ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1997, p. 1521–1530 Vol. 41, No. 7
0066-4804/97/$04.00+0

Discovery of Cyanovirin-N, a Novel Human Immunodeficiency Virus-Inactivating Protein That Binds Viral Surface Envelope Glycoprotein gp120: Potential Applications to Microbicide Development†

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Received 12 November 1996/Returned for modification 11 February 1997/Accepted 30 April 1997

We have isolated and sequenced a novel 11-kDa virucidal protein, named cyanovirin-N (CV-N), from cultures of the cyanobacterium (blue-green alga) *Nostoc ellipsosporum*. We also have produced CV-N recombinantly by expression of a corresponding DNA sequence in *Escherichia coli*. Low nanomolar concentrations of either natural or recombinant CV-N irreversibly inactivate diverse laboratory strains and primary isolates of human immunodeficiency virus (HIV) type 1 as well as strains of HIV type 2 and simian immunodeficiency virus. In addition, CV-N aborts cell-to-cell fusion and transmission of HIV-1 infection. Continuous, 2-day exposures of uninfected CEM-SS cells or peripheral blood lymphocytes to high concentrations (e.g., 9,000 nM) of CV-N were not lethal to these representative host cell types. The antiviral activity of CV-N is due, at least in part, to unique, high-affinity interactions of CV-N with the viral surface envelope glycoprotein gp120. The biological activity of CV-N is highly resistant to physicochemical denaturation, further enhancing its potential as an anti-HIV microbicide.

The main way in which human immunodeficiency virus (HIV) is transmitted is through sexual contact. Worldwide, heterosexual transmission accounts for at least 75% or more of all HIV infections (63). Although a vaccination strategy might ideally prevent sexual spread of HIV, all attempts thus far to develop a clinically effective anti-HIV vaccine have been unsuccessful. Moreover, the complexities of developing a vaccine against the highly mutable HIV are increasingly apparent (53, 55, 71). Therefore, there is now greater urgency for the discovery and development of alternative strategies other than vaccines to prevent sexual transmission of HIV (2, 51, 66, 72).

There has been growing interest in the development of antiviral microbicides, which are defined as agents that may be used ex vivo or topically to inhibit the spread of viral infection. In particular, the World Health Organization, the U.S. Department of Health and Human Services, the National Institute of Allergy and Infectious Diseases, and others, have identified as an urgent global priority the need for development of female-controlled topical microbicides that are suitable for blocking sexual transmission of HIV (23, 43, 59, 73).

Pauwels and DeClercq (61) have recently reviewed progress toward development of vaginal anti-HIV microbicides. They point to virucides, which are defined as compounds that directly interact with HIV virions to decrease or prevent infectivity, as potentially providing a "first line" microbicidal defense against sexual transmission of the virus. As further emphasized by Pauwels and DeClercq, sexual transmission of HIV involves not only cell-free virions but also HIV-infected host cells; therefore, a virucidal agent which would additionally target the cell-associated infectious process may be distinctly advantageous as an anti-HIV microbicide.

We disclose here the discovery of cyanovirin-N (CV-N), a highly potent virucidal protein that irreversibly inactivates diverse T-lymphocyte-tropic (T-tropic), laboratory-adapted strains of HIV type 1 (HIV-1), HIV type 2 (HIV-2), and simian immunodeficiency virus (SIV), as well as T-tropic, macrophage-tropic (M-tropic), and T- and M-tropic (dual-tropic) primary clinical isolates of HIV-1. Furthermore, CV-N prevents in vitro fusion and transmission of HIV-1 between infected and uninfected cells. These properties appear to be mediated through conserved interactions of CV-N with the viral surface envelope glycoprotein (herein generically termed gp120) that are distinct from the interactions of gp120 with the cellular receptor CD4 or with antibodies to known HIV-neutralizing determinants of gp120.

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† This article is dedicated to the director, staff, and benefactors of the Arizona State University Cancer Research Institute for their heroic efforts on behalf of cancer patients worldwide.

The *Nostoc ellipsosporum* extract from which the discovery of CV-N initially evolved was among a collection of cyanobacterial (blue-green algal) extracts from the U.S. National Cancer Institute's Natural Products Repository. We had selected the extract for detailed investigation both based on its strong inhibition of HIV cytopathicity in an empirical screen (6) and because there were preliminary indications that the active constituent was a protein. Antiviral proteins from any cyanobacterial source had not heretofore been described. We have been particularly interested in the elucidation of new potential therapeutic protein and peptide leads which might reveal unprecedented mechanisms of anti-HIV activity and/or serve as templates for the discovery and development of novel, small-molecule inhibitors of HIV infection. Such leads are also potentially attractive for microbicide development.

(This article is part 33 in the National Cancer Institute Laboratory of Drug Discovery Research and Development series "HIV-Inhibitory Natural Products"; for parts 31 and 32, see Currens et al. [19, 20].)

MATERIALS AND METHODS

Isolation and sequencing of natural CV-N. The *N. ellipsosporum* extract from which CV-N was isolated had been prepared originally by G. Patterson, University of Hawaii, as described elsewhere (60). Anti-HIV screening of the extract and fractions thereof and the initial testing of pure proteins were performed by the XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt) assay as described elsewhere (6, 76). Preliminary dereplication and chemical screening analyses were performed essentially as described elsewhere (5, 12, 33). Isolation of the active protein was accomplished by high-performance liquid chromatography (HPLC) on a μBondapak C_{18} matrix column (Waters Corporation, Milford, Mass.) which was eluted with a water-acetonitrile gradient. Initial sequencing employed N-terminal Edman degradation of intact protein and of numerous overlapping peptide fragments from endoproteinase digestion. Amino acid analysis was in agreement with the deduced sequence. Electrospray ionization (ESI) mass spectrometry of the reduced, HPLC-purified protein showed a molecular ion having a mass/charge ratio consistent with the calculated molecular weight. Sequence searches comparing the deduced sequence to those of known proteins and gene products were performed by using the Allprot database with the Genetics Computer Group Wisconsin package. Full details of the isolation, purification, and chemical characterization of the natural protein will be published elsewhere.

Synthesis and expression in *Escherichia coli* of a corresponding DNA coding sequence for CV-N. The deduced amino acid sequence was back-translated to a DNA sequence with an *E. coli* codon preference table and was supplemented with a termination codon and restriction sites to facilitate ligation into the expression vector pFLAG-1 (Eastman Kodak Company, Scientific Imaging Systems, Rochester, N.Y.). A CV-N coding sequence, coupled to codons for the octapeptide leader FLAG (AspTyrLysAspAspAspAspLys), was initially synthesized as 13 overlapping, complementary oligonucleotides which were assembled to form the full, double-stranded coding sequence. The synthetic DNA was amplified conventionally by PCR with appropriate primers and thermostable DNA polymerase. *E. coli* was transformed with the pFLAG-construct containing the synthetic gene ligated in the correct reading frame behind the outer membrane protein-A secretory peptide sequence of the vector. Induction of the clones with isopropyl-1-thio-β-D-galactoside resulted in expression of a corresponding, anti-HIV active FLAG-fusion protein which was purified by affinity chromatography on an anti-FLAG monoclonal antibody-linked agarose. For preparation of native recombinant protein, the FLAG-octapeptide leader sequence was deleted by site-directed mutagenesis employing a restriction site elimination maneuver. Cultures of *E. coli* were transformed with this construct and induced with isopropyl-1-thio-β-D-galactoside; periplasmic extracts were prepared and subjected to step gradient C_4 reverse-phase, vacuum-liquid chromatography to give three fractions, which were eluted with (i) 100% water, (ii) methanol-water (2:1), and (iii) 100% methanol. The anti-HIV activity was concentrated in fraction ii. Further purification of the active recombinant protein was performed by HPLC on a μBondapak C_{18} column (1.9 by 15 cm; Waters Corporation) eluted with a gradient of increasing concentrations of acetonitrile in water (0.05% trifluoroacetic acid [vol/vol] in the mobile phase). Final purification of the recombinant protein was accomplished by HPLC on a C_4 column (1 by 10 cm; Cohesive Biotechnologies, Acton, Mass.) monitored at 280 nm; gradient elution (5 ml/min) from 100% water to water-acetonitrile (7:3) was carried out over 23 min with 0.05% trifluoroacetic acid (vol/vol) in the mobile phase.

