Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 2249–2253, March 1991
Medical Sciences

A soluble factor induced by an extract from *Pinus parviflora* Sieb et Zucc can inhibit the replication of human immunodeficiency virus *in vitro*

(viral interference/lymphokine/cytokine/pine cone)

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Communicated by Bernard Roizman, December 5, 1990 (received for review September 4, 1990)

ABSTRACT **We showed that an extract (PC6) from cones of *Pinus parviflora* Sieb et Zucc induced the human T-cell line CEM to produce a pepsin-sensitive soluble factor(s) that could inhibit the replication of the type 1 human immunodeficiency virus (HIV-1) in CEM T cells, in U-937 histiocytes, in THP-1 monocytes, and in mitogen-activated human tonsillar mononuclear cells. Indirect immunofluorescence staining and polymerase chain reaction analysis of the PC6-induced CEM cells revealed the absence of known lymphokines/cytokines except granulocyte/macrophage colony-stimulating factor (GM-CSF), interleukin 3 (IL-3), transforming growth factor β_1 (TGF-β_1), and tumor necrosis factor α (TNF-α). However, functional studies with recombinant IL-3, TNF-α, and TGF-β_1 showed that these three factors did not inhibit HIV-1 replication in CEM cells. Neutralization of the PC6-induced HIV-1-inhibiting factor(s) with commercially available neutralizing antibodies to GM-CSF and TNF-α also did not abrogate the anti-HIV-1 impact. Thus, the anti-HIV-1 factor induced by PC6 may be novel. Molecular sieve separation showed that the anti-HIV-1 factor(s) is smaller than 30 kDa. Affinity chromatography using a DEAE-cellulose column enriched the factor that inhibited HIV-1.**

As our laboratory has previously reported (1), PC6, an extract from cones of *Pinus parviflora* Sieb et Zucc, can inhibit the replication of the type 1 human immunodeficiency virus (HIV-1) via two pathways. First, PC6 can bind to HIV-1 reverse transcriptase to inhibit reverse transcription of RNA to DNA. Second, PC6 modifies the intracellular microenvironment and renders the PC6-treated cells less conducive to HIV-1 replication. Pretreatment of CEM cells with PC6 did not block the absorption of HIV-1 onto the target cells (1). Yet, a single dose of PC6, used to pretreat CEM cells for 1 to 7 days, was in itself sufficient to inhibit HIV-1 replication by 80% (1). *In vivo* and *in vitro*, PC6 has been shown to possess potent immune modulatory activities (2, 3). We proposed that PC6 might induce CEM cells to produce lymphokines or cytokines, or both, that inhibited the replication of HIV-1 (1). Earlier reports have shown that lymphokines and cytokines can modulate HIV-1 replication in infected cells (4–9). Among the known lymphokines/cytokines, only the interferons (IFNs) have been shown to possess anti-HIV-1 activities (4–6), while tumor necrosis factor α (TNF-α), interleukin-6 (IL-6), and the colony stimulation factor for granulocytes and macrophages (GM-CSF) are stimulatory and can activate latent HIV-1 (7–9). We report here that PC6 can induce CEM cells to produce a soluble factor(s), HIV-1-inhibiting factor(s) (HIF), that can inhibit HIV-1 replication.

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MATERIALS AND METHODS

Virus Strains and Cell Lines. The N1T strain of HIV-1 isolated from a patient with AIDS in New York (10) was a gift from David J. Volsky (Columbia University, New York). The virus was propagated in CEM cells (1) and filtered with 0.8-μm Nalgene filters (Millipore) before use. The CD4-positive T-cell line CEM was obtained from the Centers for Disease Control. The human histiocytic lymphoma cell line U-937 and the monocytic leukemia cell line THP-1 were obtained from the American Tissue Culture Collection.

