Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 8889–8893, November 1990
Medical Sciences

Elimination of infectious human immunodeficiency virus from human T-cell cultures by synergistic action of CD4–*Pseudomonas* exotoxin and reverse transcriptase inhibitors

(AIDS/therapeutics/targeted cell killing/virus replication inhibition)

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ABSTRACT **We have previously described a recombinant protein, designated CD4(178)–PE40, consisting of the human immunodeficiency virus (HIV) envelope glycoprotein-binding region of human CD4 linked to the translocation and ADP-ribosylation domains of *Pseudomonas aeruginosa* exotoxin A. By virtue of its affinity for gp120 (the external subunit of the HIV envelope glycoprotein), the hybrid toxin selectively binds to and kills HIV-1-infected human T cells expressing surface envelope glycoprotein and also inhibits HIV-1 spread in mixed cultures of infected and uninfected cells. We now report that CD4(178)–PE40 and reverse transcriptase inhibitors exert highly synergistic effects against HIV-1 spread in cultured human primary T cells. Furthermore, combination treatment can completely eliminate infectious HIV-1 from cultures of human T-cell lines. This conclusion is based on protection of a susceptible cell population from HIV-induced killing, complete inhibition of virus protein accumulation, and elimination of HIV DNA (as judged by quantitative polymerase chain reaction analysis). The results highlight the therapeutic potential of treatment regimens involving combination of a virostatic drug that inhibits virus replication plus an agent that selectively kills HIV-infected cells.**

There is a growing appreciation that effective treatment of human immunodeficiency virus (HIV) infection may require combinations of therapeutics that attack different aspects of the infection process (1). Particularly appealing would be the use of an agent that blocks the virus replicative cycle coupled with another that selectively kills infected cells. Reverse transcriptase (RT) inhibitors such as AZT (3′-azido-3′-deoxythymidine; zidovudine) or ddI (2′,3′-dideoxyinosine) act by inhibiting synthesis of the proviral genome after the virion has entered the host cell, thereby blocking viral replication. Although this virostatic action effectively inhibits HIV spread *in vitro* and *in vivo* (2), RT inhibitors do not kill those cells that are already infected. CD4(178)–PE40 is a recombinant protein that potently and selectively kills HIV-infected cells (3–5); however, this activity can be exerted only after productive infection is established and viral gp120 begins to accumulate at the infected cell surface (5). Thus, when the hybrid toxin is added to mixed cultures of infected and uninfected T cells, some virus is produced before the infected cells are killed, and the infection eventually spreads and eliminates the susceptible cell population (4). In view of these complementary modes of action of CD4(178)–PE40 and RT inhibitors, one might predict that they would display synergistic anti-HIV effects.

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MATERIALS AND METHODS

Cell Culture and Virus Infection. Cell cultures were maintained at 37°C in 5% CO_2/95% air. Acute HIV-1 infections were generated with the LAV isolate (6). Ficoll/Hypaque-separated peripheral blood mononuclear cells were stimulated for 3 days in RPMI 1640 medium containing 10% (vol/vol) heat-inactivated fetal bovine serum and phytohemagglutinin (Boehringer Mannheim) at 5 μg/ml. For infection, the cells were washed and suspended at 1×10^7 cells per ml in RPMI 1640 medium; HIV-1 LAV_{BRU} was added at a multiplicity of 0.005 tissue culture 50% infective dose ($TCID_{50}$) per cell. After a 2-hr adsorption period the volume was raised 20-fold with RPMI 1640 medium supplemented with 10% fetal bovine serum and 10% (vol/vol) interleukin 2-containing conditioned medium (Boehringer Mannheim). The cells (2.5×10^5 cells per well) were seeded in 24-well tissue culture plates in duplicate with the indicated drug additions. Three days after infection, the cells were diluted with equal volumes of fresh medium containing the original concentrations of drugs. At 6 days the supernatants were harvested and analyzed for HIV-1 p24 activity (no p24 was detected in the supernatants at 3 days).

