HUMIC ACID

INHIBITION OF HIV-1 REPLICATION

USC SCHOOL OF MEDICINE

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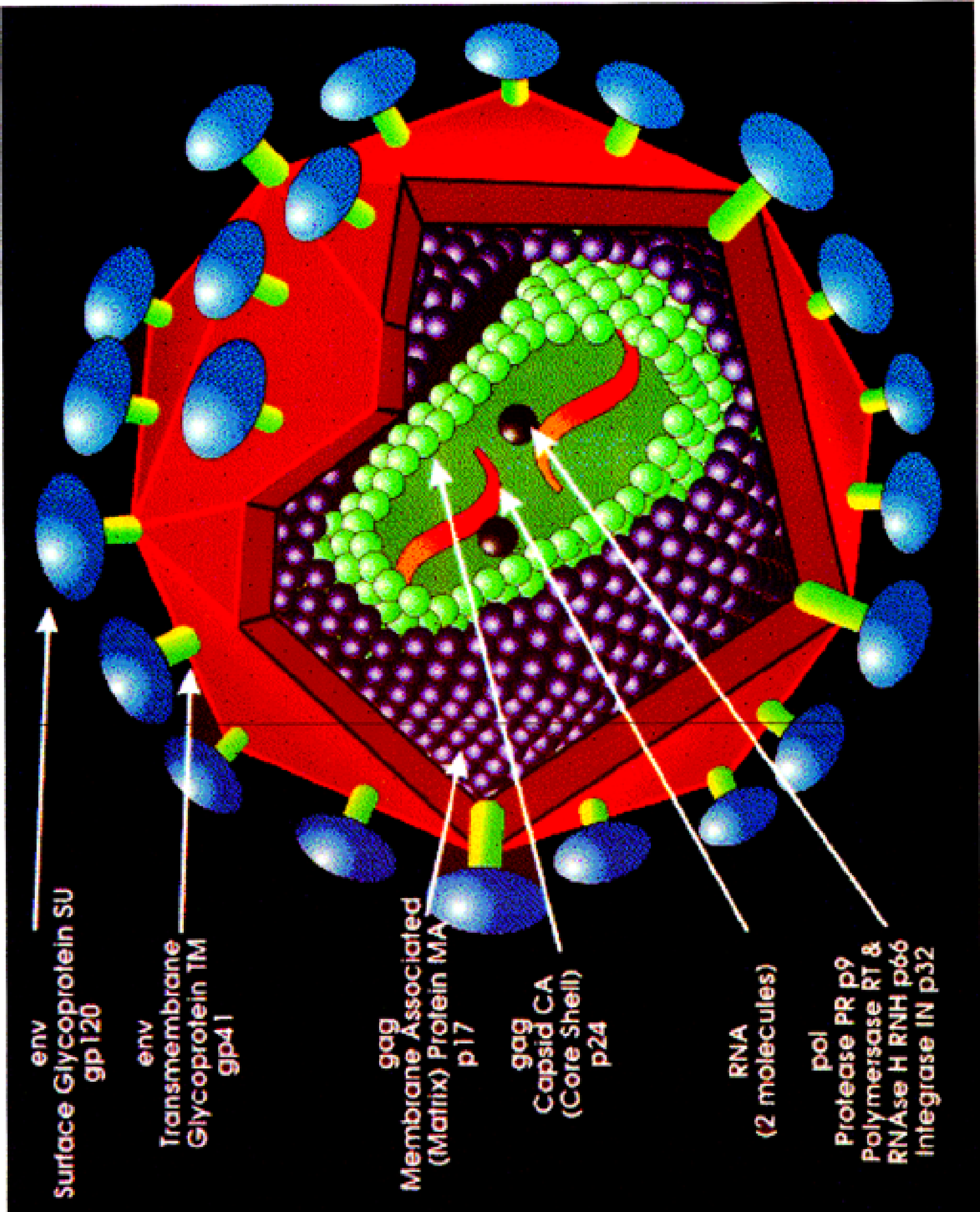
env
Surface Glycoprotein SU
gp120
env
Transmembrane
Glycoprotein TM
gp41
gag
Membrane Associated
(Matrix) Protein MA
p17
gag
Capsid CA
(Core Shell)
p24
RNA
(2 molecules)
pol
Protease PR p9
Polymersase RT &
RNAse H RNH p66
Integrase IN p32

FRONTISPIECE*: *Schematic representation of the AIDS virus.

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ABBREVIATIONS

AIDS, acquired immunodeficiency syndrome

AZT, 3'-azido-2',3'-dideoxythymidine (Zidovudin)

bp, base pair

EDTA, ethylenediaminetetraacetic acid

FBS, fetal bovine serum

GMP, good manufacturing practices

HA, humic acid

HBSS, Hank's balanced salts solution

HIV-1, human immunodeficiency virus, type 1

HPLC, high-performance liquid chromatography

IFN-γ, interferon gamma

IL-2, interleukin 2

PBMC, peripheral blood mononuclear cells

PCR, polymerase chain reaction

PHA-P, purified phytohemagglutinin

RT, reverse transcriptase

SDS, sodium dodecyl sulfate

Tris HCl, *tris*(hydroxymethyl)aminomethane hydrochloride.

SUMMARY

Human immunodeficiency virus (*HIV-1*) and acquired immunodeficiency syndrome (*AIDS*) are today relentlessly eroding entire populations and, potentially even more calamitous, threaten to become endemic in future generations. The total number of individuals infected by HIV-1 was estimated at the end of 1997 to exceed 30 million people. Of these, well over one million are children. (In the U.S., the fastest growing infected population segments are women, blacks, and hispanics.) The development of an effective treatment for HIV-1 infection and its sequelae in man is therefore considered one of society's highest priorities.

Of the treatments currently available for HIV-1 and AIDS, those based on the use of AZT and dideoxynucleosides, or newer drugs that inhibit reverse transcriptase (RT) and viral proteases, give rise to problems that limit or otherwise severely restrict their clinical use. Some are toxic; others cannot combat the rapid insurgence of virus mutation during therapy; and still others, albeit only marginally effective, are nonetheless so expensive as to be far beyond the reach of all but a handful.

Laub BioChemicals Corporation (“*Laub BioChem*”) was formed in 1995 to perform research and development on nontoxic natural-product as well as synthetic analogues of humate materials that have long been recognized as highly effective antiviral agents. Humates exhibit strong anti-HIV-1 replication activity, negligible toxicity, and immunocytoprotective effects. They offer, in effect, a means of aiding the body’s immune system to arrest the development of AIDS.

INTRODUCTION

Type-1 human immunodeficiency virus (*HIV-1*) was discovered in 1983, and is currently accepted as the etiological agent for acquired immunodeficiency syndrome (*AIDS*). 1983 also marks the beginning of the quest for the means of prevention of HIV-1 infection, as well as treatments if contracted. However, it is fair to say at this time that neither of these goals has yet been approached with any notable degree of success. In fact, the HIV-1 drugs currently recommended [*e.g.*, 3'-azido-2',3'-dideoxythymidine (*AZT*) and other nucleoside analogs; HIV-1 protease inhibitors; and antisense RNA] exhibit only limited effectiveness in reducing virus replication and restoration of the immune system. Indeed, these drugs appear to be efficacious only with patients who still have relatively intact immune systems. The treatments currently available are further limited by various host immunologic deficits, low therapeutic indices of the drugs themselves, and the evolution of drug-resistant strains of viruses. The need for development of new classes of HIV-1 drugs therefore remains.

This report documents the results obtained to date of studies of a unique class of materials that potentially represent co-therapeutic agents for AIDS. It was found in this work that both synthetic as well as naturally-occurring humic substances extracted from soil are in fact capable of inhibiting HIV-1 replication. The findings are based on the in vitro monitoring of cell viability; HIV-1 p24 expression; reverse transcriptase (*RT*) levels in cell culture supernatants; and molecular assays of viral *m*RNA.