Chemicals, Antibodies, and Chromatography Materials. PC6, an extract from cones of *P. parviflora* Sieb et Zucc, was prepared as described (1, 11). Briefly, pine cones were finely minced, washed extensively with alcohol, and boiled in water for 4 hr three times. The residue collected was extracted twice with 1% NaOH for 4 hr at 20°C, and PC6 was precipitated from the liquid phase at pH 5.0 with acetic acid. The PC6 did not contain any protein (11). Monoclonal antibodies to human IFN-α, -β, and -γ were from Green Cross (Osaka, Japan). Commercially available rabbit antibodies specific to human IL-1 to IL-4, IL-6, GM-CSF, G-CSF, M-CSF, TNF-α and -β were purchased from Genzyme. These antibodies were used for neutralization and cytochemical studies carried out according to the manufacturer's instructions. Recombinant human IFN-α and transforming growth factor β_1 (TGF-β_1) were obtained from Collaborative Research. Recombinant human GM-CSF, TNF-α, and IL-3 were purchased from Genzyme. Ammonium sulfate was obtained from Sigma. Immobilized pepsin crosslinked to 6% beaded agarose was from Pierce. DEAE-cellulose was obtained from Whatman. The molecular sieves with cutoffs of 30 kDa (Centricon-30 and SIY30) and 3 kDa (Centricon-3 and SIY3) were obtained from Amicon. Gel filtration standards were from Bio-Rad.

RNA Isolation, Reverse Transcription, and Polymerase Chain Reaction (PCR). Total RNA was extracted with 4 M guanidine thiocyanate as described (12) and was digested with RNase-free DNase (Worthington) at 200 μg/ml for 1 hr at 37°C to give RNA completely free of DNA. The RNA was then extracted with phenol/chloroform, 1:1 (vol/vol), and precipitated with ethanol. Reverse transcription of RNA (10

Abbreviations: GM-CSF, granulocyte/macrophage colony-stimulating factor; HIV-1, human immunodeficiency virus type 1; HIF, HIV-1-inhibiting factor(s); IFN, interferon; IL, interleukin; PCR, polymerase chain reaction; PHA, phytohemagglutinin; $TCID_{50}$, tissue culture mean infectious dose; TGF-β_1, transforming growth factor β_1; TMC, tonsillar mononuclear cells; TNF-α, tumor necrosis factor α.

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μg) was performed with 0.5 μg of oligo(dT) and the Red Module kit (InVitrogen, San Diego) to give cDNA templates for PCR amplification with paired oligonucleotide primers for known lymphokines/cytokines. Paired primers were produced in accordance with sequences contained within the GenBank nucleic acid database. The primer pairs produced PCR products that spanned the following sequences: IL-1α, nucleotides 582-1121 (accession no. M15329); IL-1β, nucleotides 524–896 (accession no. M15330); IL-2, nucleotides 293–4497 (accession no. K02056); IL-3, nucleotides 10–463 (accession no. M14743); IL-4, nucleotides 64–525 (accession no. M13982); IL-5, nucleotides 45–449 (accession no. X04688); IL-6 (IFN-β_2), nucleotides 399–701 (accession no. M18403); IL-7, nucleotides 385–917 (accession no. J04156); IFN-α4B, nucleotides 1021–1286 (accession no. X02955); IFN-γ, nucleotides 109–609 (accession no. X13274); TNF-α, nucleotides 796–2592 (accession no. X02910); TNF-β, nucleotides 1276–2226 (accession no. M16441); TGF-β_1, nucleotides 1859–2017 (accession no. X02812); GM-CSF, nucleotides 16–447 (accession no. M11734); and β-actin, nucleotides 587–1146 (accession no. X00351), which was used as an internal control. The paired primer sequences used for IL-8 were as described (13). PCR amplification of the cDNA was performed in a 100-μl reaction mixture containing 1.25 mM of each of the four dNTPs, 50 mM KCl, 10 mM Tris·HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 0.6 μg each of the paired primers, and 2.5 units of *Thermus aquaticus* DNA polymerase (United States Biochemical). Each cycle comprised 1 min of denaturation (94°C), 1 min of annealing (55°C), and 2 min of extension (72°C). After 30 cycles, the amplified DNA was electrophoresed on a 1.5% agarose gel containing 0.5 μg of ethidium bromide per ml, and the DNA was visualized under ultraviolet light.