Comparisons of natural and recombinant CV-N. The identity of the recombinant protein with the natural protein was initially established by HPLC retention, ESI mass spectrometry, and amino acid analysis. Further confirmation of identity was provided by N-terminal sequencing of the first 25 amino acids of each protein. The anti-HIV activities of the natural and recombinant proteins were compared as described below.

Anti-HIV assays. Descriptions and sources of the T-tropic virus strains, primary isolates, and cell lines used in these studies have been previously published (8, 9). The HIV-1 dual-tropic isolate 89.6 (18) and the M-tropic isolates Ba-L (27) and Ada-M (29) were obtained from the National Institute of Allergy and Infectious Diseases (NIAID) AIDS Research and Reference Program (for further details of virus strains, isolates and cell lines, see the NIAID Program Catalogue (73a), and pertinent other original citations therein). The M-tropic isolate SKLA was obtained from a pediatric patient of the University of Alabama (Birmingham) Pediatric AIDS clinic by cocultivation of the patient's peripheral blood mononuclear cells with uninfected normal donor cells; the virus pool was expanded and frozen in liquid nitrogen after one passage (7a).

For antiviral assays, peripheral blood lymphocytes (PBL) and macrophages (MAC) were isolated as necessary following Ficoll-Hypaque centrifugation as described elsewhere (28). The soluble CD4 (sCD4) reference standard used in the antiviral assays (7) was obtained from Genentech, Inc., San Francisco, Calif. Tests of CV-N against herpesvirus type 1, cytomegalovirus, and adenovirus type 5 were performed by conventional assays (38, 64).

For comparisons of the anti-HIV activities of the natural and recombinant proteins, bioactivity stability studies, concentration dependency studies, delayed-addition studies, and CV-N–gp120 titration studies (see below), a modified XTT assay as described by Gulakowski et al. (32) was used. When appropriate, antiviral effects were also assayed either by an enzyme-linked immunosorbent assay (ELISA) of the HIV core antigen (p24) or by determination of supernatant reverse transcriptase (RT) as described elsewhere (8, 9, 32). Antiviral assays used in the range-of-activity studies of the various laboratory strains and primary isolates of virus were as described elsewhere (8, 9).

Evaluation of direct cytotoxicity of CV-N to uninfected host cells. Five-milliliter aliquots of CEM-SS or phytohemagglutinin-stimulated PBL suspensions containing 2×10^6 cells/ml of culture medium were added to 25-cm^2 tissue culture flasks. A concentrated solution of CV-N (2 mg/ml) in phosphate-buffered saline (PBS) or PBS alone was added to the flasks to yield a final CV-N concentration of 0, 3,000, or 9,000 nM. Following incubation for 48 h at 37°C, the cells were washed free of CV-N by three centrifugation steps, resuspended in fresh medium, and placed in 25-cm^2 flasks to monitor posttreatment viability and proliferation. Every 2 days, the cells were enumerated and assayed for viability by trypan blue exclusion or by a two-color fluorescence cell viability assay as described elsewhere (45). The cells were supplied with fresh medium on days 4 and 8 following CV-N washout.

Biological-activity stability studies for CV-N. An aliquot (120 μl) of a solution (100 μg/ml) of CV-N in PBS was immersed in a boiling water bath for 15 min and then tested for anti-HIV activity. Solutions of CV-N were individually treated with 0.5% sodium dodecyl sulfate (SDS), 8 M guanidine HCl, or 0.5% H_2O_2 (1.0 mM sodium acetate buffer [pH 4.0]) at room temperature for 90 min. The reagents were removed by use of a 3-kDa ultrafilter (Amicon, Inc., Beverly, Mass.) and three 1-ml PBS wash steps. The 3-kDa retentates were then tested in the XTT assay. A solution of CV-N in Tris buffer (pH 8.2) was reacted with an excess of β-mercaptoethanol in the dark under N_2, after which an excess of the alkylating agent vinyl pyridine was added to the reaction mixture. After 90 min, the remaining reagents were removed by 3-kDa ultrafiltration with three 1-ml PBS washes. Reduced, alkylated CV-N was recovered in the 3-kDa retentate and tested in the XTT assay. The identity of the reaction product as the pyridylethyl cysteine derivative of CV-N was confirmed by ESI mass spectrometry.

Effects of CV-N on cell-to-cell fusion and virus-to-cell and cell-to-cell infectivity. Cell-to-cell fusion and cell-to-cell virus transmission assays were performed initially with a cocultivation assay system comprised of uninfected CEM-SS cells and CEM-SS cells chronically infected with HIV-1, as previously described (9). Other virus-to-cell and cell-to-cell fusion studies were performed with β-galactosidase (β-Gal) cells as described elsewhere (9, 16, 39). The β-Gal cells were $CD4^+$ HeLa derived and engineered for a Tat protein-induced transactivation of a β-Gal gene driven by the HIV-1 long terminal repeat promoter (39). All of the aforementioned assays were performed in triplicate, and the mean values were calculated; the corresponding standard errors averaged ≤15% of the respective means; for graphical presentation, the data points were converted to percentages of the appropriate controls.

Direct effects of CV-N on virus versus cells. A quantitative infectivity assay (58) was performed as previously described (44) to assess the direct effects of CV-N on cell-free virions. Briefly, aliquots of cell-free $HIV\text{-}1_{IIIB}$ stocks were preincubated with and without CV-N (20 nM) for 90 min at 37°C. The control and CV-N-treated virus preparations were then ultracentrifuged to remove free CV-N, followed by assaying of viral infectivity. A similar study was also performed with virus-free CEM-SS cells preincubated with and without CV-N, which was followed by gentle centrifugation and multiple washes to remove free CV-N and then assaying of cellular infectibility by intact infectious $HIV\text{-}1_{IIIB}$.

Ultrafiltration and SDS-PAGE study of CV-N–gp120 interactions. Solutions of CV-N and native gp120 ($HIV\text{-}1_{IIIB}$ gp120; Advanced BioTechnologies, Inc., Columbia, Md.) were prepared in PBS and incubated together, as appropriate, for 90 min and then passed through 50-kDa cutoff centrifugal ultrafilters (Amicon, Inc.). After being washed three times with PBS, the 50-kDa filtrates were concentrated with 3-kDa ultrafilters. The 50- and 3-kDa retentates were analyzed

by SDS-polyacrylamide gel electrophoresis (PAGE) on an 8 to 20% gradient gel stained with Coomassie brilliant blue.