Human T-cell lines A3.01 (7) and H9/HTLV-IIIB (ref. 8; a gift from M. Robert-Guroff, National Cancer Institute, Bethesda, MD) were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 10 mM Hepes, penicillin (100 units/ml), and streptomycin (0.1 mg/ml). The conditions for viral infection and maintenance of cultures are indicated for each experiment. Spread of infection was monitored by assaying virus-mediated cell killing and RT in the medium.

Assays. Virus production was monitored by assaying the cell culture medium either for HIV-1 p24 using an antigen-capture ELISA (DuPont) or for RT (9). HIV-1 DNA was assayed using a polymerase chain reaction (PCR)-amplification method (30 cycles) with SK38/SK39 primers from the *gag* gene of HIV-1 (10). Amplified product was hybridized in solution to a ^{32}P-labeled SK19 probe, resolved from free probe on a 10% polyacrylamide gel, and detected by autoradiography. The PCR was used to quantitate numbers of infected cells in samples of cell cultures by determining the end-point dilutions that still gave a positive signal.

Relative viable cell numbers were determined by the modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-

Abbreviations: HIV, human immunodeficiency virus; RT, reverse transcriptase; AZT, 3′-azido-3′-deoxythymidine; ddI, 2′,3′-dideoxyinosine; PCR, polymerase chain reaction; sCD4, soluble CD4.

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zolium bromide (MTT) oxidation procedure (11), as described (4, 5), and are expressed for each experimental sample as the percent of the value obtained with the virus-free control.

Analysis of Drug Interactions. Drug interactions were analyzed using the COMBO program package, which operates on mainframes and personal computers in the MLAB computing environment (Civilized Software, Bethesda, MD). The methods are described in detail elsewhere (12, 13). Briefly, the data were fitted by iteratively reweighted nonlinear least squares regression to a "robust potentiation" model defined by the implicit equation

$$1 = (1/z - 1)^{-1/B[1]}\left\{\frac{C[1]}{IC_{50}[1]}\left[1 + \left(\frac{C[2]}{PC_{50}[2]}\right)^{BP[2]}\right]\right\} + (1/z - 1)^{-1/B[2]}\left\{\frac{C[2]}{IC_{50}[2]}\left[1 + \left(\frac{C[1]}{PC_{50}[1]}\right)^{BP[1]}\right]\right\},$$

where $z = (a - y)/(a - d)$; y is the p24 level; C[1] and C[2] are the drug concentrations; a is p24 in the absence of drug; d is p24 at indefinitely high drug levels; $IC_{50}[1]$ and $IC_{50}[2]$ are the doses for 50% viral inhibition with drug 1 alone and drug 2 alone, respectively; B[1] and B[2] are the corresponding logistic slope parameters; $PC_{50}[1]$ is the potentiation parameter for drug 1 on drug 2, and $PC_{50}[2]$ is the reverse; BP[1] and BP[2] are the corresponding slopes for the potentiation effect. Including all four potentiation-associated parameters in the fit simultaneously did not significantly improve the quality of fit. Hence, calculations with either $PC_{50}[1]$ or $PC_{50}[2]$ set to infinity (i.e., with all potentiation attributed to one of the drugs) are presented here. Weights for the fitting procedure were determined from the error structure of the data set itself using a kernel algorithm based on Gaussian windows to estimate the relationship between variance and p24 level (12, 13). Parameter d was fixed at zero. Distribution-free conservative confidence limits for the model parameter estimates were obtained by a Monte Carlo resampling technique related to the bootstrap method of Efron (14).

Reagents. CD4(178)–PE40 (donated by S. Johnson, Upjohn Laboratories, Kalamazoo, MI) was expressed in *Escherichia coli* and purified by methods similar to those described (3). Soluble CD4 (sCD4, donated by S. Johnson, Upjohn Laboratories, Kalamazoo, MI) produced by transformed CHO cells contained all four extracellular domains of CD4 and was confirmed to display high-affinity binding to gp120. AZT was obtained from Sigma and ddI was a gift from H. Mitsuya (National Cancer Institute, Bethesda, MD).