MATERIALS AND METHODS

Materials

Jurkat and HUT 78 cells were obtained from the American Type Culture Collection (Rockville, MD); fresh normal peripheral blood mononuclear cells (*PBMC*) were donated by members of the natural-product research team; and HIV-1 infected blood samples were donated by AIDS patients from the greater Los Angeles area (informed consent was obtained in each case). The naturally-occurring humic substance, *HA*, upon which the synthetic humates are based, was obtained from Aldrich Chemical (Milwaukee, WI).

Methodologies Employed for Drug Testing

Both the natural-product material, HA, as well as its synthetic counterparts, were tested in vitro using two widely-accepted systems that enable the monitoring of HIV-1 replication. In the first system Jurkat cells were transfected with an HIV-1 molecular construct; while in the second, HUT 78 cells were chronically infected with a laboratory strain of HIV-1. Both infected cell cultures produced high levels of HIV-1 (approximately 1 x 10^7 particles/mL, as measured by electron microscopy).

In addition, HA and the synthetic humates were also tested with three other systems. The first made use of freshly obtained peripheral blood mononuclear cells that were infected with HIV-1_{4105}; the second utilized U937 cells (aprotypic monocytic cell line) that were also infected with HIV-1_{4105}; while the third comprised PBMC isolated from the freshly drawn heparinized blood of HIV-1 infected patients.

Cell Cultures. Established cell lines (Jurkat, HUT 78, and U937 cells) were subcultured every fifth day using RPMI-1640 medium supplemented with 2 *mM* L-glutamine and 15% *v/v* fetal bovine serum (*FBS*; ICN, Costa Mesa, CA).

PBMC obtained fresh from the heparinized blood of normal as well as HIV-1 infected donors were separated against a Ficoll-Hypaque gradient, and were then stimulated for 48 h with 5 μg/mL purified phytohemagglutinin (*PHA-P*; Burroughs-Wellcome, SC). The medium was replaced with RPMI-1640, 2 *mM* L-glutamine, 15% *v/v* FBS, and 10% *v/v* interleukin 2 (*IL-2*) every 3 days.

Cell counts were determined with a Coulter particle counter (Coulter Corporation, Hialeah, FL).

The presence of HIV-1 was established by transmission and retransmission of HIV-1 from naturally-infected PBMC to PHA-P stimulated normal PBMC (1 x 10^6 cells/mL) by incubating the latter with culture supernatants of the former for 2 h at 37°C; or by co-culturing PBMC in short-term cell cultures.

Infection of Jurkat, HUT 78, and PBMC Cell Cultures. Prior to studying the inhibitory effects of humates on HIV-1 replication, Jurkat cell culture supernatants were first tested for HIV-1 p24 production to establish a pretreatment baseline. Next, on the day prior to transfection, the growth medium was changed and the cell number was adjusted to 1.5 x 10^6 cells/mL. Ten million Jurkat cells were then stimulated to take up 20 μg of the HIV-1 plasmid construct, pNL4-3, in RPMI-1640 (Invitrogen electroporator; San Diego, CA). After electroporation, the cell number was adjusted to 1 x 10^6 cells/mL and the cells were then cultured in complete medium comprised of RPMI-1640 supplemented with 2 *mM* L-glutamine,

15% *v/v* fetal bovine serum, and 1% *v/v* Pen-Strep (100 *U* Penicillin + 100 µg Streptomycin/mL; ICN). The cells were monitored for approximately four weeks prior to use in order to ensure stable HIV-1 production.

HUT 78 cells were utilized for efficacy testing of humates in a manner essentially identical to that described for Jurkat cells. The PBMC from normal donors that were subsequently infected with HIV-1_{4105} were cultured in the same way.

Efficacy Testing of Humates on Infected Cell Cultures. Two days prior to treatment of infected cells with humates, and after confirming the level of virus production, equal volumes of transfected cells were admixed with normal, untreated cells to bring the level of virus production to within the range of the HIV-1 p24 immunoassay. After 24 h, the equivalent of approximately 25-50 µg/mL HA or synthetic humate was added to the blend. Cells were later collected and counted, while supernatants were assayed as described below. *m*RNA and total DNA were also extracted in the usual way.

In tests utilizing infected PBMC, humates were added only after confirming HIV-1 production. (Untreated infected cells and uninfected normal cells served as positive and negative controls, respectively.)

Assays

HIV-1 Reverse Transcriptase (RT) Activity. Culture supernatants were routinely assayed for RT activity using either Mg^{2+} or Mn^{2+} together with $dt_{15}.rA_n$ or $dt_{15}.dA_n$ as exogenous template primers for viral and cellular polymerase activities, respectively.

p24 Production. HIV-1 p24 was detected using a solid-phase assay designed for HIV-1 antigens (*HIVAG-1*; Abbott Laboratories, Abbott Park, IL; Abbott Quantum II ELISA reader and data reduction module 1.21).

Polymerase Chain Reaction (PCR): DNA-PCR. Cells were washed in Hank's balanced salts solution (*HBSS*; ICN) and lysed in buffer comprised of 10 *mM* *tris*(hydroxymethyl)aminomethane hydrochloride (*Tris HCl*), pH 8.0; 10 *mM* ethylenediaminetetraacetic acid (*EDTA*), pH 8.0; and 0.7% *w/v* sodium dodecyl sulfate (*SDS*). DNA was extracted into phenol-chloroform, precipitated with ethanol, and then dissolved in Tris/EDTA buffer (ICN) to a concentration of 3×10^4 cells/5 µL. Five microliters of the DNA solution was used for PCR amplification: tight-fitting tubes were employed with a Perkin-Elmer (Norwalk, CT) Cetus Gene Amp PCR system. The reaction was allowed to proceed at 95°C for 15 sec and then at 60°C for 1 min; the cycle was iterated 33 times.

Polymerase Chain Reaction (PCR): RT-PCR. Messenger RNA (m*RNA*) was extracted using Quick Prep micro purification kits (Pharmacia; Piscataway, NJ); following which complementary DNA (c*DNA*) synthesis was carried out using First-Strand *c*DNA kits (Clontech, Palo Alto, CA). Twenty microliters of 1:100 diluted *c*DNA was then added to 30 μL of PCR reaction mixture. The reaction was carried out for 15 sec at 95°C and then for 1 min at 60°C for 30 cycles (25 cycles was equivalent to β-actin). PCR products were analyzed on 2% *w/v* agarose gel media (FMC Bioproducts, Rockland, ME).

***DNA and* m*RNA*.** Following treatment with humates, HIV-1 infected Jurkat cells were assayed for provirus and for viral *m*RNA expression. Three different primer pairs were used for RT-PCR assay, as detailed in Table I. Two primer pairs were specific for HIV-1. One set amplified a 160-base pair (*bp*) fragment spanning the major splice donor site in the 5' region of the virus, which permitted the detection of unspliced HIV-1 *m*RNA. The second primer pair amplified a 131-bp fragment generated following splicing, with subsequent elimination of an intron between the two coding exons of *tat* and *rev*. Multiply-spliced HIV-1 *m*RNA species could thereby be detected. The last pair amplified the β-actin *m*RNA fragment, and served as an indicator of relative RNA quantity.

Drug Preparation

Humates, which are dark-brown, water-soluble amorphous solids, were separately dissolved in distilled water and filtered through 0.2-μm Nalgene filters (Rochester, NY). Appropriate dilutions were then made in complete culture medium for cell lines or PBMC, whichever was to be tested. Liquid-chromatographic fractions of natural-product humic acid (see below) were diluted directly.