CV-N–gp120 titration study. PBS solutions (60 μl) containing 3 μg of CV-N were preincubated with or without various amounts of native gp120 (0.25 to 5.0 μg of gp120) at room temperature for 90 min. Appropriate serial dilutions (to yield a final highest test concentration equivalent to 46 nM CV-N) were then prepared and tested in the XTT assay. Control PBS solutions containing identical amounts of gp120 (but no CV-N) were similarly assayed.

Preparation of anti-CV-N polyclonal antibodies. Three New Zealand White rabbits were initially immunized with 100 μg of CV-N in Freund's complete adjuvant. Three follow-up booster injections of 50 μg of CV-N in Freund's incomplete adjuvant were administered at 28-day intervals. The immunoglobulin fraction of the resultant immune serum of each rabbit was isolated by protein A-Sepharose (BioRad Laboratories, Hercules, Calif.) affinity chromatography in accordance with the manufacturer's instructions. Reactivity of the polyclonal antibodies for CV-N was demonstrated by Western blot analyses with 1:1,000 to 1:5,000 dilutions of the rabbit immunoglobulin fractions.

ELISA studies of CV-N–gp120 interactions. Each well of a 96-well protein-adsorbing plate (Maxisorp Immunoplate; Nalge NUNC, International, Naperville, Ill.) was incubated with 100 ng of native gp120 in PBS at room temperature for 2 h. The plate was then washed three times with PBS augmented with 0.5% Tween 20 (TPBS) and blocked with a 1% solution of bovine serum albumin (BSA). A control plate was incubated with BSA only. After washing three times with TPBS, 0.5-$\log_{10}$ serial dilutions of CV-N were added to triplicate wells of both the gp120-treated plate and the BSA-treated control plate, and the plates were incubated for 1 h. The plates were then washed three times with TPBS, and the bound CV-N was determined by incubation first with a solution of the anti-CV-N rabbit polyclonal antibody preparation (1:1,000 dilution), washing three times with TPBS, and then incubation with goat-anti-rabbit antibodies conjugated to alkaline phosphatase (Boehringer Mannheim, Indianapolis, Ind.). After a final set of three washes with TPBS, absorbance at 405 nm was measured for each well following addition of the alkaline phosphatase substrate.

To determine the relative affinities of CV-N for a series of standard proteins, 100 ng of the following proteins per well was bound to a 96-well protein-adsorbing plate: native gp120 (HIV-1_{IIIB} gp120); gp130 and gp140 (SIV_{mac} 239 gp130 and SIV_{mac} 1A11 gp140, respectively, obtained from the AIDS Research and Reference Program, NIAID, NIH); rgp120 (recombinant, nonglycosylated HIV-1_{SF2} gp120 [70] obtained from K. Steimer, Chiron Corp.); p24 (HIV-1_{IIIB} p24, obtained from NEN-Dupont, Boston, Mass.); sCD4 (obtained from R. Sweet, SmithKline Beecham, Philadelphia, Pa.); and human serum albumin, human orosomucosoid, aprotinin, and BSA (all obtained from Sigma Corp., St. Louis, Mo.). The plate was blocked with BSA and washed as described above and then incubated for 1 h with 20-ng/well CV-N followed by washing three times with TPBS. The amount of bound CV-N was determined as described above.

Immunoblot study of CV-N–gp120 interactions. HIV-1_{MN} produced in CEM-SS cells was isolated by sucrose gradient centrifugation and lysed with 4× Laemmli (40) sample buffer. Viral lysates (30 μg) were subjected to SDS-PAGE on a preparative 6 to 18% gradient gel and blotted onto a polyvinylidine difluoride membrane. Membrane strips were incubated for 1 h in a solution of CV-N (1 μg/ml) in PBS, while control membranes were incubated in PBS only. After washing three times with TPBS, pairs of the control and CV-N-treated membranes were incubated either with the anti-CV-N polyclonal antibody preparation (1:1,000 dilution) or with an anti-gp120 monoclonal antibody (3F5-D5-P8, kindly provided by Steve Nigida, Jr., Science Applications International Corporation, Frederick, Md.). After an incubation for 1 h, all membranes were washed three times with TPBS and treated with either goat-anti-rabbit or goat-anti-mouse antibodies conjugated to horseradish peroxidase. After a final three washes with TPBS, antibodies were visualized by incubating the membranes in a solution of 3-amino-9-ethyl carbazole.

Studies of binding interactions of CV-N, anti-gp120 monoclonal antibodies, and sCD4 on gp120. The following reagents were obtained through the AIDS Research and Reference Reagent Program, NIAID: mouse monoclonal antibodies to the third hypervariable (V3-loop) epitope of gp120, including 489.1 (57) and R/V3-50.1 (22, 78), from Repligen Corp., Cambridge, Mass.; 257-D-IV (30, 31), from Susan Zalla-Pazner; 0.5β (49) from Shuzo Matsushita; IIIB-V3-21, IIIB-V3-01, and IIIB-V3-13 (41, 42) from John Laman; 4G10 (74, 75) from Albrecht von Brunn, courtesy of the MRC AIDS Directed Progamme; a human monoclonal antibody, IgG1b12, to the HIV-1 gp120-CD4 binding site of gp120 (3, 10, 11, 65) from Dennis Burton and Carlos Barbas; and, recombinant sCD4 and CD4 antiserum (T4-4) from R. Sweet (SmithKline Beecham).

ELISA plates were treated as described above with gp120 (100 ng/well) and then with either 1:50, 1:100, or 1:500 dilutions of gp120-specific monoclonal antibodies (257-D-IV, IIIB-V3-13, 4G10, or IgG1b12) or with serial dilutions of sCD4 (10,000, 1,000, 100, 10, and 1 ng/well). After washing three times with TPBS, the plates were incubated with 100 ng of CV-N per well for 1 h followed by three washes with TPBS. For detection of CV-N and sCD4, anti-CV-N and anti-sCD4 polyclonal antibody preparations (1:1,000 dilutions) were added to the appropriate wells, followed by 1 h of incubation and three washes with TPBS. Appropriate secondary antibodies and substrate were then added to all wells, and binding was measured by determining the absorbance at 405 nm as described above.

In other experiments, gp120-treated plates and control plates were incubated with either PBS alone or PBS with CV-N (20 ng/well) for 1 h at room temperature. Following removal of the CV-N, 1:500 dilutions each of sCD4 and the above-referenced gp120-specific monoclonal antibodies (489.1, R/V3-50.1, 257-D-IV, 0.5β, IIIB-V3-21, IIIB-V3-01, IIIB-V3-13, 4G10, and IgG1b12) were added to triplicate wells, and the plates were incubated for 1 h, followed by washing three times with TPBS. An anti-sCD4 polyclonal antibody preparation (1:1,000 dilution) was added to the sCD4-treated wells, followed by 1 h of incubation and an additional wash three times with TPBS. The plate was then treated with appropriate secondary antibody-alkaline phosphatase conjugates. Following a final set of three washes with TPBS and addition of alkaline phosphatase substrate, the plates were incubated for 15 min and then quenched by the addition of 100 μl of 0.1 M EDTA. Antibody binding was measured by determining absorbance at 405 nm.