RESULTS AND DISCUSSION

Synergistic Action of CD4(178)–PE40 and RT Inhibitors in Primary T Cells. In the experiment shown in Table 1, human peripheral blood mononuclear cells were infected with HIV-1 in the absence of drugs and then cultured in the presence of various combinations of CD4(178)–PE40 and AZT or ddI. The range of RT inhibitor concentrations used overlapped with plasma levels achieved in clinical studies (2). Control experiments (data not shown) indicated that none of these drugs, alone or in combination, were toxic to uninfected cells at the concentrations employed. In the absence of drugs, virus spread resulted in the appearance of HIV-1 p24 core protein in the culture medium (Table 1) and elimination of most of the CD4-positive cells (as determined by fluorescence-activated cell sorter analysis; data not shown) within 6 days. Each drug alone inhibited p24 production in a dose-dependent manner.

Table 1. Effects of combinations of CD4(178)–PE40 and RT inhibitors on HIV-1 replication in human peripheral blood mononuclear cells

	Relative p24 production, % of control						
CD4(178)–	No RT	ddI			AZT		
PE40, nM	inhibitor	1 μM	5 μM	25 μM	20 nM	100 nM	500 nM
0	100	129	55	0.1	71	4	0.5
2	66	48	9	0.1	18	2	0.2
10	43	24	3	<0.1	5	0.3	<0.1
50	17	8	0.3	<0.1	2	0.2	<0.1

Human peripheral blood mononuclear cells were cultured and infected with HIV-1. Mean p24 levels at day 6 were obtained by averaging levels obtained from replicate wells (quadruplicate for no drug addition; duplicates for drug-treated cultures; 34 total wells). Values are expressed as percent of control wells (no drug, p24 content = 45 ng/ml).

The data in Table 1 were analyzed for possible synergy by using the COMBO program package (12, 13); Table 2 gives the resulting parameter estimates. Fits were done in two ways: (*i*) with CD4(178)–PE40 as the potentiating agent and (*ii*) with the RT inhibitor as the potentiating agent. Of particular interest are the potentiation factors (i.e., the PC_{50} values). $PC_{50}[1]$ quantifies the potentiating effect of drug 1 on drug 2; $PC_{50}[2]$ quantifies the potentiating effect of drug 2 on drug 1. More explicitly, $PC_{50}[1]$ is defined as that concentration of drug 1 required to increase the apparent potency of drug 2 (i.e., decrease its apparent IC_{50}) by a factor of 2 (beyond what would be expected on the basis of the intrinsic activity of drug 1). The lower the value of PC_{50}, the stronger the potentiation; additivity corresponds to $PC_{50} \rightarrow \infty$. Table 2 shows that CD4(178)–PE40 potentiated each RT inhibitor ($P < 0.01$) and that each RT inhibitor potentiated CD4(178)–PE40 ($P < 0.01$). In each case, the PC_{50} value was at least several times lower than the corresponding IC_{50} value, indicating strong potentiation. This mutual capacity to potentiate corresponds to "synergy."

Fig. 1 *Upper* and *Lower*, respectively, illustrates the relationships graphically for the potentiation of CD4(178)–PE40 by ddI and AZT. Fig. 1 *Left* shows the three-dimensional dose–response surface for the indicated pair of drugs, based on the best fit of the experimental data. Inward bowing of the isoeffect contours toward the origin indicates synergistic drug interactions. Fig. 1 *Right* shows the appearance of the surface if the effects of the drugs had been simply additive [i.e., if there had been no potentiation ($PC_{50}[2] \rightarrow \infty$)].

The strong synergy between CD4(178)–PE40 and RT inhibitors may have clinical implications, since combination therapy with these agents may minimize problems associated with *in vivo* toxicity and immunogenicity of these compounds and, possibly, the generation of drug-resistant variants.