Preparation of Soluble Factor(s) with Anti-HIV-1 Activity. Unless indicated otherwise in the text, anti-HIV-1 factor(s) was produced in CEM cells (1.5×10^5 cells per ml) maintained in medium [RPMI-1640 with antibiotics and 5% (vol/vol) newborn calf serum] and induced with 5–10 μg of PC6 per ml. As the control, CEM cells were maintained in medium alone. After incubation for 3 days, the cell-free supernatant fluids were collected, clarified at 9000 × *g*, and filtered through a 0.22-μm Nalgene filter before assay. To prepare PC6-free supernatant fluids, the pellets of treated cells were washed twice, suspended at 1×10^7 cells per ml in serum-free medium, and incubated for an additional 16 hr. Supernatant fluids from the cultures were then processed as described above.

Assay for HIV-1 Replication. The ability of PC6 and supernatant fluids produced by PC6-induced CEM cells to inhibit HIV-1 replication was assayed as described (1) and quantitated with an ELISA test specific for the HIV-1 p24 gag protein.

RESULTS

It has been suggested (1) that PC6 may induce CEM cells to produce a lymphokine that inhibits HIV-1 replication in CEM cells. To confirm this hypothesis, we collected culture supernatant fluids from uninfected CEM cells that had been treated with various doses of PC6 for 3 days and then tested these cell-free supernatant fluids to see if they could inhibit HIV-1 replication in freshly infected CEM cells. Fig. 1 shows that HIV-1 replication was inhibited when infected CEM cells were maintained in medium containing 3–30% of such a supernatant. Supernatant fluids collected from CEM cultures not induced by PC6 did not inhibit HIV-1 replication (data not shown). The ability of the PC6-induced supernatant fluids to inhibit HIV-1 replication was not restricted to CEM cells. Viral replication was also inhibited in U937 histiocytes and THP-1 monocytes (Fig. 1). Thus, the cell-free supernatant fluid of uninfected CEM cells induced by PC6 possessed anti-HIV-1 activity. This inhibitory activity could not have been mediated by PC6 that might have been present because 3% transfer of a supernatant fluid (containing 3% of PC6 at 5 μg/ml—i.e., 0.15 μg/ml) was able to inhibit HIV-1 in infected CEM cells, whereas twice this amount of PC6 (0.3 μg/ml) added directly to the infected cells for 5 days could not. The anti-HIV-1 activity could be found in supernatant fluids collected from uninfected CEM cells treated with 5 μg of PC6 per ml for 1–7 days. A 10% volume transfer of the 1-, 3-, and 7-day-treated supernatant fluids inhibited HIV-1 replication in CEM cells by 97%, 98%, and 98%, respectively (data not shown).