Table I. Sequences for Primer Sets Used for HIV-1 Assays

List of Sequences for DNA-PCR Primers in 5' to 3' Orientation

Primer	Sequence
M667:	GGC TAA CTA GGG AAC CCA CTG
AA55:	CTG CTA GAG ATT TTC CAC ACT GAC
M661:	CCT GCG TCG AGA GAG CTC CTC TGG
SK38:	ATA ATC CAC CTA TCC CAG TAG GAG AAA T
SK39:	TTT GGT CCT TGT CTT ATG TCC AGA ATGC
Primer Set for β-Globulin:	
LA1:	ACA CAA CTG TGT TCA CTA GC
LA2:	CAA CTT CAT CCA CGT TCA CC

List of Sequences for RT-PCR Primers in 5' to 3' Orientation

Primer	Sequence
Unspliced (US):	TCT CTA GCA GTG GCG CCC GAA CA
	TCT CCT TCT AGC CTC CGC TAG TC
Multiply Spliced 9 (MS):	CTT AGG CAT CTC CTA TGG CAG GAA
	TTC CTT CGG GCC TGT CGG GTC CC
β-Actin:	CGA GCA CAG AGC CTC GCC TTT GC
	CAT AGG AAT CCT TCT GAC CCA TG

HIV-1 proviral DNA synthesis was assessed using primers amplifying the early, intermediate, and late portions of resultant material. M667/AA55 was used for early synthesis, SK38/SK39 for intermediate synthesis, and M667/M662 for late-stage synthesis. Primers for β-globulin and β-actin were employed for comparative purposes.

HPLC Fractionation of Natural-Product Humic Acid

The Hewlett-Packard (Avondale, PA) liquid chromatography system consisted of a Model HP 1050 dual pump unit, an HP UV/Vis detector employed at 340 nm, and an HP 3396-II integrator/plotter. The mobile phase was a pH gradient from 3 to 13 at a flow rate of 1.5 mL/min. Injections were made from a Rheodyne Model 7125 valve (Cotati, CA) via a 10-μL loop onto the HPLC column, 150 x 4.1 mm internal diameter, containing 10-μm reverse-phase particles.

Individual solutions of crude HA were prepared by the addition of 5-10 g to 100 mL dilute base, pH ca. 8-10. Each was allowed to stand overnight, and was then centrifuged at 3000 rpm for 10 min

RESULTS

Natural-Product Humic Acid Inhibition of HIV-1 Replication in Jurkat and HUT 78 T Cell Lines

During the first five days of treatment of Jurkat cells with natural-product HA, the total cell numbers in the infected and uninfected cultures increased similarly (cell viability remained greater than 95%; Figs. 1A,B). However, in repeated experiments the HIV-1 p24 expression was dramatically reduced by days 6-8 (Fig. 2A). (The data also showed a transient increase in the production of p24 on the second day following HA treatment, but this stimulatory effect could not be reproduced in subsequent experiments.)

The maximum inhibitory effect of natural-product HA was obtained at 50 μg/mL, as judged by the reduction achieved in RT activity (Fig. 2B). Later experiments showed that 25 μg/mL of HA as well as synthetic humates was in fact sufficient for complete inhibition of virus production. The latter amount was therefore used as a standard dose in all subsequent studies. By way of comparison, AZT inhibited HIV-1 replication roughly to the same extent, but with considerable cytostasis, at a dose of 50 μg/mL.

HA inhibition of HIV-1 replication both in Jurkat and HUT 78 cells required that the substance be present in the culture medium. HIV-1 production otherwise gradually resumed, and eventually attained levels comparable to those exhibited by untreated HIV-1 producing cells.

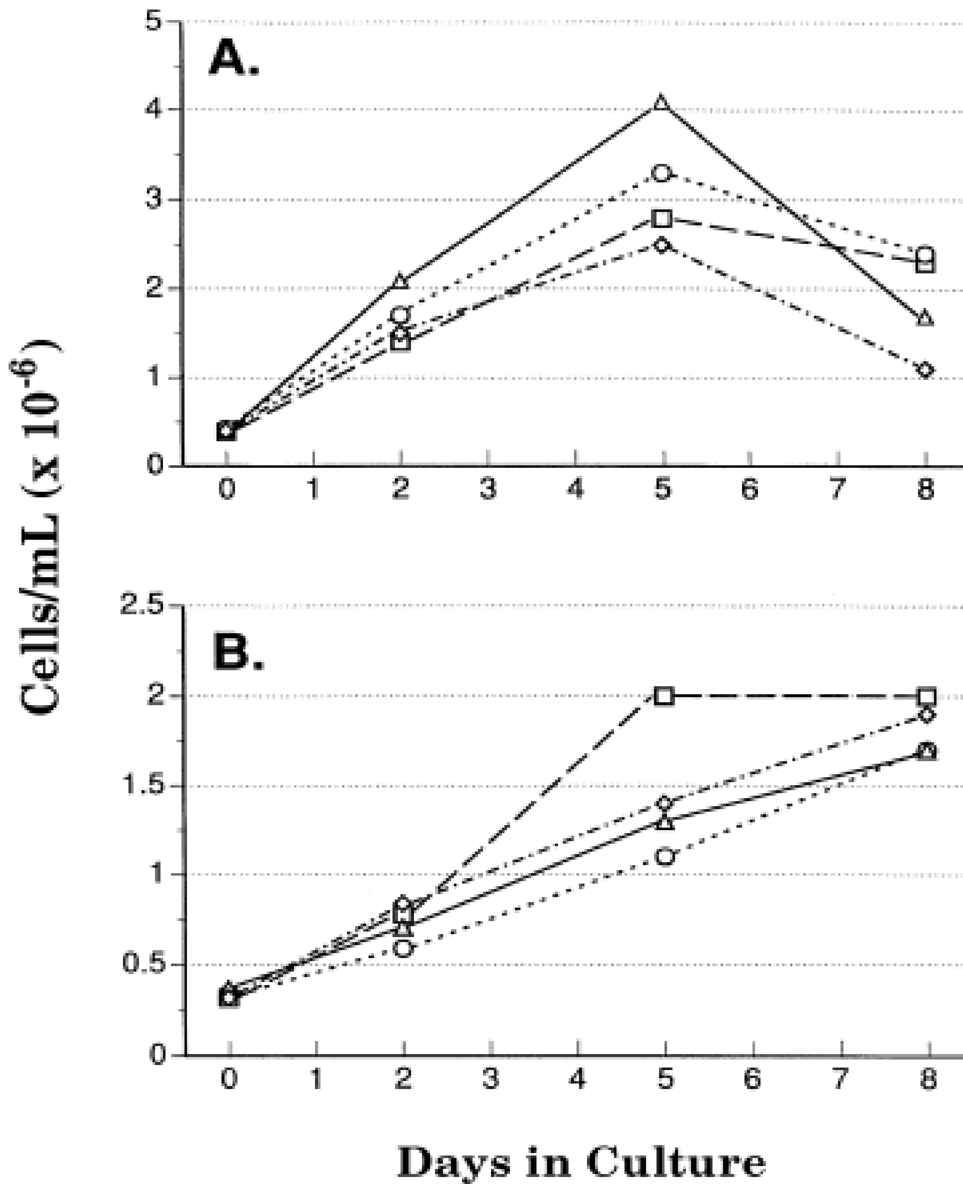

Figure 1. Effect of natural-product humic acid on the proliferation of Jurkat cells. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Viable cell numbers were determined manually by Trypan Blue dye exclusion. Symbols correspond to humate doses of 0 ([ ]---[ ]), 100 (O---O) 200 (Δ----Δ), and 500 (◊----◊) µg/mL. ***(A)*** Uninfected Jurkat cells. ***(B)*** HIV-1 infected Jurkat cells.