Elimination of Infectious HIV from Cultures of Human T-Cell Lines by Combination Treatment with CD4(178)–PE40 Plus RT Inhibitors. To test whether combination treatment with CD4(178)–PE40 plus RT inhibitors might eliminate HIV infection in culture, we conducted experiments with continuous human T-cell lines, which can be propagated for prolonged periods. In the experiment shown in Fig. 2, susceptible A3.01 cells were acutely infected with HIV-1 and subsequently cultured in the presence of the indicated drug combinations; the cultures were diluted every 2 or 3 days to maintain the cell densities in the range of exponential growth. At day 31, drug treatment was terminated and the cultures were continued for an additional 3 weeks. Viable cell number and RT activity in the supernatant were used as measures of HIV spread. In the absence of drugs, most of the A3.01 cells were killed by the virus between 11 and 16 days, with a concomitant peak of RT activity. CD4(178)–PE40 alone de-

Table 2. Parameter values obtained for analysis of synergy between CD4(178)–PE40 (CD4-PE40) and dideoxynucleoside drugs using the "robust potentiation" model

Drug 1	Drug 2	$IC_{50}[1]$, nM	$IC_{50}[2]$, nM	B[1]	B[2]	BP[1]	BP[2]	$PC_{50}[1]$, nM	$PC_{50}[2]$, nM	95% limits on PC_{50}, nM	F*	*P* value for additivity†
CD4–PE40 ← ddI		3.9	4900	0.67	5.4	—	1.8	—	750	320–1100	10.7	<0.01
CD4–PE40 → ddI		2.9	4800	0.59	2.7	0.54	—	0.92	—	0.08–8.8	8.1	<0.01
CD4–PE40 ← AZT		6.2	29	0.74	2.6	—	1.7	—	3.9	0.03–10	9.6	<0.01
CD4–PE40 → AZT		5.9	29	0.69	2.5	0.50	—	0.90	—	≈0–9.6	9.3	<0.01

←, Calculation for potentiation of drug 1 by drug 2; →, the reverse. Confidence limits were calculated for all parameters, but only those for the potentiation parameter are shown. Calculations were done using all 34 points except for one obvious outlier in the no-drug quadruplicate for AZT. Inclusion of that point made only minor differences in parameter estimates.

*Pseudo F statistic relative to best additive model fit, with 28 and 26 degrees of freedom for the experiments with ddI and 29 and 27 degrees of freedom for the experiments with AZT.

†Two-tail test; hypothesis of additivity rejected at the 1% level.

layed virus-induced cell killing and suppressed free virus production; however, the bulk of the cell population eventually succumbed. AZT alone provided considerable protection against HIV spread as long as it was maintained in the culture, but cell death and virus production rapidly ensued upon cessation of treatment. We conclude that the infected cells continued to replicate along with the uninfected population during the AZT treatment, such that infected cells were still present at the time of cessation of drug treatment, despite the repeated dilutions of the cultures. Inclusion of sCD4 along with AZT did not enhance the antiviral effect. In contrast, treatment with a combination of CD4(178)–PE40 and AZT completely protected the culture from HIV spread. Thus, cell death and virus production were prevented while the drugs were present and after treatment was terminated. Moreover, analysis of the cells remaining 14 days after