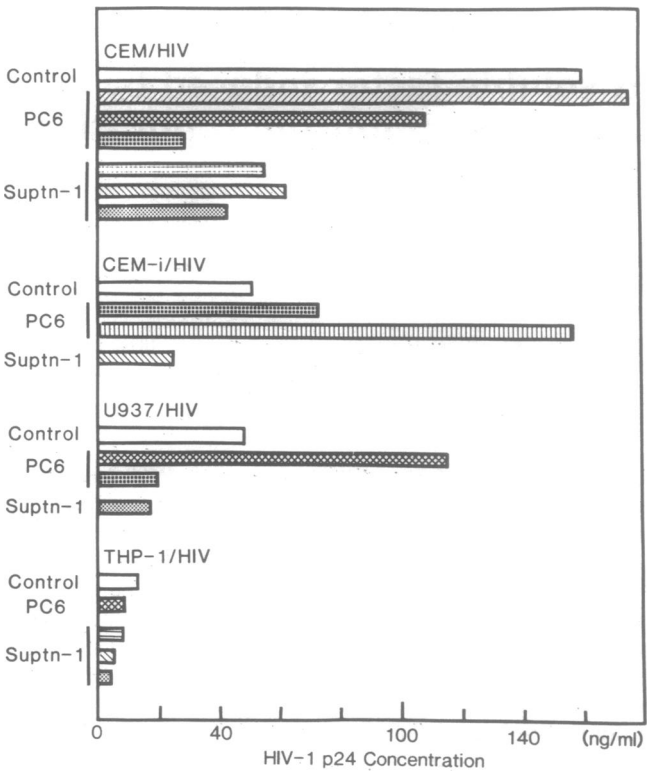

FIG. 1. Impact of PC6-induced supernatant fluids on HIV-1 replication in HIV-1-infected CEM, CEM-i, U-937, and THP-1 cells. Target cells (1×10^6 cells) were incubated with HIV-1 (2×10^5 $TCID_{50}$) for 1 hr at 37°C. The infected cells were washed extensively and maintained in medium containing various concentrations of a PC6 or 1–30% cell-free supernatant fluid (Suptn-1) from PC6-induced CEM cells containing anti-HIV-1 activity. Suptn-1 was derived from an uninfected CEM culture treated with 5 μg of PC6 per ml for 3 days as described. Untreated infected cells served as negative controls. Positive controls included infected cells maintained in medium containing 30, 10, 3, or 0.3 μg of PC6 per ml. Unless stated otherwise in the text, test samples and controls were performed in duplicate cultures. In all experiments, the infected cells (1.5×10^5 cells per ml) were incubated at 37°C for 5 days. The culture supernatants containing HIV-1 were collected and filtered through 0.45-μm filters. The concentration of HIV-1 in each supernatant was determined by a commercially available p24 capture assay (Coulter) as described (1). CEM-i is permissive to HIV-1, but PC6 cannot inhibit HIV-1 replication in these cells. PC6: ▨, 0.3 μg/ml; ▩, 3 μg/ml; ▩, 10 μg/ml; ▩, 30 μg/ml. Suptn-1: ▤, 1%; ▩, 3%; ▧, 10%; ▨, 30%.

Two sets of experiments confirmed that contaminating PC6 in the supernatant fluids was not responsible for the impact on HIV-1 replication. First, we used a PC6-resistant CEM subline developed in our laboratory, CEM-i, to test the anti-HIV-1 activity of the supernatant fluids. The CEM-i cells were permissive to HIV-1, but PC6 did not inhibit HIV-1 replication in these cells. Fig. 1 shows that 30 μg of PC6 per ml failed to inhibit HIV-1 replication in the CEM-i cells. In contrast, 10% vol transfer of a cell-free supernatant fluid derived from wild-type CEM cells treated with 5 μg of PC6

Table 1. Impact on HIV-1 replication in CEM cells and in mitogen-activated human tonsillar mononuclear cells (TMC) by PC6-induced culture supernatant fluids

Exp.	Cells	Culture treatment: Medium	Conc., %	HIV-1 p24, ng/ml	% inhibition
A	Infected TMC	Untreated control		18.0	0
		+ supernatant 9	30	8.3	54
B	Infected CEM cells	Untreated control		51.0	0
		+ supernatant 9	30	25.2	51
		+ PC6-free supernatant 9	30	23.3	54
C	Infected CEM cells	Untreated control		915.3	0 (0)
		+ PC6-free supernatant 9	50	309.1 (245.5)	66 (73)
		(or <30-kDa permeate)	30	538.1 (577.3)	41 (37)
			20	712.4 (770.4)	22 (24)
			10	1055.0 (818.4)	(11)