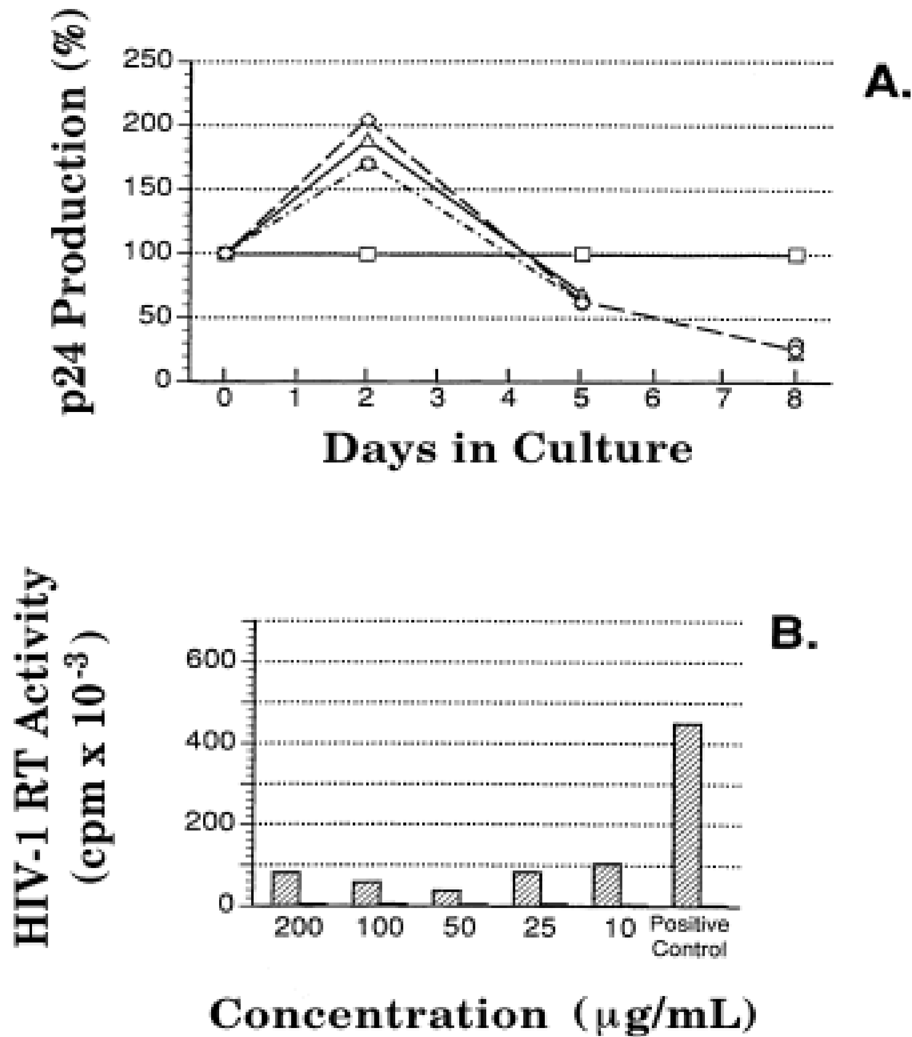

Figure 2. Effect of natural-product HA treatment on HIV-1 replication in Jurkat cells transfected with the HIV-1 molecular construct, pNL4-3. (***A***) Kinetics of HIV-1 p24 production by infected Jurkat cells. Relative p24 production (HA-treated/HA-untreated cells, x 100) is shown plotted against the period of treatment. Symbols correspond to HA doses of 0 ([ ]), 100 (◊), 200 (O), and 500 (Δ) µg/mL. (***B***) Reduction in HIV-1 RT levels released by Jurkat cells in culture supernatants on day 6 of treatment with HA. The RT activity in 5-µL samples of 30-fold concentrated cell culture media was tested using synthetic template-primer sets of oligo $dt_{15}.rA_n$ (▨) and oligo $dt_{15}.dA_n$ (■) [DNA polymerase (*DNA-P*) and purified HIV-1 RT were used as controls for cellular and viral enzymes, respectively]. Supernatant from infected untreated cells was used as a positive control.

Experiments similar to those described above were carried out with HUT 78 cells stably infected with HIV-1_{4105}. The cells were treated with a range of concentrations of HA. p24 expression as well as HIV-1 RT activity were inhibited roughly to the same degree as was found for infected Jurkat cells. The viability of HUT 78 cells following HA treatment was identical to that of untreated control cells.

Natural-Product Humic Acid Inhibition of HIV-1 Replication in Normal PBMC

Over the course of 18 days no significant difference in the viability of transfected PBMC cells was observed when treated either with IL-2 or with humic acid. However, the combination of IL-2 with HA had a synergistic suppressive effect on cell proliferation, as shown in Fig. 3A.

HA by itself reduced RT expression to background levels, Fig. 3B, and HIV-1 p24 expression to below detection levels, Fig. 3C.

HA was also tested in combination with interferon gamma (*IFN*-γ), as the latter is known to potentiate the effects of chemotherapeutic regimens as well as inhibit HIV-1 replication by enhancing the lysis of infected cells. It was observed that IFN-γ alone had no significant effect on p24 production, nor did it have any synergistic effect when used in combination with HA (Fig. 3D).

Natural-Product Humic Acid Inhibition of HIV-1 Replication in Infected PBMC

The ability of natural-product HA to block the replication of wild-type viruses in PBMC from HIV-1 infected individuals was studied in a parallel set of experiments. PBMC from 16 infected donors at various stages of the disease were treated. HA was found to reduce both HIV-1 RT activity and p24 production to background levels in all cases (Figs. 4A,B).

Inhibition of HIV-1 replication in PBMC from infected individuals could have been biased by the intrinsic fragility of the infected donor cells, low levels of virus specific markers, or both. To rule out these possibilities, cells from HIV-1 positive donors were co-cultured with fresh normal PBMC that had been preactivated with PHA-P. HA was then added 48 h post co-cultivation. As before, treatment with HA strongly inhibited HIV-1 RT activity and p24 production (Figs. 4C,D). These results confirmed that HA effectively inhibits the replication of a variety of HIV-1 isolates transmitted into normal PBMC.

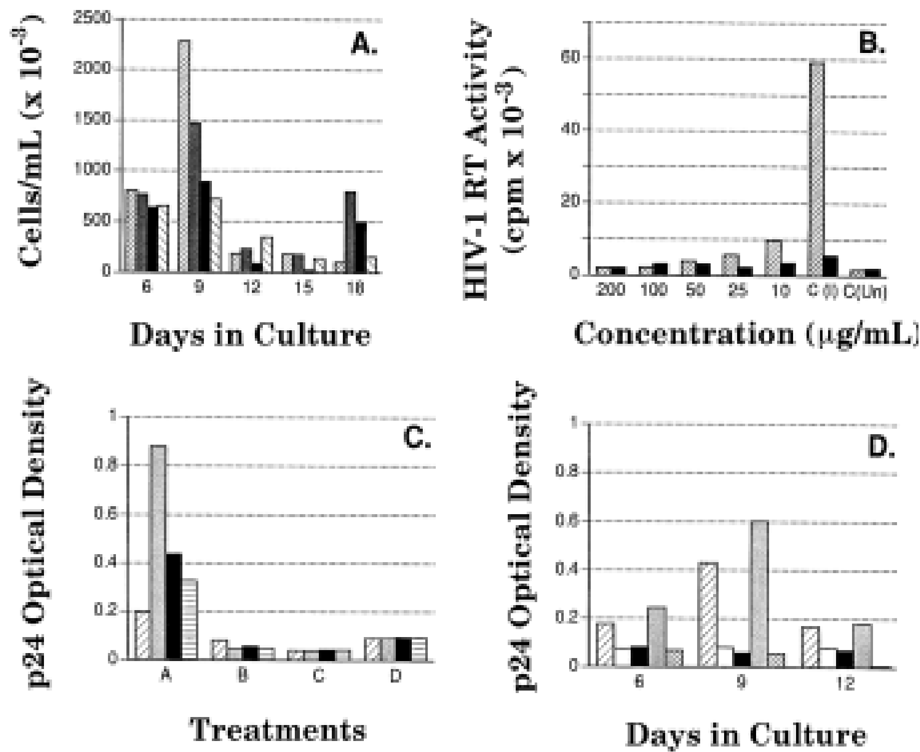

Figure 3. HA inhibition of HIV-1 replication in PBMC from normal donors. (***A***) Viability of HIV-1_{4105} infected PBMC treated with 25 μg/mL HA alone, or in combination with IL-2. Symbols: HA + IL-2 ( ), IL-2 ( ), HA ( ), and no treatment ( ). (***B***) Expression of RT by infected PBMC treated with 10, 25, 50, 100, and 200 μg/mL HA. Cell culture supernatants were harvested on day 6. Symbols: oligo $dt_{15}.rA_n$ ( ) and oligo $dt_{15}.dA_n$ ( ) [HIV-1 RT and DNA-P were used as controls]. C(I) is the control for supernatant from HIV-1 infected cells that were not treated with HA, while C(UN) is the control for supernatant from uninfected and untreated cells. (***C***) Production of HIV-1 p24 by infected PBMC treated with HA. Cell culture supernatants were the same preparations as those used in the RT determinations (***B***). Symbols correspond to harvests from days 6 ( ), 9 ( ), 12 ( ), and 15 ( ). Indicated experimental groups are: *A*, infected untreated cells; *B*, HA-treated infected cells; *C*, uninfected cells; and *D*, the cut-off value (defined by the manufacturer as the value below which all specimens are considered unreactive). (***D***) Effect of IFN-γ on HIV-1 infected normal PBMC. IFN-γ was used at a concentration of 100 U/mL together with HA at 50 and 25 μg/mL. Cell culture supernatants were harvested on days 6, 9, and 12; and then assayed for HIV-1 p24. Symbols: IFN-γ ( ), IFN-γ + HA (50) ( ), IFN-γ + HA (25) ( ), untreated infected cells ( ), and HA treatment only (25) ( ).