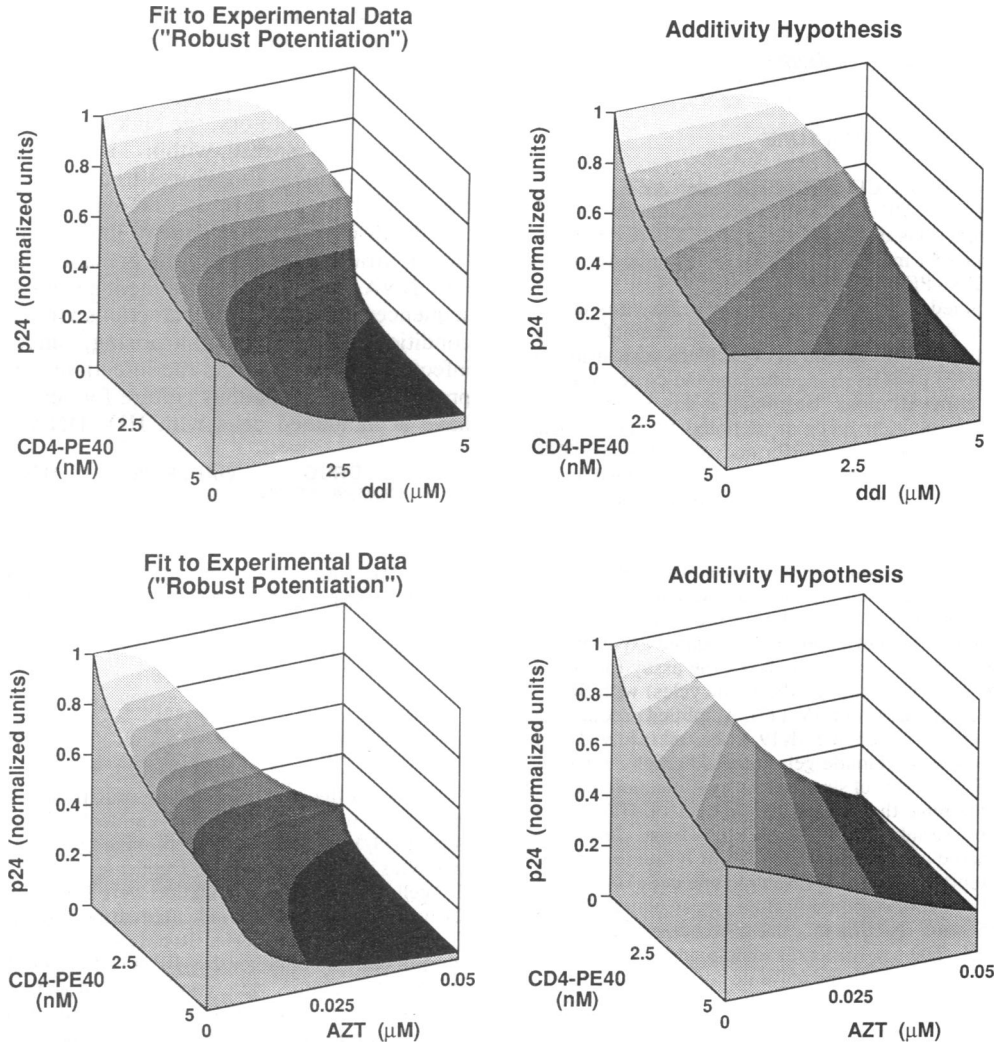

FIG. 1. Synergistic interaction of CD4(178)–PE40 (CD4–PE40) with ddI and with AZT in primary T-cell cultures. The dose–response surfaces were constructed from the parameter estimates in Table 2. (*Upper*) Potentiation of CD4(178)–PE40 by ddI. (*Lower*) Potention of CD4(178)–PE40 by AZT. (*Left*) Best fits of the experimental data using the "robust potentiation" model (12, 13). (*Right*) Surfaces that would have been obtained in the absence of synergy. Similar dose–response surfaces were obtained for potentiation of each RT inhibitor by CD4(178)–PE40 (data not shown).