The CEM cells or freshly isolated TMC were incubated with HIV-1 at 37°C for 4 hr. After extensive washing, infected CEM cells were cultured in medium alone or in medium containing 30% PC6-induced culture supernatant fluid. Infected TMC were cultured at 1×10^6 cells per ml in phytohemagglutinin (PHA)-containing medium (1 μg of PHA per ml) alone or in the same medium containing 30% PC6-induced culture supernatant fluid. After 5 days of incubation, virus concentration in the cell-free culture supernatant was assayed by a p24 assay as described in Fig. 1. The percentage inhibition of HIV-1 replication is given by [T/C] × 100, where T is the amount of p24 in the test cell-free culture fluid, and C is the amount of p24 in the control cell-free culture fluid. A PC6-containing supernatant fluid was prepared by treating uninfected CEM cells with 10 μg of PC6 per ml for 3 days, and the cell-free culture supernatant fluid was recovered as described to give PC6-containing supernatant 9 (see experiments A and B). The pellet of treated cells was washed extensively before reseeding at 1×10^7 cells per ml in serum-free medium without PC6. After 16 hr, PC6-free supernatant fluid was collected to give PC6-free supernatant 9 (see experiments B and C). The PC6-free supernatant 9 was allowed to pass through a 30-kDa molecular sieve. The retentate gave molecules > 30 kDa. The permeate contained molecules < 30 kDa ("<30 kDa permeate"; see experiment C). Anti-HIV-1 activity was titrated in HIV-1-infected CEM cells as described above. Anti-HIV-1 activity of the <30-kDa permeate is given in parentheses in experiment C. The fraction with molecules > 30 kDa had no anti-HIV-1 activity even when transferred at 100% equivalent.

per ml was able to inhibit HIV-1 replication by 54% in these CEM-i cells (Fig. 1). In the second set of experiments, the contaminating PC6 was removed (to give PC6-free supernatant fluids) prior to assay for anti-HIV-1 activity. The characteristic absorption spectrum of PC6 was not observed when the PC6-free supernatant fluids (e.g., PC6-free supernatant 9 in Table 1, experiment B) were scanned over the range of UV and visible light (190–900 nm). Table 1, experiment B shows that 30% volume transfer of a PC6-free supernatant fluid (PC6-free supernatant 9) inhibited HIV-1 replication by 54%, similar to that obtained with the PC6-containing supernatant fluid (i.e., 51% inhibition by supernatant 9). Taken together, these results suggest that PC6 treatment of CEM cells induced the production of a soluble mediator(s) that could inhibit HIV-1 replication in CEM cells.

To further study the anti-HIV-1 factor, the PC6-treated CEM culture supernatant fluid was fractionated by molecular size with a 30-kDa molecular sieve. The unfractionated supernatant fluid and its fractions were then titrated for their anti-HIV-1 activity. Most, if not all, of the anti-HIV-1 activity was found in the fraction containing molecules with molecular weights < 30,000 (Table 1, experiment C). The fraction containing molecules with molecular weights > 30,000 had no anti-HIV-1 activity even when transferred at 100% equivalent. Anti-HIV-1 activity of the supernatant fluid was not restricted to HIV-1-infected laboratory cell lines; it also inhibited HIV-1 replication in freshly collected human TMC activated by PHA (Table 1, experiment A). The same supernatant fluid, when used at an equivalent concentration, did not inhibit the mitogenic response of TMC to PHA as assessed by cellular incorporation of [^{3}H]thymidine (data not shown).