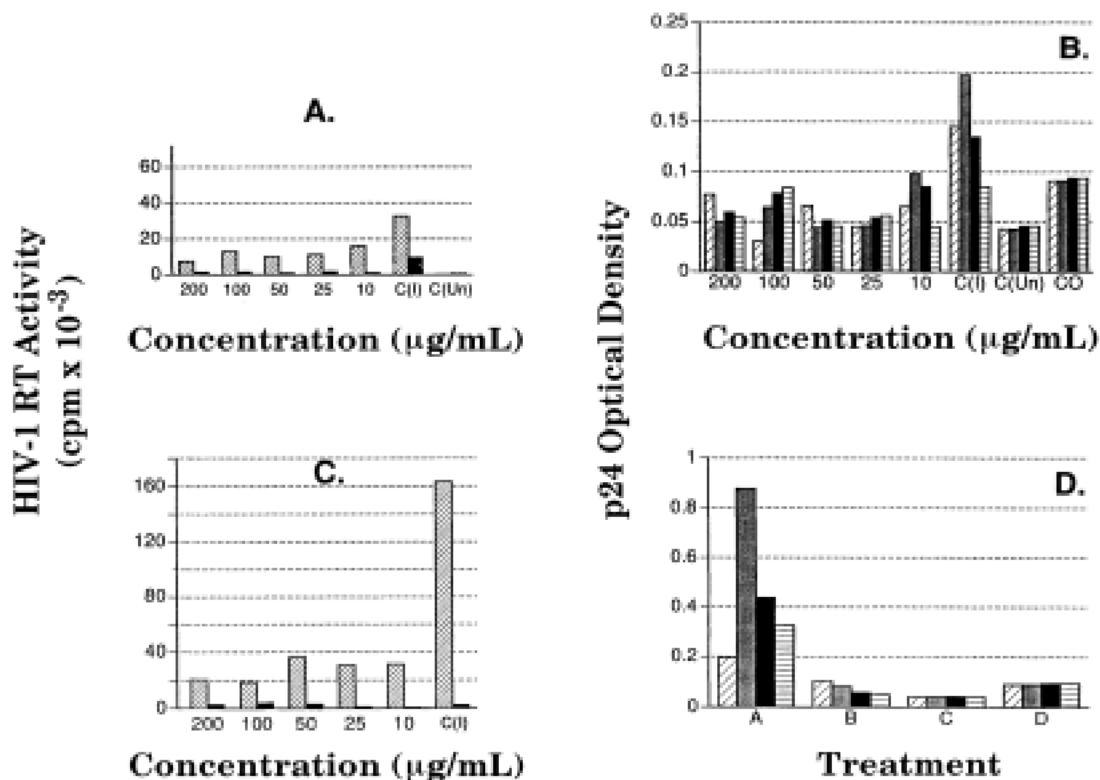

Figure 4. Natural-product HA inhibition of HIV-1 replication in PBMC of HIV-1 positive donors. (***A***) Expression of HIV-1 RT activity. Cells from HIV-1 positive donors were cultured in the same way as those obtained from uninfected normal donors. Culture supernatants were harvested on day 6 and assayed for HIV-1 RT activity (HIV-1 RT and DNA-P were used as controls). Symbols: exogenous primer-templates $dt_{15}.rA_n$ ( ) and $dt_{15}.dA_n$ ( ). (***B***) p24 production. Bars correspond to harvests of culture supernatants on days 6 ( ), 9 ( ), 12 ( ), and 15 ( ). C(I) represents the positive control for infected cells; C(UN) that for uninfected cells; and CO the negative control. (***C***) HA inhibition of HIV-1 replication after in vitro virus transmission from infected to uninfected PBMC: effect on HIV-1 RT activity. Cell culture supernatants from suspensions of 1 x 10^6 cells/mL were harvested on day 6 [HIV-1 RT ( ) and DNA-P ( ) were used as controls]. C(I) is the control for infected untreated cells. (***D***) HA inhibition of HIV-1 replication after in vitro virus transmission from infected to uninfected PBMC: effect on p24 production. Cells were treated with IL-2 in the presence or absence of 25 µg/mL HA. Cell culture supernatants were harvested for p24 assay on days 6 ( ), 9 ( ), 12 ( ), and 15 ( ). Treatments correspond to: *A*, IL-2; *B*, IL-2 + HA; *C*, negative control; and *D*, the cut-off value (*cf.* Fig. 3C).

HPLC Fractionation of Natural Product Humic Acid Activity

The HPLC elution pattern of natural-product HA is shown in Fig. 5. Each of the five major peaks was comprised of complex mixtures of compounds. Even so, three of the five exhibited appreciable levels of anti-HIV-1 activity. Each was therefore tested in vitro on HIV-1_{4105} infected Jurkat cells. None had any appreciable effect on cell viability (Fig. 6A). However, HIV-1 RT activity and p24 production were both reduced to background levels by each of the active fractions, as shown in Figs. 6B,C. Similar results were obtained for HIV-1_{4105} infected PBMC from normal donors.

When separate batches of Jurkat cells infected with pNL4-3 virus were treated individually with each of the three active HA fractions, multiply-spliced HIV-1 *m*RNA decreased dramatically. In contrast, treatment with the remaining two HPLC fractions had little effect on the level of HIV-1 specific *m*RNA transcript (Fig. 7A.1). None of the HPLC fractions had much effect on the level of unspliced HIV-1 *m*RNA.

To follow the kinetics of HIV-1 DNA synthesis in cells treated with different fractions of HA, primers amplifying early, intermediate, and full-length DNA were employed as described earlier and as detailed in Table I. For the most part, the HPLC fractions had little effect on the pattern of proviral DNA in infected Jurkat cells, as shown in Fig. 7B. However, a decrease in proviral DNA was found using primer set M667/AA55. A decrease in specific PCR production was observed as well (Fig. 7B.1). PCR analysis employing a second set of primers (SK38/SK39) gave similar results (Fig. 7B.2). The late set (M667/M661) additionally yielded reduced DNA product (Fig. 7B.3).

Lack of a specific signal in lane 4 of Fig. 7B (corresponding to HPLC fraction 4) might be said to be due to the presence of DNA polymerase inhibitors. To rule out this possibility, a cell- and DNA-free PCR analysis was performed with the pNL4-3 HIV-1 plasmid, together with the intermediate primer set, using diluted HPLC fraction 4 in place of distilled water. It was observed that fraction 4 did indeed inhibit PCR amplification of the HIV-1 plasmid, as illustrated in Fig. 7C. These data, together with the observations recounted earlier, thus point to the specific inhibition by HA HPLC fraction 4 of the amplification of DNA extracted from HIV-1 infected cells.

