium. We have found a similar enhanced susceptibility to vaginal SHIV89.6P infection in progesterone-pretreated macaques. This stock of SHIV89.6P requires a dose of 10 TCID50 to infect 50% of methoxyprogesterone-pretreated macaques. The challenge with 5000 TCID50 (500 MID50) used in this study was a very large dose of virus exposure for methoxyprogesterone-treated macaques. Just 1 ml of each gel containing 0.5% CV-N was sufficient to protect five of six drug-treated macaques while all of the placebo gel and untreated controls became infected following challenge with a high dose of a very pathogenic virus. We did not evaluate the efficacy of CV-N against cell-associated challenge. While the role of cell-associated transmission remains an area of debate, it is encouraging in this regard that CV-N is effective against cell-to-cell spread of the virus in vitro.

In the present study, our choice of challenge virus was based on experience with our SHIV89.6P stock, which proved to be highly infectious by several routes and in several macaque species. In the case of methoxyprogesterone-treated macaques, we found the vaginal application of this stock to be a most stringent vaginal challenge. We are aware of a controversy regarding the appropriateness of this SHIV89.6P as a challenge virus in nonhuman primate models. Clearly, the use of our SHIV89.6P stock to challenge CV-N-treated macaques in this study was appropriate as indicated by the rapid infection outcome in all of our placebo gel and virus control macaques. However, our in vitro results indicate that CV-N potently blocks virus entry of both SHIV89.6P and SHIV162P3 in macaque PBMC cultures and targeted cell lines. Thus, as noted in our companion paper, we would predict similar results from macaque experiments using SHIV162P3 as the challenge virus.

For in vivo studies, we used 0.5, 1, and 2% concentrations of CV-N, which corresponded to protein solutions of 5, 10, and 20 mg/ml. Theoretically, the antiviral efficacy of 0.5, 1, and 2% CV-N gels is, respectively, about 5000, 11,000, and 22,000 times that of in vitro ED99 doses for SHIV89.6P. The rationale for the use of such high concentrations of CV-N in macaque studies has been described in our companion article, although the rectum and vagina have different complex environments. Under normal circumstances, there is a combination of physical, microbiological, and chemical barriers in the vaginal lumen of both humans and nonhuman primates. CV-N is a small protein whose antiviral potency could be reduced if CV-N binds with other proteins or undergoes other physical or chemical degradation in the vaginal environment. To compensate for such loss, we used very high concentrations of CV-N (i.e., 0.5–2%) for this initial study of its efficacy potential as a vaginal microbicide. Our rationale for using high concentrations was also to determine whether such high CV-N levels were safe. On the basis of these promising results, we are optimistic that future in vivo studies will demonstrate that gel formulations containing less than 0.5% CV-N are both safe and effective.

In the present study, intravaginal SHIV challenge of macaques pretreated with CV-N gel was used as a model to study the possible efficacy of this microbicide for the prevention of HIV transmission by vaginal intercourse. In addition to the strong efficacy of pretreatment with CV-N gel for the preven-
tion of SHIV89.6P infection in this model, pretreatment of HIV gp120 with CV-N can block gp120 interaction with CD4 and cell-associated CCR5 coreceptor. In an ectocervical explant culture system, CV-N potently blocked infection of CCR5-using HIV-1BaL strain. We have also observed CV-N to effectively inhibit viral replication of CCR5-using SHIV162P3 in vitro (C.-C. Tsai, et al., unpublished data). Moreover, in vitro studies indicate that CV-N is fully active against representative strains of all known HIV clades and no CV-N-resistant virus has emerged after numerous tests (M. R. B. et al., unpublished observations). Together, these results suggest that CV-N is a promising agent for development as a topical vaginal microbicide for the prevention of sexual transmission of HIV infection.

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