To determine if the anti-HIV-1 factor (<30 kDa) found in the supernatant fluids of PC6-activated CEM cell cultures is a known lymphokine/cytokine, we used a panel of commercially available lymphokine/cytokine-specific antibodies to stain CEM cells treated with 10 μg of PC6 per ml for 1–3 days. Untreated CEM cells served as controls. Indirect immunofluorescence staining of acetone-fixed cell smears revealed the presence of IL-3 in the untreated CEM cells, while IL-3, TNF-α, and GM-CSF were found in a small proportion (<10%) of the PC6-treated CEM cells. Other lymphokines/cytokines, including IFN-α, -β, and -γ, were not detected. PCR analysis of the PC6-treated CEM cells with a panel of lymphokine/cytokine-specific oligonucleotide primers (see *Materials and Methods* for the primers used) also confirmed this observation and detected mRNAs coding for GM-CSF, TNF-α, and IL-3 (data not shown). Additionally, PCR also detected the mRNA coding for TGF-β_1. Four hours of preincubation with neutralizing antibodies specific to GM-CSF (Table 2, experiment A) and TNF-α (Table 2, experiment B) before testing failed to abrogate the anti-HIV-1 activity found in the supernatant fluids from PC6-treated CEM cell cultures. Furthermore, recombinant human TNF-α (Table 2, experiment C), IL-3 (Table 2, experiment C), and TGF-β_1 (Table 2, experiment D) tested against acutely infected CEM cells did not inhibit HIV-1 replication. These results suggest that the HIF produced by the PC6-treated CEM cells was unrelated to IL-3, TNF-α, TGF-β_1, and GM-CSF. Taken together, these results also suggest that the HIF produced by CEM cells treated with PC6 might have been a novel lymphokine or cytokine.

To enrich HIF, supernatant fluids from CEM cells treated with 10 μg of PC6 per ml for 3 days were chromatographed on a DEAE-cellulose column (pH 7.4). A typical elution profile of HIF with 0.05–0.6 M NaCl is shown in Fig. 2. The fractions eluted with 0.25 and 0.35 M NaCl were found to have the highest HIF activity (Fig. 2 and Table 3, experiment

Table 2. HIV-1 replication in HIV-1-infected CEM cells in the presence of recombinant human TNF-α, IL-3, and TGF-β_1 (rhTNF-α, rhIL-3, and rhTGF-β_1) and HIF neutralized with anti-GM-CSF and anti-TNF-α antibodies

Exp.	Culture treatment: Medium	Conc.	HIV-1 p24, ng/ml	% inhibition
A	Untreated control		132.0	0
	+ anti-GM-CSF Ig	1 μg/ml	118.0	11
	+ supernatant 3	10%	62.1	53
	+ supernatant 3 and anti-GM-CSF Ig	10% 1 μg/ml	42.2	68
B	Untreated control		85.0	0
	+ anti-TNF-α Ig	1 μg/ml	86.3	0
	+ supernatant 4	10%	11.7	87
	+ supernatant 4 and anti-TNF-α Ig	10% 1 μg/ml	5.1	94
C	Untreated control		235.3	0
	+ rhTNF-α	0.2 ng/ml	236.7	0
		0.5 ng/ml	272.6	0
	+ rhIL-3	500 cfu/ml	226.2	4
		2500 cfu/ml	214.1	9
D	Untreated control		152	0
	+ rhTGF-β_1	10.0 ng/ml	183	0
		2.0 ng/ml	182	0

The supernatant fluids used to inhibit HIV-1 replication were prepared as described. HIV-1-infected CEM cells were cultured as described in Table 1 in the media indicated above. After 5 days, the amounts of HIV-1 p24 in the cell-free culture fluids were quantitated, and the percentage inhibition of HIV-1 replication was calculated as described in the legend to Table 1.