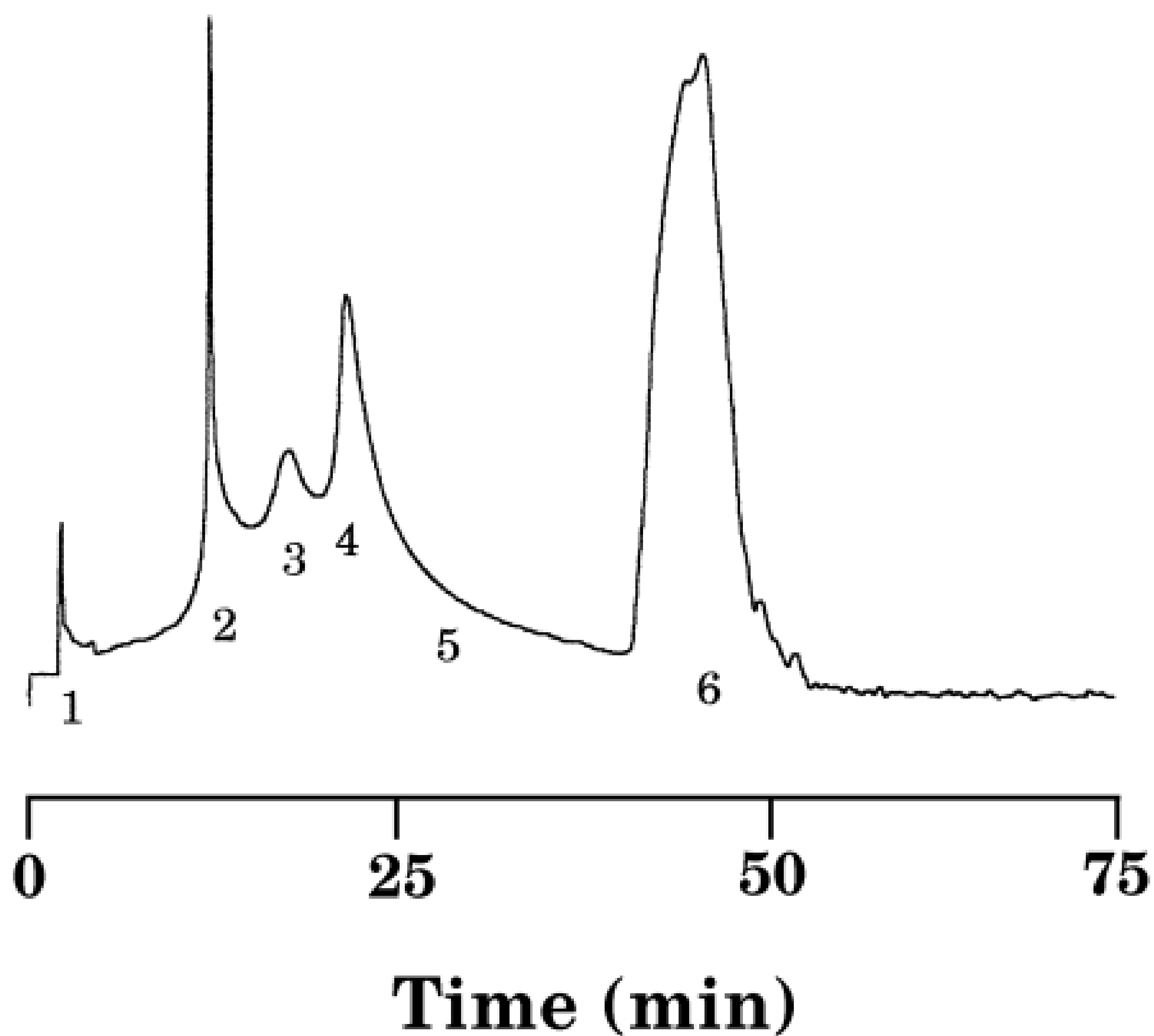

Figure 5. HPLC elution pattern obtained for natural-product HA. Fractions 1-5 were tested for efficacy. Fraction 6 reflects a column wash with 90-100% *v/v* methanol.

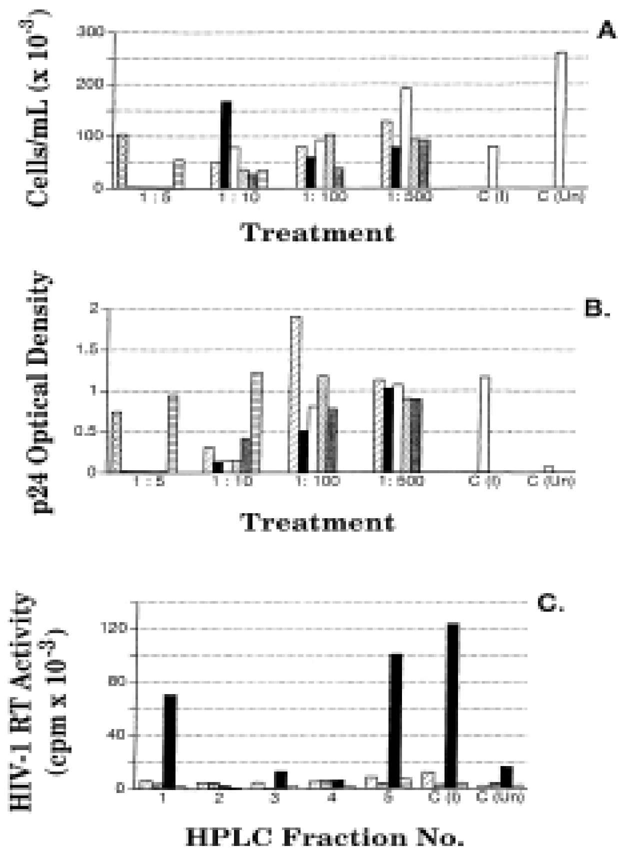

Figure 6. Effects of HPLC fractions of HA on cell viability and HIV-1 replication. (***A***) Viability of HIV-1 infected PBMC from normal donors after treatment with 5 μg/mL HPLC fractions. Fractions were diluted 1:5, 1:10, 1:100, and 1:500 with cell culture medium and were then added to suspensions of infected cells. Treatments were maintained continuously for 6 days. Symbols: untreated cells ([X]); treatment with HPLC fractions 1 (◩), 2 (■), 3 (□), 4 (◪), and 5 (▩); and with buffer (⊟). C(I) represents the control for infected cells, while C(UN) is the control for uninfected cells. (Buffer was tested at 1:5 and 1:10 dilutions only.) (***B***) Inhibition of p24 production. HPLC fractions were diluted 1:5, 1:10, 1:100, and 1:500 with cell culture medium. Symbols: untreated cells ([X]); treatment with HPLC fractions 1 (◩), 2 (■), 3 (□), 4 (◪), and 5 (▩); and with buffer (⊟). C(I) is the control for infected cells, and C(UN) that for uninfected cells. (Buffer was tested at 1:5 and 1:10 dilutions only.) Cell viability and p24 expression were determined using cells and cell culture supernatants from the same flasks. (***C***) Inhibition of RT expression. Cells obtained from the same normal donors as in Figs. 4A,B were treated with HPLC fractions 1-5 at 1:10 dilution. Cell culture supernatants were harvested on days 6 and 9. C(I) is the control for infected cells, and C(UN) is the control for uninfected cells. Symbols: day 6, $dt_{15}.rA_n$ (◩) and $dt_{15}.dA_n$ (▩); day 9, $dt_{15}.rA_n$ (■) and $dt_{15}.dA_n$ (⊟).