Nucleotide sequence accession number. The DNA sequence reported in this paper has been deposited in the GenBank database (accession no. L48551).

								Leu	**Gly**	**Lys**	**Phe**	**Ser**	**Gln**	**Thr**	**Cys**	**Tyr**	**Asn**	**Ser**	**Ala**
								5′-CTT	GGT	AAA	TTC	TCC	CAG	ACC	TGC	TAC	AAC	TCC	GCT
								3′-GAA	CCA	TTT	AAG	AGG	GTC	TGG	ACG	ATG	TTG	AGG	CGA
Ile	**Gln**	**Gly**	**Ser**	**Val**	**Leu**	**Thr**	**Ser**	**Thr**	**Cys**	**Glu**	**Arg**	**Thr**	**Asn**	**Gly**	**Gly**	**Tyr**	**Asn**	**Thr**	**Ser**
ATC	CAG	GGT	TCC	GTT	CTG	ACC	TCC	ACC	TGC	GAA	CGT	ACC	AAC	GGT	GGT	TAC	AAC	ACC	TCC
TAG	GTC	CCA	AGG	CAA	GAC	TGG	AGG	TGG	ACG	CTT	GCA	TGG	TTG	CCA	CCA	ATG	TTG	TGG	AGG
Ser	**Ile**	**Asp**	**Leu**	**Asn**	**Ser**	**Val**	**Ile**	**Glu**	**Asn**	**Val**	**Asp**	**Gly**	**Ser**	**Leu**	**Lys**	**Trp**	**Gln**	**Pro**	**Ser**
TCC	ATC	GAC	CTG	AAC	TCC	GTT	ATC	GAA	AAC	GTT	GAC	GGT	TCC	CTG	AAA	TGG	CAG	CCG	TCC
AGG	TAG	CTG	GAC	TTG	AGG	CAA	TAG	CTT	TTG	CAA	CTG	CCA	AGG	GAC	TTT	ACC	GTC	GGC	AGG
Asn	**Phe**	**Ile**	**Glu**	**Thr**	**Cys**	**Arg**	**Asn**	**Thr**	**Gln**	**Leu**	**Ala**	**Gly**	**Ser**	**Ser**	**Glu**	**Leu**	**Ala**	**Ala**	**Glu**
AAC	TTC	ATC	GAA	ACC	TGC	CGT	AAC	ACC	CAG	CTG	GCT	GGT	TCC	TCC	GAA	CTG	GCT	GCT	GAA
TTG	AAG	TAG	CTT	TGG	ACG	GCA	TTG	TGG	GTC	GAC	CGA	CCA	AGG	AGG	CTT	GAC	CGA	CGA	CTT
Cys	**Lys**	**Thr**	**Arg**	**Ala**	**Gln**	**Gln**	**Phe**	**Val**	**Ser**	**Thr**	**Lys**	**Ile**	**Asn**	**Leu**	**Asp**	**Asp**	**His**	**Ile**	**Ala**
TGC	AAA	ACC	CGT	GCT	CAG	CAG	TTC	GTT	TCC	ACC	AAA	ATC	AAC	CTG	GAC	GAC	CAC	ATC	GCT
ACG	TTT	TGG	GCA	CGA	GTC	GTC	AAG	CAA	AGG	TGG	TTT	TAG	TTG	GAC	CTG	CTG	GTG	TAG	CGA
Asn	**Ile**	**Asp**	**Gly**	**Thr**	**Leu**	**Lys**	**Tyr**	**Glu**											
AAC	ATC	GAC	GGT	ACC	CTG	AAA	TAC	GAA-3′											
TTG	TAG	CTG	CCA	TGG	GAC	TTT	ATG	CTT-5′											

FIG. 1. Amino acid sequence of CV-N and the corresponding DNA coding sequence.

RESULTS

Isolation, purification, amino acid sequencing, and comparison of antiviral activities of natural and recombinant CV-Ns. Our preliminary analyses of the crude *N. ellipsosporum* extract indicated that its anti-HIV activity was not due to the presence of sulfolipids (33), sulfated polysaccharides (5), or any other known anti-HIV chemical or mechanistic class. Furthermore, the elution behavior of the anti-HIV activity in our chemical screening protocols (12) suggested that the active constituent was macromolecular, possibly proteinaceous. Anti-HIV bioassay-guided fractionation of the extract led to the isolation and purification of CV-N. A unique 101-amino-acid sequence (Fig. 1) was deduced for the natural CV-N by conventional chemical methods. For confirmation, a corresponding DNA coding sequence (Fig. 1) was synthesized and then expressed in *E. coli*. ESI mass spectrometric analysis indicated that the recombinant protein product was comprised mostly of the full-length CV-N (i.e., 101 residues), plus as much as 25 to 30% of a truncated cyanovirin missing the first two N-terminal residues (leucine and glycine), presumably due to proteolytic cleavage within the host bacterium. Otherwise, the natural and recombinant CV-Ns were indistinguishable, both in chemical composition and in biological activity. Searches of available databases did not reveal any homologies of greater than eight contiguous amino acids or >20% total sequence homology between CV-N and any amino acid sequences of known proteins or transcription products of known nucleotide sequences. Throughout purification and analysis, the biological activity of CV-N showed a remarkable degree of stability. Unbuffered storage solutions of the protein withstood multiple freeze-thaw cycles and dissolution in organic solvents (CH_3CN, MeOH, and dimethyl sulfoxide). In addition, treatment of CV-N with high salt (8 M guanidine HCl), detergent (0.5% SDS), 0.5% H_2O_2 or boiling (15 min in H_2O) did not result in significant loss of anti-HIV activity. Only when the cysteine residues of CV-N were reduced and alkylated with vinyl pyridine was the antiviral activity abolished (data not shown).

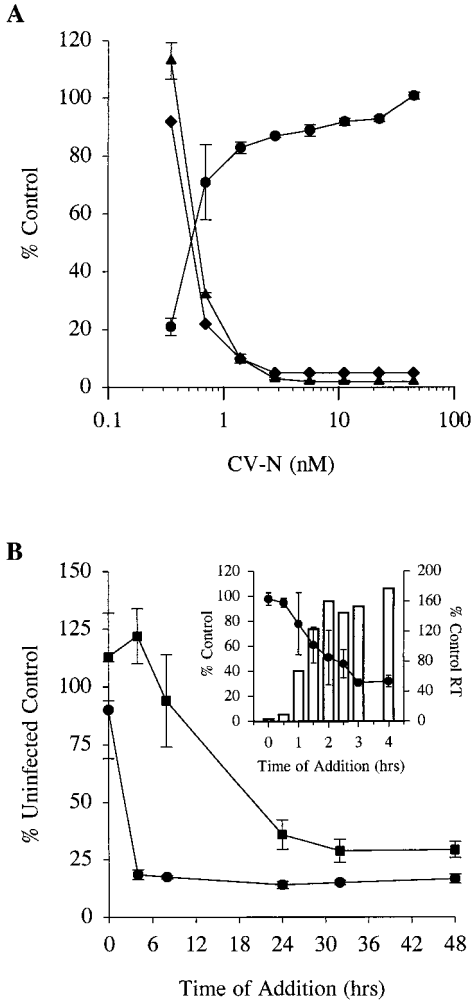

FIG. 2. Anti-HIV activity of CV-N in CEM-SS cells infected with HIV-1_{RF}. (A) Concentration-dependent effects of CV-N on cellular viability assessed by XTT-tetrazolium (●) and on viral reproduction as assessed by supernatant RT activity (▲) and p24 (◆). (B) Delayed-addition effects. Introduction of CV-N (●) or the reference compound ddC (■) (10 nM and 5 μM concentrations, respectively) was delayed by various times after initial infection, followed by 6 days of incubation and then XTT assaying of cellular viability (line graphs) and RT (open bars [inset]). Points are averages (± standard deviations) of at least triplicate determinations.