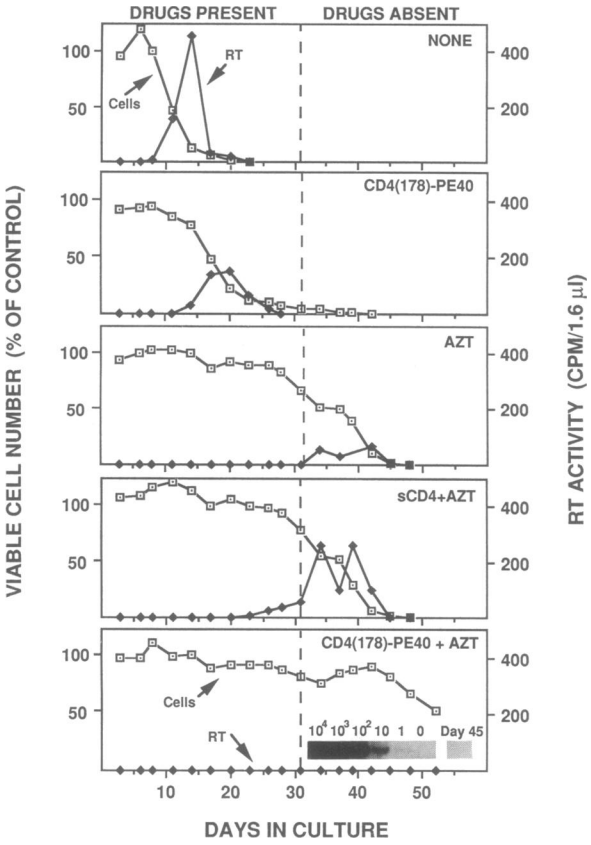

FIG. 2. Effects of sCD4, CD4(178)–PE40, and AZT on HIV spread. Approximately 2×10^5 A3.01 cells were mixed with 1×10^3 tissue culture 50% infectious doses ($TCID_{50}$) of HIV-1 in duplicate wells in a total volume of 1 ml in the presence of the indicated drug combinations [CD4(178)–PE40 or sCD4 at 10 nM; AZT at 1 μM]. A control culture contained 2×10^5 A3.01 cells with no virus in the absence of drugs. On day 3 and every 2 or 3 days thereafter, the cultures were diluted by transferring 80 μl of each cell suspension to new wells containing 920 μl of fresh medium and the corresponding drugs at the original concentrations. Beginning at day 31, the drugs were omitted from the dilution medium (denoted by the vertical dashed lines). RT assays (◆) were performed using the [^{32}P]TTP method (13). Relative viable cell numbers (⊡) were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) oxidation procedure (14) as described (2, 3) and are expressed for each experimental sample as the percent of the value obtained with the virus-free control. The gradual decline in relative cell number after cessation of drug treatment in the lowest panel is due to variation introduced by the repeated dilution process, not to HIV spread; this phenomenon was not observed in repeated experiments. The autoradiograph in the lowest panel shows the assay for HIV-1 DNA by using a PCR-amplification method (30 cycles) with SK38/SK39 primers from the *gag* gene of HIV-1 (7). Amplified product was hybridized in solution to a ^{32}P-labeled SK19 probe and resolved from free probe in a 10% polyacrylamide gel; autoradiographs exposure was 12 hr. On the left is a series of signals from amplification reaction mixtures, each containing the indicated numbers of HIV-1 proviruses, obtained by serial dilution of a lysate from chronically infected U1 cells (mixed with an uninfected A3.01 lysate to maintain constant DNA content). The lane representing one copy of provirus was obtained by diluting the standard lysate to a calculated value of 0.1 copy per amplification reaction mixture and choosing a lane that gave a positive signal. The experimental sample on the right shows the absence of a signal using a volume of lysate corresponding to 75% of the cells remaining at day 45 in the cultures that had received CD4(178)–PE40 plus AZT during the treatment phase; no signal was observed even in autoradiographs exposed four times longer.

cessation of drug treatment indicated that no HIV DNA could be detected using a PCR-amplification technique sensitive enough to detect provirus from a single infected cell (Fig. 2 *Bottom Insert*). The dilution protocol was presumably responsible for the removal of HIV DNA from killed infected cells.

Additional insight was gained from similar studies using chronically infected cells instead of free virus as the infectious input. In the experiment shown in Fig. 3, HIV-infected H9/HTLV-IIIB cells and uninfected A3.01 cells were mixed at a ratio of 1 to 10; cultures were maintained for 32 days in the presence of the indicated drug combinations. With sCD4 alone, the infection spread and the viable cell number was reduced to a level corresponding to the initial infected cell input; CD4(178)–PE40 alone only partially inhibited cell killing resulting from virus spread. By contrast, when either CD4 derivative was used in combination with AZT or ddI, the cultures were completely protected over this time period, consistent with results of the previous experiment with free virus.