A). For example, when tested at dilutions of 1:50 and 1:10, an enriched HIF preparation inhibited HIV-1 replication by 77% and 94%, respectively (Table 3, experiment A). This degree of HIV-1 inhibition is higher than that observed with 1000 units of IFN-α per ml, which served as the positive inhibition control (Table 3, experiment A). Titration studies showed that the ion-exchange column greatly enriched the HIF (compare experiment C in Table 3 with experiments B and C in Table 1). A 51% inhibition of HIV-1 was achieved when HIF-2 enriched from supernatant 9 was tested at a dilution of 1:100 (Table 3, experiment C) as compared with 30% vol

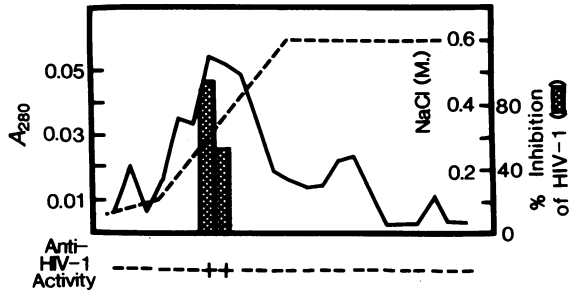

FIG. 2. DEAE-cellulose column chromatography profile and anti-HIV-1 activity of HIF. Cell-free supernatant fluid from uninfected CEM cells induced by 10 μg of PC6 per ml was passed through a 30-kDa molecular sieve under 18 psi (1 psi = 6.89 kPa). Fractions < 30 kDa were concentrated by retention with a 3-kDa molecular sieve at 18 psi before precipitation with ammonium sulfate at a final concentration of 30%. The precipitate recovered after centrifugation at 9000 × *g* was redissolved in a minimal volume of elution buffer [10 mM Tris, pH 7.4/50 mM KCl/0.1 mM dithiothreitol/50 mM NaCl] and chromatographed through a 15 × 300 mm DEAE-cellulose column by elution buffer (pH 7.4). The collected fractions (10 ml per fraction) were desalted by use of Centricon-3 centrifugation and assayed for their protein concentration (determined by absorption at A_{280}) and anti-HIV-1 activities. Anti-HIV-1 activity in each fraction was assayed at a dilution of 1:30, and the percentage inhibition of HIV-1 replication was calculated as in Table 1.

Table 3. Impact of enriched HIF on HIV-1 replication in HIV-1-infected CEM cells

Exp.	Culture treatment: Medium	Conc.	HIV-1 p24, ng/ml	% inhibition
A	Control		426.3	0
	+ HIF-1	2%*	97.2	77
		10%*	23.9	94
	+ rhIFN-α	100 U/ml	266.0	38
		1000 U/ml	171.3	60
B	Control		79.6	0
	+ HIF-1	1%*	37.7	53
		3%*	16.3	80
	+ Pepsin-digested HIF-1	1%*	65.9	17
		3%*	48.6	39
C	Control		273.0	0
	+ HIF-2	0.2%*	212.4	22
		1.0%*	134.0	51
		3.3%*	19.6	93
		10.0%*	17.7	94

The HIF in cell-free supernatant fluids was enriched by DEAE-cellulose chromatography as described in Fig. 2. Anti-HIV-1 activity in culture media containing the enriched HIF-1 (2% or 10%) or a recombinant human IFN-α (rhIFN-α) preparation (experiment A), containing the enriched HIF-1 (1% or 3%) or pepsin-digested HIF-1 (experiment B), and containing the enriched HIF-2 (0.2%, 1%, 3.3%, or 10%) (experiment C) was determined in HIV-1-infected CEM cells, and the percent inhibition of HIV-1 replication was calculated as described in Table 1. The HIF-1 and the HIF-2 were enriched from two separately prepared supernatant fluids. The HIF-2 was enriched from supernatant 9 shown in Table 1. Pepsin digestion of HIF-1 was performed by treatment with immobilized pepsin crosslinked to 6% beaded agarose (pH 7.2) for 48 hr at 4°C with constant agitation.
*Dilutions of HIF-1 or HIF-2 in media: 2% = 1: 50 dilution, and 0.2% = 1:500 dilution. U, units.