TABLE 1. Range of antiviral activity of CV-N

Virus	Target cell[a]	Tropism[b]	EC_{50} (nM)[c,d]
HIV-1 laboratory strains			
RF	CEM-SS	T	0.1
RF	U937	T	0.5
IIIB	CEM-SS	T	0.4
IIIB	MT-2	T	0.4
MN	MT-2	T	2.3
G910-6	MT-2	T	5.8
A17	MT-2	T	0.8
214	CEM-SS	T	0.4
SK1	CEM-SS	T	4.8
205	CEM-SS	T	0.8
G1	CEM-SS	T	0.9
HIV-1 primary isolates			
WEJO	PBL	T	6.7
VIHU	PBL	T	5.5
BAKI	PBL	T	1.5
WOME	PBL	T	4.3
89.6	PBL	T and M	7.3
89.6	MAC	T and M	36.8
Ba-L	MAC	M	17.0
Ada-M	MAC	M	1.7
SLKA	MAC	M	5.5
HIV-2			
ROD	CEM-SS	T	7.6
MS	CEM-SS	T	2.3
SIV			
$Delta_{B670}$	174 × CEM	T	11.0

[a] Target cells used in the in vitro anti-HIV assay.
[b] T, T-tropic (a T-tropic virus is defined experimentally by its ability to efficiently infect immortalized, CD4 receptor-positive T-cell lines, as well as primary lymphocytes, but not primary macrophages, in vitro); M, M-tropic (an M-tropic virus is defined experimentally by its ability to infect primary macrophages and lymphocytes, but not T-cell lines, in vitro); T and M, dual-tropic virus, (i.e., which has both T-tropic and M-tropic characteristics).
[c] Mean 50% effective concentration (EC_{50}) values were determined from concentration-response curves from eight dilutions of the test agent (triplicate wells/concentration). G910-6 is a nucleoside-resistant strain; A17 is a pyridinone-resistant strain. HIV-1 89.6, Ba-L, Ada-M, and SLKA were tested in human primary MAC cultures by p24 ELISA. Other primary isolates were tested in human PBL cultures by supernatant RT activity. All other assays employed XTT-tetrazolium. Standard errors averaged less than 10% of the respective means.
[d] Microscopic examination of cells in the antiviral assays at the highest tested concentrations of CV-N (45 to 400 nM) revealed little or no evidence of direct cytotoxicity of CV-N.

Concentration and time dependency of antiviral activities of CV-N. Potent cytoprotective and antireplicative activities of CV-N were examined in further detail by using HIV-1_{RF} in CEM-SS cells. CV-N elicited a striking, concentration-dependent inhibition of virus-induced cell killing, along with concomitant decreases in supernatant RT and viral core antigen (p24) (Fig. 2A). By microscopic examination, there was no indication of direct toxicity of CV-N to the uninfected control cells at the highest concentrations of CV-N tested (1.1 μM; data not shown; see additional cytotoxicity evaluations below). Delayed-addition experiments revealed further that to afford maximum antiviral activity, CV-N had to be added to cells before or shortly after addition of virus. For example, in marked contrast to the reference compound dideoxycytidine (ddC; an inhibitor of RT), delay of addition of CV-N by only 3 h resulted in little or no antiviral activity (Fig. 2B).

Range of antiretroviral activity of CV-N. The range of activity of CV-N against diverse $CD4^+$-tropic immunodeficiency retroviruses in various target cells was remarkable (Table 1). CV-N potently inhibited all tested laboratory strains of HIV-1, HIV-2, and SIV; moreover, CV-N showed comparable (generally low-nanomolar) antiviral potency against T-tropic, M-tropic, and dual-tropic HIV-1 primary isolates (Table 1). This was in contrast to the control, recombinant sCD4 which, as expected (53), was inactive at the highest concentrations tested (100 nM) against the primary isolates (data not shown). Antiviral specificity of CV-N (5 μg/ml) was evidenced by its lack of activity (data not shown) against three unrelated human viruses (herpesvirus type 1, cytomegalovirus, and adenovirus type 5) tested in conventional assays (38, 64). Microscopic examination revealed no indication of direct lethality of CV-N to the host cells by the highest concentrations tested (45 to 400 nM) in the anti-HIV (or SIV) assays represented in Table 1. More direct evaluations of cytotoxicity against selected representative host cells were performed in further detail as follows.

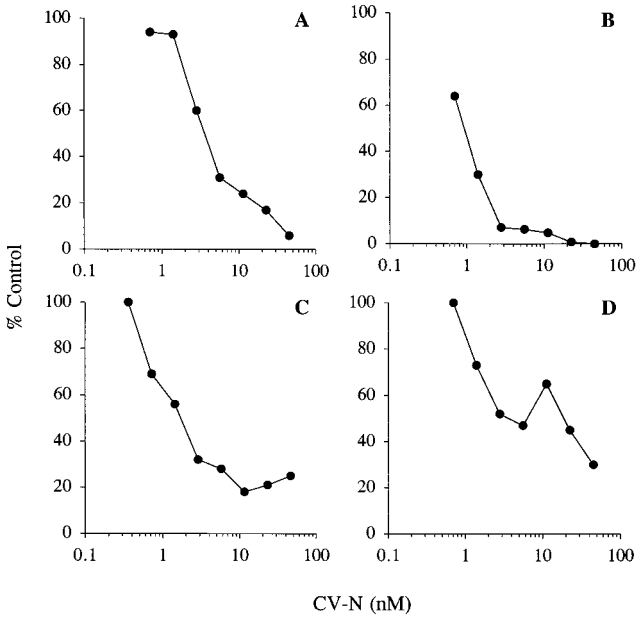

FIG. 3. Effects of CV-N on virus-cell and cell-cell interactions. (A) Effect of CV-N on syncytium formation between uninfected and chronically HIV-1-infected CEM-SS cells in a cocultivation assay. (B) Effect of CV-N on cell-to-cell virus transmission in the CEM-SS cocultivation assay. (C) Effect of CV-N on HIV-1_{IIIB} infectivity of $CD4^+$ β-Gal cells. (D) Effect of CV-N on the fusion of $CD4^+$ β-Gal cells with HIV-1 Env-expressing HL2/3 cells. Points are averages of duplicate determinations.