Dramatic differences between the effects of sCD4 and CD4(178)–PE40 were revealed when quantitative PCR amplification was used to estimate the amount of HIV-1 DNA and the corresponding ratios of infected to uninfected cells remaining after combination drug treatment. When the treatment included sCD4 plus an RT inhibitor, this ratio was not reduced below the starting level, indicating that the treatment was merely virostatic. By contrast, combinations of CD4(178)–PE40 plus RT inhibitors reduced the infected to uninfected cell ratio $\approx 10^4$ times. When the drug treatments were terminated (data not shown), results similar to those in Fig. 2 were obtained. Thus, in cultures initially exposed to sCD4 plus RT inhibitors, the bulk of the cell population was killed by virus spread within 11 days after cessation of treatment; whereas in cultures treated with CD4(178)–PE40 plus RT inhibitor, there was no evidence of virus-mediated cell death during a subsequent 36-day culture period. The lack of viral spread despite the presence of low quantities of HIV DNA after the drug treatment suggested that these HIV sequences did not express infectious virus under our culture conditions. This is not surprising, since the chronically infected cell line used as the infectious challenge had been propagated in continuous culture for several years and may have accumulated cells with HIV DNA in a defective or

DRUG COMBINATION	VIABLE CELLS % of control	HIV-1 DNA lysate dilution 10^0 10^{-1} 10^{-2} 10^{-3} 10^{-4}	
sCD4 + AZT	96		(10^{-1})
CD4(178)-PE40 + AZT	95		(10^{-5})
sCD4 + ddI	100		(10^{-1})
CD4(178)-PE40 + ddI	98		(10^{-5})
sCD4	13	n.d.	
CD4(178)-PE40	30	n.d.	

FIG. 3. Effects of combination drug treatment on HIV spread in mixed cultures of infected and uninfected human T-cell lines. Infected H9/HTLVIIIB cells were mixed with uninfected A3.01 lymphocytes at a ratio of 1 to 10. Duplicate 1-ml samples containing 2×10^5 cells of this mixed population were seeded in individual wells of 24-well plates, and the indicated drug combinations were added [CD4(178)–PE40 or sCD4 at 10 nM; AZT At 1 μM; ddI at 10 μM]. A control culture contained 2×10^5 A3.01 cells only, in the absence of drugs. The cultures were continued and analyzed for cell viability and the presence of HIV-1 DNA as described in the legend for Fig. 2. The autoradiographs show hybridization of an HIV-specific probe (SK19) to products amplified with the PCR from serial 1:10 dilutions of cell lysates from the drug-treated cultures (n.d., not determined). In the amplification reaction, the undiluted samples contained DNA from 3×10^5 cells. The values in parentheses indicate the approximate ratios of infected to uninfected cells at day 32, calculated by determining the maximum dilution still giving a positive signal.

latent state. Such cells would not be killed by CD4(178)–PE40 (5).

Several additional analyses (not shown) were considered in evaluating the success of combination treatment with CD4(178)–PE40 and RT inhibitors in eliminating infectious HIV in the experiments shown in Figs. 2 and 3. We observed that a single infectious unit of HIV-1 was sufficient to kill a fresh A3.01 cell population within 14–21 days, a period during which no cell killing or virus production was observed after termination of the combination drug treatment. We also detected no virus growth when the cells remaining after combination treatment were mixed with fresh A3.01 cells. The treatment did not select for an HIV-resistant subpopulation, since the cells remaining 3 weeks after drug removal were still CD4-positive (as judged by immunofluorescence analysis) and they could be readily killed by addition of a small number of chronically infected H9/HTLVIIIB cells. Based on these findings, we conclude that the HIV-infected cells were selectively eliminated from the culture by the synergistic anti-HIV action of CD4(178)–PE40 and RT inhibitors and that there was no infectious virus present in the culture at the time of cessation of drug treatment.

The ability of simultaneous treatment with an RT inhibitor and CD4(178)–PE40 to eliminate infectious HIV from T-cell cultures highlights the therapeutic potential of treatment regimens involving combinations of a virostatic drug plus an agent capable of selectively killing HIV-infected cells.

We thank P. B. Robbins for excellent technical assistance, M. A. Martin for the use of his equipment, B. Bunow for extensive work on the synergy analysis, and L. R. Muenz for advice on the statistics. This work was supported in part by the National Institutes of Health Intramural AIDS Targeted Antiviral Program.

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