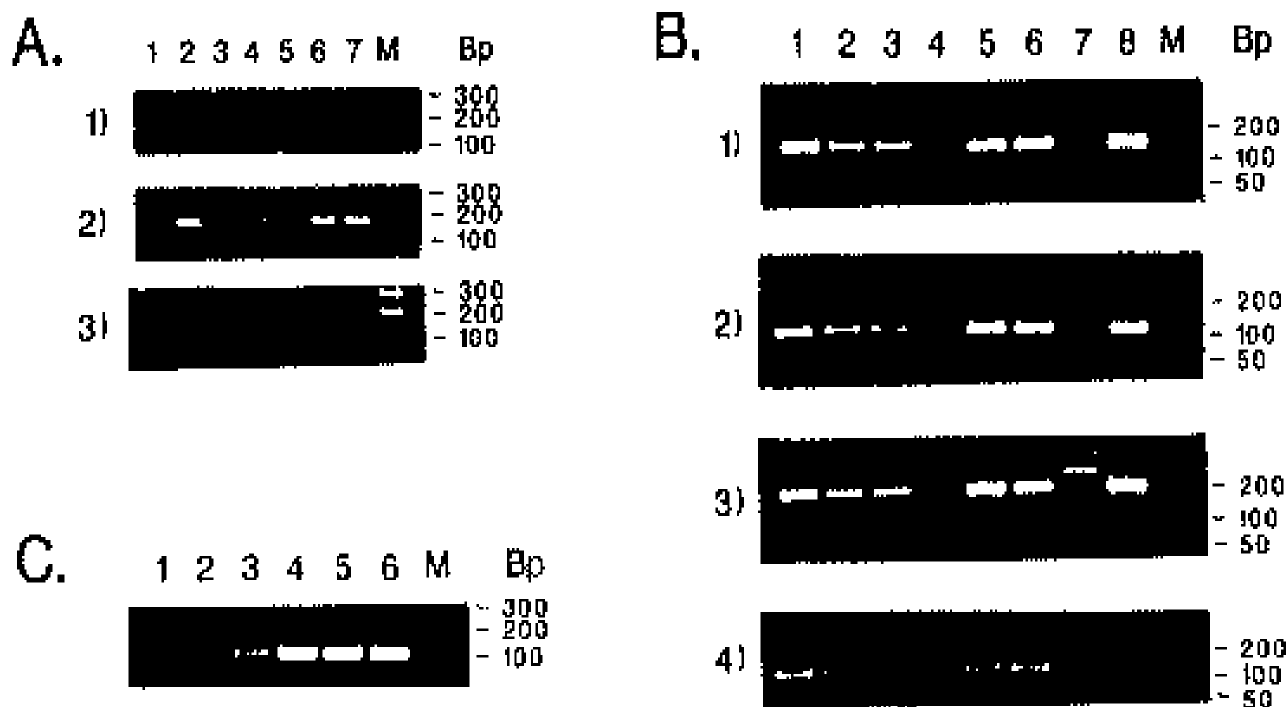

Figure 7. Effects of HPLC fractions of natural-product humic acid on HIV-1 *m*RNA and proviral DNA in Jurkat cells transfected with pNL4-3. (***A***) RT-PCR of *m*RNA was performed using two pairs of primers that discriminated multiply-spliced (***A.1***) and unspliced (***A.2***) HIV-1 *m*RNA. β-Actin (***A.3***) was used as a quantitation control. Lane 1 is the negative control; lanes 2-6 correspond to HA HPLC fractions 1-5; lane 7 is *m*RNA from untreated cells; and M refers to the molecular marker. Sizes are indicated in the right-hand margin. (***B***) HIV-1 proviral DNA synthesis was tested using the primers detailed in Table I. Primer set M667/AA55 was used for early synthesis (***B.1***); SK38/SK39 for the intermediate stage (***B.2***); and M667/M661 for late synthesis (***B.3***). Primers LA1 and LA2 were used for amplification of β-globulin as a quantitation control (***B.4***). Lanes 1-5 correspond to HA HPLC fractions 1-5; lane 6 is for DNA from untreated cells; lane 7 is the negative control (DNA from uninfected cells); and lane 8 is the positive control, pNL4-3 plasmid DNA. (***C***) Blocking of PCR amplification by HPLC fraction 4 in a treated cell- and DNA-free system. DNA from pNL4-3 was used as the template and SK38/SK39 as the set of primers. HA HPLC fraction 4 at ca. 25 μg/mL was diluted 1:10, 1:100; 1:200, 1:500, and 1:1000 with distilled water, corresponding to lanes 1-5. Lane 6 is PCR with distilled water only.

Synthetic Humic Acid Inhibition of HIV-1 Replication in Infected PBMC

The HPLC elution pattern of synthetic humic acid is provided in Fig. 8. As shown, the major peak corresponds nearly exactly to the active fraction 4 of natural-product HA (Fig. 5). Accordingly, several forms of synthetic humate were tested for efficacy with infected PBMC using the procedures described earlier for natural-product HA with infected PBMC (Fig. 6). The results are depicted in Fig. 9. The data labeled F3 and F4 reflect treatment with materials derived from two distinct synthetic routes to the synthetic material. F5 is identical to F4 except that the synthetic humate was first freeze-dried from aqueous solution and then reconstituted in appropriate medium. Also shown for comparison are the data for solutions of natural-product HA dissolved neat (F1), and following freeze-drying (F2). The positive and negative controls, +0 and -0, reflect p24 expression for infected and uninfected cells, respectively.

DISCUSSION

It is apparent from the results presented here that both natural-product HA, long recognized as an antiviral agent, as well as the synthetic analog of its most active HPLC fraction, effectively inhibit the replication of HIV-1 in constitutively-infected T-cell lymphoma and monocytic cell lines, as well as in PBMC from normal or HIV-1 infected individuals (that is, involving various non-laboratory strains of HIV-1).

Some literature reports attribute the HA inhibition of HIV-1 infection to prevention of the binding of HIV-1 to a specific receptor. In this work, HIV-1 *m*RNA was reduced both in freshly infected PBMC as well as in Jurkat cells in the presence of both natural-product and synthetic humates. These results suggest that the antiviral activity of humates may also be due to the blocking of transcriptional and/or post-transcriptional processes, with the consequence that HIV-1 RT expression and p24 production are both reduced. In fact, in every instance investigated, the production of p24 as well as the RT activity released by the cells was markedly decreased or blocked altogether. (Consistent with these data, natural-product HA was found to inhibit synctia formation in a model system. The substance may therefore limit the production of fusogenic proteins as well.) Humates also appear to suppress the events that result in the predominant expression of multiply-spliced HIV-1 *m*RNA, which initiate soon after HIV-1 infection occurs.