Evaluation of cytotoxicity of CV-N to uninfected host cells. The direct cytotoxicity of CV-N against a representative cell line (CEM-SS) and against freshly isolated PBL was evaluated by using trypan blue exclusion and by a two-color fluorescence cell assay (45) to monitor cellular viability. Cultures of CEM-SS cells or PBL treated with a 48-h continuous exposure to either 3,000 or 9,000 nM CV-N showed only slightly diminished (e.g., by ≤10%) viable cell numbers compared to the corresponding control incubations (data not shown). Removal of CV-N from the CEM-SS cell cultures resulted in almost immediate recovery of proliferation of the cells to control levels. Similarly, removal of CV-N from the PBL resulted in essentially complete recovery of the control proliferative level, albeit requiring a somewhat longer recovery time (8 to 10 days) (data not shown).

Inhibition by CV-N of cell-to-cell fusion and virus-to-cell and cell-to-cell transmission of viral infection. Cocultivation of chronically infected and uninfected CEM-SS cells with CV-N did not inhibit viral replication (not shown) but did cause a concentration-dependent inhibition of cell-to-cell fusion (Fig. 3A) and virus transmission (Fig. 3B). Similar results were obtained from binding and fusion inhibition assays employing β-Gal indicator cells. CV-N inhibited the cell-free HIV-1_{IIIB} fusion and infection of β-Gal cells in a concentration-dependent manner (Fig. 3C); likewise, CV-N inhibited fusion of the $CD4^+$ β-Gal cells with HIV-1 envelope-expressing HL 2/3 cells in a concentration-dependent manner (Fig. 3D).

Direct effects of CV-N on virus versus cells. Uninfected CEM-SS cells preincubated with CV-N (20 nM, 90 min) and then centrifuged and washed to remove the CV-N retained normal susceptibility to HIV infection (data not shown). In contrast, the infectivity of cell-free virus similarly pretreated and then ultracentrifuged to remove CV-N was essentially abolished (data not shown). These results indicated that CV-N behaved as a virucide, i.e., acting directly on some component(s) of the virus to irreversibly inactivate and prevent its infectivity toward otherwise susceptible host cells.

TABLE 2. Effect of preincubation of CV-N with virus-free gp120 on HIV-1-inactivating activity[a]

Test group	EC_{50} (nM)[b]
CV-N control	0.16
CV-N + 0.25 μg of gp120	0.17
0.25 μg of gp120	Inactive
CV-N + 0.50 μg of gp120	0.31
0.50 μg of gp120	Inactive
CV-N + 0.75 μg of gp120	0.44
0.75 μg of gp120	Inactive
CV-N + 3.0 μg of gp120	Inactive
3.0 μg of gp120	1.7
CV-N + 5.0 μg of gp120	Inactive
5.0 μg of gp120	2.4

[a] Solutions of 3 μg of CV-N in PBS were preincubated with or without increasing amounts of native HIV-1_{IIIB} gp120, and appropriate dilutions (to yield an equivalent highest test concentration of 46 nM CV-N) were tested for anti-HIV activity in the XTT assay. Control solutions containing the appropriate amounts of gp120 alone were tested in parallel.

[b] EC_{50}, 50% effective concentrations. Values shown for CV-N–gp120 mixtures are based on CV-N concentrations only.

Ultrafiltration–SDS-PAGE studies of CV-N–gp120 interactions. We surmised that all of the aforementioned antiviral, anticytopathic, and direct virucidal effects of CV-N might be explained by a disruption of the normal function of gp120 somewhere in the virus-to-cell and cell-to-cell attachment and/or fusion process(es). To explore this further, we first looked for an interaction of CV-N directly with native, fully glycosylated, soluble gp120 in solution phase. Control experiments demonstrated that gp120 was retained on a 50-kDa cutoff ultrafilter, while CV-N eluted through a 50-kDa filter but was retained on a 3-kDa ultrafilter. SDS-PAGE analyses of the 50- and 3-kDa retentates from a preincubated mixture of CV-N and gp120 showed that CV-N was retained on the 50-kDa filter in the presence of gp120, indicating that CV-N bound directly to gp120, forming a complex that could not pass through the 50-kDa ultrafilter (data not shown).

CV-N–gp120 titration study. Other assays were conducted to determine the effect of exogenous, virus-free gp120 on the anti-HIV activity of CV-N. As shown in Table 2, addition of increasing amounts of the free glycoprotein caused a concentration-dependent decrease in the antiviral effects of CV-N. Although both CV-N and virus-free gp120 individually inhibited the infectivity of the intact HIV-1 (free gp120 presumably competing with the virus-associated gp120 for the CD4 receptor), the complex of CV-N and gp120 was inactive. Furthermore, treatment of the above-described, 50-kDa ultrafilter-retained CV-N–gp120 complex with strong denaturants, including 6 M guanidine-HCl and 0.1% SDS, did not result in the release of any CV-N detectable by anti-HIV bioassaying (data not shown). In contrast, pretreatment of CV-N with sCD4 did not diminish the anti-HIV activity of CV-N or result in a CV-N–sCD4 complex (data not shown).

ELISA studies of CV-N–gp120 interactions. Further evidence for direct interaction of CV-N and gp120 was obtained in an ELISA experiment. CV-N was specifically bound to gp120-treated microtiter plate wells in a CV-N concentration-dependent manner but was not bound to BSA-treated control wells (Fig. 4). The bound CV-N was detected with rabbit polyclonal anti-CV-N antibodies.

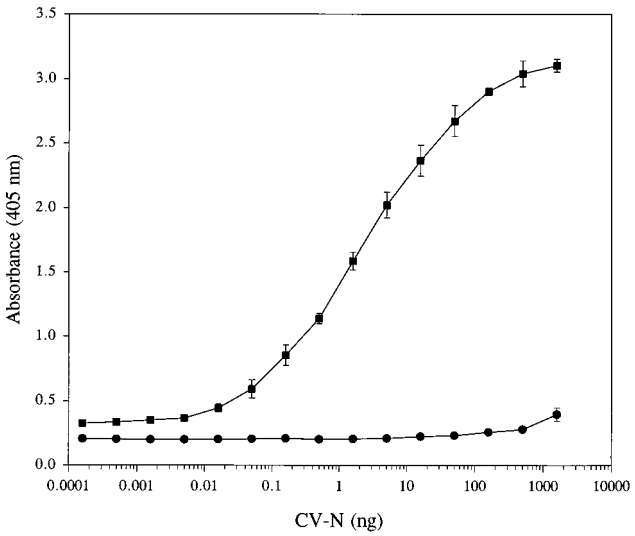

FIG. 4. ELISA study of concentration-dependent binding of CV-N to gp120. CV-N at 0.5-$\log_{10}$ serial dilutions was added either to 100-ng/well native gp120 (■) or to BSA-coated control wells (●). Anti-CV-N polyclonal antibodies were used to detect the bound CV-N as indicated by absorbance at 405 nM. Points are averages (± standard deviations) of triplicate determinations.

Additional evidence of specificity of association of CV-N with native HIV-1 gp120 and with analogous SIV glycoproteins (gp130 and gp140) was demonstrated in a similar ELISA format (Fig. 5). Recombinant, nonglycosylated HIV-1_{SF2} gp120 showed considerably less binding to CV-N than did the native gp120. There was little or no detectable interaction between CV-N and sCD4 or with other reference proteins including p24, BSA, aprotinin, human serum albumin, and orosomucosoid (Fig. 5).