transfer of the unfractionated supernatant 9 (Table 1, experiments B and C). To demonstrate that HIF is a protein, an aliquot of an enriched HIF was treated with immobilized pepsin crosslinked to 6% beaded agarose (pH 7.2) at 4°C for 48 hr with constant agitation. The immobilized pepsin was removed by centrifugation, and the pepsin-digested HIF was filtered through a 0.22-μm filter before testing for its anti-HIV activity in the presence of 2% newborn calf serum. Table 3, experiment B shows that HIF is a protein and was pepsin sensitive. The HIF also had no IFN-like activity. It did not inhibit HSV-1 replication in human embryonic lung cells when tested at a concentration that gave 94% inhibition of HIV-1. In contrast, IFN-α at 10 and 100 units/ml inhibited herpes simplex virus type 1 plaque formation in human embryonic lung cells by 36% and 62%, respectively, whereas 100 units of IFN-α per ml inhibited HIV-1 replication by 38%.

DISCUSSION

Among the natural host cells for HIV-1, actively proliferating cells from the lymphoid and the myeloid elements are most sensitive to productive, cytocidal HIV-1 infection (14–16). These cell types are also exquisitely sensitive to regulation by interleukins and cytokines (17–20). Hence, it is not surprising that HIV-1 replication and production in these cells can be regulated by interleukins and cytokines. Previous reports have shown that GM-CSF (7–9), TNF-α (8, 9) and IL-6 (9) can activate latent HIV-1 in ACH-2 T cells and/or U1 histocytic cells. Among the interleukins and cytokines, only the IFNs (4–6), especially IFN-α (4, 6), have been reported to be effective in inhibiting HIV replication. Here, we show that PC6 can induce uninfected CEM cells to produce a soluble factor(s) (HIF) that can inhibit HIV-1 replication in CEM cells. Since the HIF is pepsin sensitive and there are no

proteins in PC6 (11), we have ruled out the possibility that HIF is PC6 or a metabolite of PC6.

Immunofluorescence staining and PCR analyses revealed that uninfected CEM cells treated with PC6 were positive for GM-CSF, IL-3, TGF-β_1, and TNF-α but not for any of the IFNs or other known lymphokines/cytokines. Moreover, neutralization and functional studies showed that HIF was not related to GM-CSF, TGF-β_1, IL-3, and TNF-α. Since GM-CSF and TNF-α were produced by PC6-induced CEM cells and are most likely present in the crude supernatant fluids we tested, results presented in Fig. 1 and Table 1 suggest that the HIF could override the previously reported (7–9) positive impact of GM-CSF and TNF-α on HIV-1 activation. Our studies also show that HIF is a potent inhibitor of HIV-1 as compared with IFN-α. From these results, it appears that HIF may be a novel factor. The HIF also inhibits HIV-1 in TMC but has little impact on the mitogenic response of these cells to PHA.

The inhibition of HIV-1 by PC6-induced HIF is in accord with our original hypothesis that PC6 can induce CEM cells to produce factors that render the intracellular microenvironment less conducive to HIV-1 replication (1). Additionally, HIF inhibition of HIV-1 replication by treatment of the infected cells 4 hr after infection—i.e., after the reverse transcription of viral genomic RNA to DNA is virtually completed (21)—suggests that HIF might interfere with the replicative life cycle of HIV-1. We have shown (21) that PC6 interferes with HIV-1 replication by inhibiting HIV-1 LTR-driven gene transcription. Whether HIF interferes with this pathway awaits further studies. If HIF is as active *in vivo* as it is *in vitro*, it has the potential to be used as a potent anti-HIV-1 therapeutic agent. However, neither the normal function(s) of HIF nor the prevalence of HIF in normal and in infected persons during the acute, chronic, and late phase of HIV-1 infection is known and awaits further studies when the HIF gene and highly purified HIF protein become available.

We thank J. Donovan and Dr. Y. T. Wu for technical assistance. This work was supported by a U.S. Public Health Service Grant U01 AI27280 from the National Institutes of Health, and a grant for enhancement of AIDS research (LP116) from the State of Florida.

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