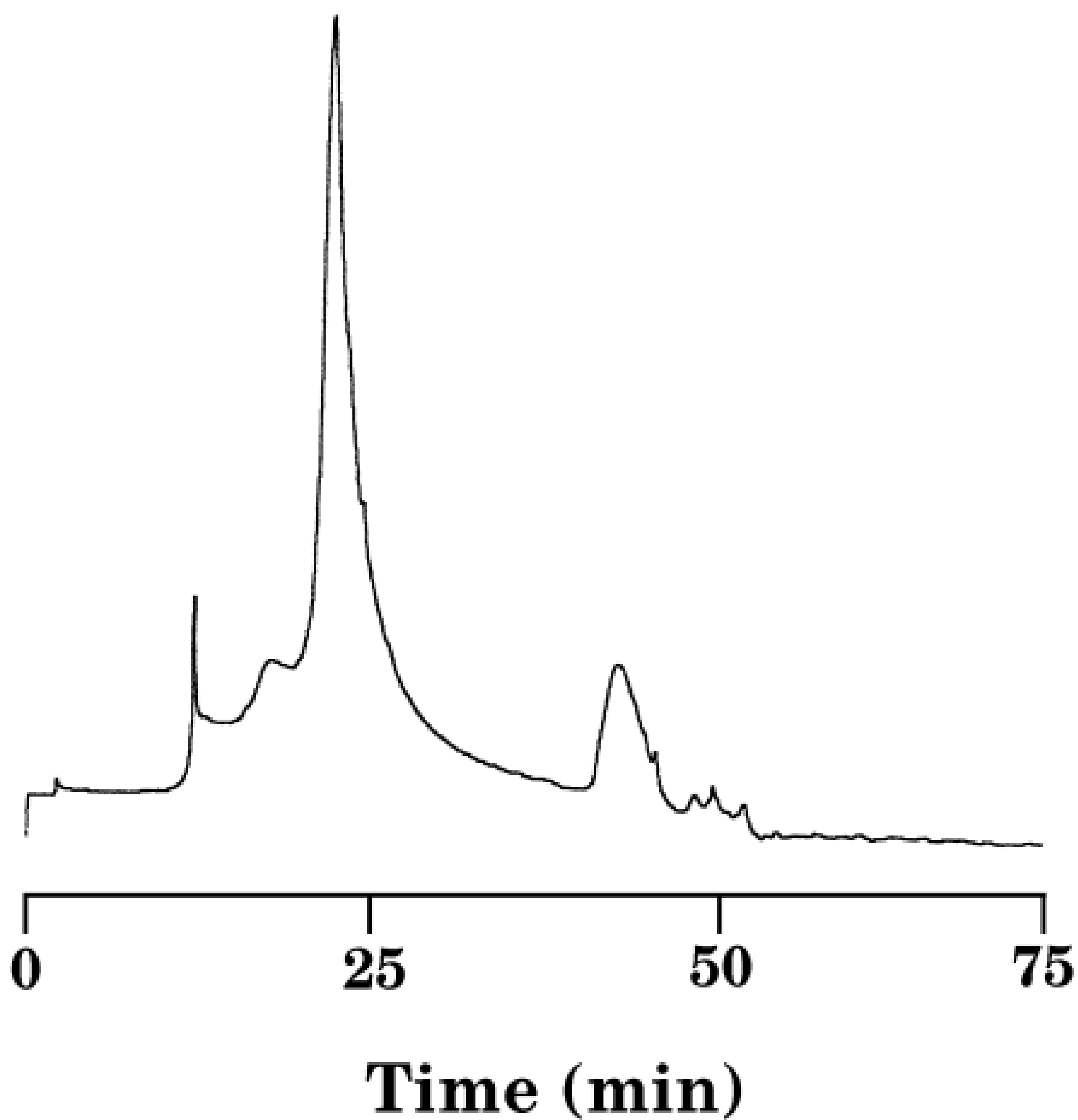

Figure 8. HPLC elution pattern obtained for synthetic humic acid. The column and conditions were identical to those employed in Fig. 5. The retention time of the large peak corresponds to that of fraction 4 of natural-product HA.

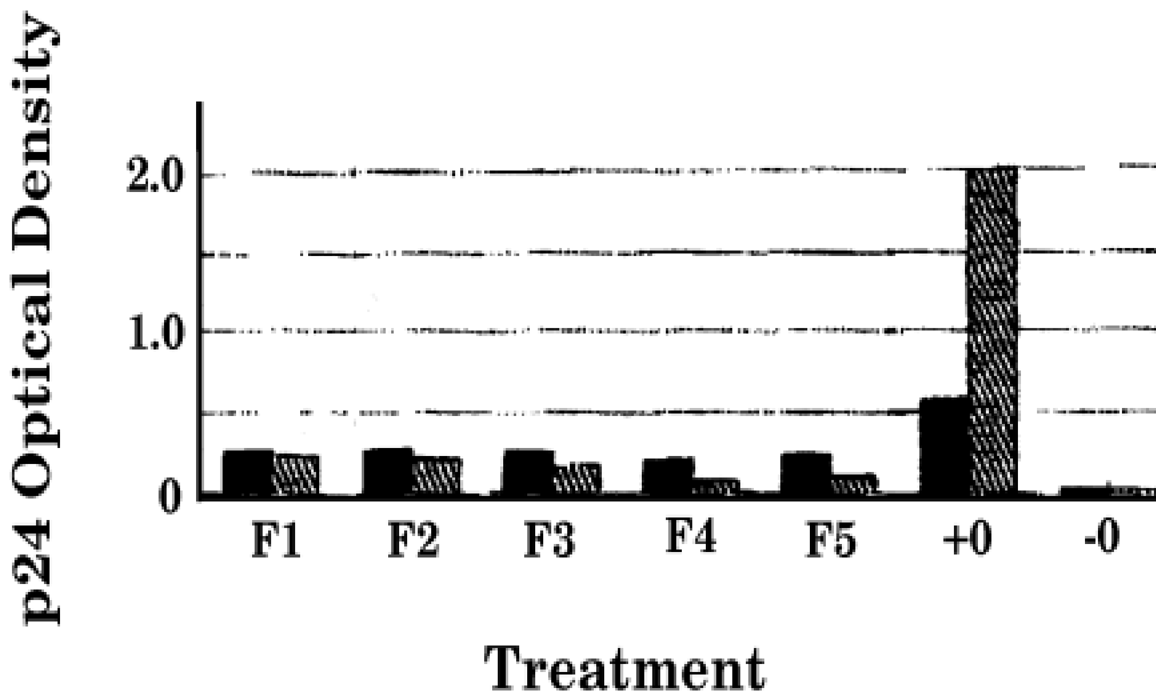

Figure 9. Effects of natural-product humic acid compared with those of synthetic humate on HIV-1 replication in infected PBMC (see also Fig. 6). Substances tested: *F1*, ca. 25 μg/mL solution of natural-product HA; *F2*, freeze-dried and redissolved natural-product HA; *F3,F4*, ca. 25 μg/mL solutions of humate synthesized via different routes; *F5*, freeze-dried and redissolved synthetic humate F4; *+0*, infected cells without drug treatment; *-0*, uninfected cells. Solid bars: cells harvested on day 6; hashed bars, day 8.

AZT, an RT inhibitor, is currently the anti-HIV-1 compound most commonly-employed (whether alone or in admixture) for the treatment of HIV-1 infection. The effects of AZT were therefore compared with those of HA HPLC fraction 4 on the de novo infected monocytic U937 cell line. Fraction 4 was found on a weight basis to be substantially more effective than AZT (Table II). In fact, in U937 cells infected with HIV-1_{4105} and then treated after stable levels of virus production had been established, HA as well as synthetic humic acid inhibited HIV-1 p24 production at half the concentration of AZT (25 μg/mL versus 50 μg/mL). Indeed, at the concentration necessary for the same anti-RT action as HA and synthetic humate, AZT was found to be toxic, as reflected by the much lower residual cell numbers in the respective cultures (Table II).

Careful inspection of Fig. 9 reveals little change in p24 expression from day 6 to day 8 with natural-product HA (F1,F2). However, synthetic humate (F4-F6) gave notable reductions in p24 expression from day 6 to day 8. This result may portend efficacy for short-term medication with synthetic humic acid, as opposed to the potential requirement for habitual treatment with natural-product HA. [In addition, there are of course considerable practical advantages to synthetic humic acid over natural-product HA, including good manufacturing practices-level (*GMP*) control over its source, purity, and availability.]

Finally, the ability to inhibit the replication of wild-type strains is an obvious (and, arguably, the most important) prerequisite in order that any HIV-1 drug be considered useful. The results obtained to date have shown that HA as well as synthetic humic acid do in fact inhibit the replication of HIV-1 wild-type strains, in addition to assaulting in a unique way the intracellular replicative events of the virus. At the same time, the materials provide substantial cytoprotection. Humic substances thus appear to hold considerable promise as a co-treatment for HIV-1 infection and related sequelae in man. Synthetic humic acid appears to be particularly attractive in this regard.

Table II. Comparison of Efficacy of Natural-Product Humic Acid HPLC Fraction 4 with that of AZT for Inhibition of Virus Production from HIV-1$_{4105}$ Infected U937 Cells

Group	Cell Count, x 10^{-6} [a]	Relative HIV-1 p24 Production, x 10^{2}
A	1.7	10.9
B	1.4	3.5
C	1.2	3.5
D	1.7	3.9
E	1.8	4.0
F	0.6	4.2

[a] Number of viable cells determined by the Trypan Blue dye exclusion test. Initial density of all cell cultures was 1 x 10^{6}.

Cells and media were harvested on day 6 and assayed for cell viability and p24 production. Groups correspond to: ***A***, HIV-1 infected untreated U937 cells; ***B***, uninfected U937 cells; ***C, D, E***, HIV-1 infected U937 cells treated with HA HPLC fraction 4 (100, 50, and 25 μg/mL, respectively); and ***F***, HIV-1 infected U937 cells treated with AZT (50 μg/mL).