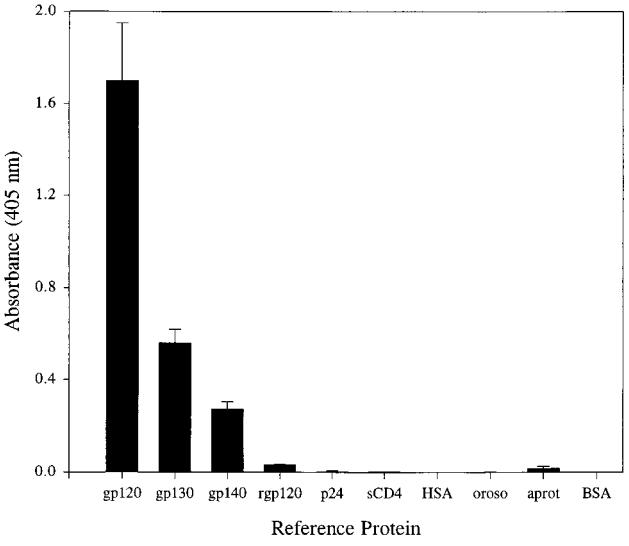

FIG. 5. ELISA of the binding of CV-N to reference proteins. Proteins were bound to a 96-well plate, and the plate was incubated with CV-N. Bound CV-N was visualized with anti-CV-N polyclonal antibodies as indicated by absorbance at 405 nm. gp120, HIV-1_{IIIB} gp120; gp130, SIV_{mac}239 gp130; gp140, SIV_{mac}1A11 gp140; rgp120, recombinant, nonglycosylated HIV-1_{SF2} gp120; p24, HIV-1_{IIIB} p24; HSA, human serum albumin; oroso, human orosomucosoid; aprot, aprotinin. Error bars are standard deviations based on at least triplicate determinations.

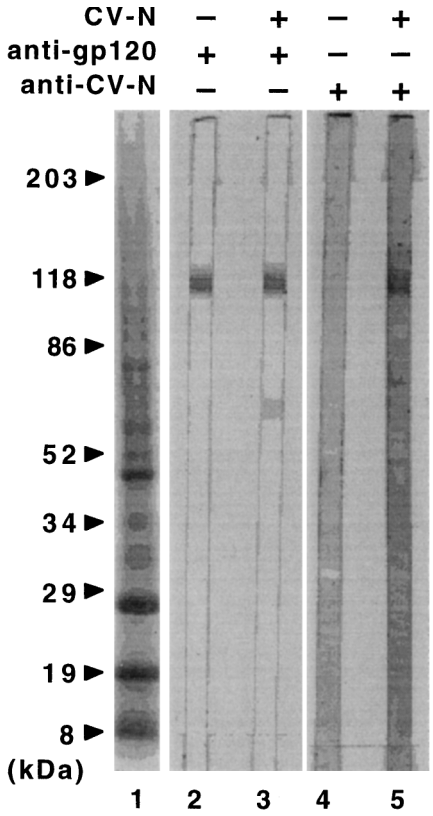

FIG. 6. Immunoblot analysis of CV-N–gp120 binding specificities in HIV-1_{MN} viral lysates. A lysate from HIV-1_{MN} was separated by preparative SDS-PAGE and blotted onto a polyvinylidine difluoride membrane. Strips of the membrane were incubated with either PBS (lanes 2 and 4) or CV-N in PBS (lanes 3 and 5) followed by either anti-gp120 antibodies (lanes 2 and 3) or anti-CV-N antibodies (lanes 4 and 5). A Coomassie-stained gel of the viral lysate is pictured in lane 1, with molecular mass markers shown to the left of the gel.

Immunoblot study of CV-N–gp120 interactions. Viral lysates, which were derived from HIV-1_{MN} propagated in CEM-SS cells, were used in immunoblot analyses to further ascertain the binding specificity of CV-N for viral proteins. CV-N was most prominently bound to the gp120 in these lysates (Fig. 6). This was consistent with the earlier viral infectivity results and with the ELISA experiments in which CV-N was bound both to native HIV-1 gp120 and SIV gp130 and gp140 and, albeit to a diminished extent, to recombinant nonglycosylated HIV-1 gp120, but not to other reference proteins. Together with the ultrafiltration studies, the CV-N–gp120 titration studies, and the ELISA studies, these results demonstrated considerable specificity for the binding of CV-N to gp120 in its native form, in its SDS-PAGE-denatured form, and in its nonglycosylated recombinant form.

ELISA studies of binding interactions of CV-N, anti-gp120 monoclonal antibodies, and sCD4 on gp120. Prebinding of gp120 with sCD4 or with any of the four tested anti-gp120 monoclonal antibodies (31) did not block the subsequent binding of CV-N with gp120 (data not shown). Furthermore, gp120 pretreated with high (20-ng/well) concentrations of CV-N retained its binding affinity for all of the seven tested mouse monoclonal antibodies that target the immunodominant, third hypervariable (V3) epitope of gp120; similar pretreatment of gp120 with CV-N did not inhibit gp120 binding with sCD4 or with IgG1b12, a human monoclonal antibody specific for the CD4 binding site of gp120 (data not shown). Thus, the binding

interaction of gp120 with CV-N appeared distinct from its interaction with either neutralizing antibodies or sCD4.

DISCUSSION

The discovery of CV-N was based on an empirical screening strategy (6) employing cultured human lymphoblastoid cells infected in vitro with HIV-1 (6, 76). The physiological role of CV-N in *N. ellipsosporum* is unknown, and the effects of this novel protein against HIV and SIV could have been neither anticipated nor predicted from any known protein sequence or from current knowledge of any viral or cellular molecular target. Present data indicate that the antiviral, anticytopathic, and direct virucidal activities of CV-N against HIV-1 are attributable, at least in part, to unique and effectively irreversible interactions of CV-N directly with the viral surface envelope glycoprotein gp120 that render the latter incapable of mediating virus-to-cell or cell-to-cell fusion.

Evidence for a direct interaction of CV-N with gp120 converged from several different experimental protocols. Interaction of virus-free gp120 with CV-N resulted in a gp120–CV-N complex which was highly resistant to dissociation and which was devoid of antiviral activity against intact HIV-1. ELISA studies likewise revealed a high-affinity interaction of CV-N with virus-free gp120 but not with nonenvelope viral proteins or other standard reference proteins. Immunoblots from whole-virus lysates of HIV-1 that had been electrophoresed, treated with CV-N, and probed with anti-gp120 or anti-CV-N antibodies showed preferential binding of CV-N to gp120 but not to other viral proteins.

Additional ELISA experiments showed that pretreatment of virus-free gp120 with anti-gp120 monoclonal antibodies directed either to the V3 loop or the CD4 binding site did not block subsequent binding of the gp120 with CV-N and vice versa. Similarly, pretreatment of virus-free gp120 with sCD4 did not block subsequent binding of gp120 with CV-N and vice versa. Thus, the binding of CV-N with virus-free gp120 appeared distinct from that of either sCD4 or antibodies directed to known neutralizing determinants of gp120. Consistent with this view were the potent inhibitory effects of CV-N against primary HIV-1 isolates which are highly resistant (53, 55, 71) to neutralization by sCD4 or by most anti-gp120 antibodies.

CV-N did not block the binding of virus-free gp120 with sCD4, suggesting that CV-N's antiviral effects against the intact virus might be manifest after the initial virus-to-cell attachment phase, but prior to completion of viral entry and intracellular initiation of the viral replicative process. Consistent with this possibility were results of delayed-addition studies wherein CV-N yielded no anti-HIV effect if introduced more than 3 h following exposure of host cells to virus. Thus, CV-N's anti-HIV effects were expressed only within the general timescale of the initial binding and/or fusion process, significantly preceding the reverse transcription step. The reference RT inhibitor ddC could be added much later than CV-N and still produce substantial anti-HIV activity in the study protocol employed. Further studies addressing the effects of CV-N on intact virus and virus-associated gp120 will be necessary to define more precisely the mechanism of CV-N's inhibition of gp120-mediated viral and cellular interactions.

The molecular details underlying gp120-mediated, virus-to-cell and cell-to-cell fusion are believed to share many features in common (for reviews, see references 56 and 68). Interaction of the cellular receptor CD4 with the viral gp120 induces conformational changes in gp120, as well as exposure of the viral transmembrane glycoprotein gp41, which may facilitate the fusion process. However, the binding of gp120 and CD4 is not alone sufficient for fusion or infection of $CD4^+$ host cells by HIV-1. Recent investigations have revealed that there are obligate interactions of HIV-1, HIV-2, and SIV with one or more cellular coreceptors to fully enable CD4-dependent virus-to-cell and cell-to-cell fusion and viral entry into the cell; coreceptors identified to date are from the seven-transmembrane-domain, G-protein-coupled family of cell surface receptors; the physiological ligands for these receptors are members of the CC- or CXC-chemokines (for reviews pertinent to HIV-1, see references 4, 17, 62, and 77; for HIV-2 and SIV, see also references 13, 15, 25, and 48). Chemokine receptors have also been shown to mediate cellular entry of certain forms of HIV that are capable of CD4-independent infection of cells (25).

It will be of interest to determine if CV-N disrupts any chemokine receptor interactions that occur subsequent to, in addition to, or in lieu of the viral gp120 interaction with cellular CD4. However, given current observations, it seems that any blocking of coreceptor functions on $CD4^+$ cells by CV-N would not be specific to any presently known coreceptor(s), since the inactivating effect of CV-N is conserved across a wide range of T-tropic, M-tropic, and dual-tropic forms of HIV-1, as well as HIV-2 and SIV, which use CD4 as a primary receptor but which do not necessarily share a common coreceptor preference (4, 13, 15, 17, 25, 48, 54, 62, 77). Furthermore, based on the genetic diversity represented in the forms of virus tested to date, it does not seem likely that the HIV-1-inactivating activity of CV-N will be substantially restricted between or within any known HIV-1 clade(s) (for reviews of HIV-1 clades, geographic distributions, and implications thereof, see references 26, 69, and 79).

Experimentally, CV-N may provide a useful new reagent to help further define essential steps in the gp120 mediated virus-to-cell and cell-to-cell fusion processes. Furthermore, elucidation of the molecular details of the gp120–CV-N interaction may allow the design of small-molecule mimetics of CV-N which might have therapeutic or immunoprophylactic potential. CV-N might also be incorporated directly into the design of high-throughput primary screening assays to enable an empirical search for novel, small-molecule antagonists of gp120-mediated fusion processes.

Further investigations of potential therapeutic applications of CV-N per se, as well as functional (i.e., gp120-targeting and/or HIV-inactivating) derivatives or fragments thereof (hereafter generically termed cyanovirins) and the corresponding DNA coding sequences, are warranted. Possible applications of CV-N to targeting of cytotoxic or immunological agents to HIV-infected cells will be of additional interest. Although systemic immunogenicity of the highly foreign CV-N protein may be anticipated in vivo, the significance thereof, either in the context of established or incipient HIV infection, is unknown. Moreover, the issue of immunogenicity may be irrelevant to acute or limited-term applications, in which the aim is to abort the initial establishment of HIV infection in individuals inadvertently exposed to HIV either sexually or otherwise (e.g., health-care workers exposed percutaneously and infants born to HIV-infected mothers).

More immediate opportunities for development of cyanovirin-based microbicides or for other ex vivo applications requiring HIV inactivation merit attention. The latter possibilities might include, for example, solution- or solid-phase CV-N compositions, matrix-anchored CV-N, or other cyanovirin-based technologies to inactivate and/or remove infectious HIV from medical equipment, supplies, or fluids such as blood or blood products and tissues or cells. The remarkable robustness of the HIV-inactivating activity of CV-N against physicochemical degradation and the demonstrated feasibility of genetically

transforming foreign host cells to produce the functional protein add further interest.

The use of genetically engineered microorganisms for large-scale production of CV-N or another functional cyanovirin(s) should provide a ready source of material for further development and investigation of conventional microbicidal formulations and strategies for topical prophylaxis against various modes of sexual transmission of HIV infection. Less conventional, probiotic or biotherapeutic strategies built on concepts recently reviewed by other authors (14, 24, 37) that use genetic engineering approaches to enhance microbial interference also merit consideration. For example, selected nonpathogenic microorganisms, particularly bacteria and yeasts, that normally colonize mucosal surfaces of body cavities such as the genitourinary tract, mouth, colon, and rectum are amenable to genetic modification. Such microorganisms, which might be made to express a selected antiviral protein in situ, might thereby produce a persistent mucosal barrier to viral entry. For instance, lactobacilli are prominent normal inhabitants of the vagina, and the genetic engineering of lactobacilli to produce foreign proteins is feasible (36, 46, 52). Intravaginal administration and/or long-term establishment of a lactobacillus population engineered to express CV-N might thereby create a local mucosal environment hostile to infectious HIV. It is of further interest that a large clinical trial has been initiated recently under auspices of the NIAID to explore the potential anti-HIV prophylactic efficacy of intravaginal suppository administration of live, lactobacillus cultures (23, 35, 59). That study is based on observations of in vitro anti-HIV effects of certain normal (nonengineered) H_2O_2-producing strains of lactobacilli (35). Such bacterial strains, as well as strains that do not necessarily produce H_2O_2 yet which have optimal mucosal adherence properties and/or other attributes for probiotic or biotherapeutic applications (1, 24, 50), might therefore be appropriate candidates for genetic modifications with cyanovirin-coding sequences. Whether in any such application the host would develop a mucosal immune response to CV-N is unknown, as is the clinical significance thereof.

In summary, the discovery of CV-N provides a novel lead for further investigation of new potential therapeutic and preventive strategies against HIV infection. The attributes described herein qualify CV-N as a particularly attractive candidate for microbicide development. Especially pertinent to this view is the potent virucidal activity of CV-N against M-tropic as well as T-tropic primary isolates of HIV-1. There is persuasive evidence that M-tropic forms of HIV-1 are critically involved in sexual transmission of infection (e.g., see references 21, 34, 47, and 67), and this has not heretofore been a prominent consideration in the selection or development of candidate anti-HIV microbicides.

ACKNOWLEDGMENTS

We thank R. Bernal, A. Smythe, K. Garvey, M. Merges, S. Conley, R. Moran and J. Mariner for technical input into this research. We thank R. Wittes, E. Sausville, R. Gallo, L. Arthur and P. Fischinger for reviews of the work prior to submission.

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