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Combinations of Isoprinosine and 3′-Azido-3′-Deoxythymidine in Lymphocytes Infected with Human Immunodeficiency Virus Type 1

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Since clinical trials are being planned with the immunomodulating drug isoprinosine combined with the antiviral drug 3′-azido-3′-deoxythymidine (AZT) in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex, it is important to determine the type of antiviral interaction produced by these drugs in vitro. Such a combined modality may not only produce enhanced antiviral effects but also may have a valuable immunorestorative action. The interaction of several ratios of AZT and isoprinosine on the replication of human immunodeficiency virus type 1 in human peripheral blood mononuclear cells was determined by reverse transcriptase assay of disrupted virus obtained from supernatants of cells that were exposed to virus and the drugs separately and in combination and by a human immunodeficiency virus type 1 p24 enzyme immunoassay of the same supernatants. The correlation between the reverse transcriptase and enzyme immunoassay data was high. The antiviral activity of AZT alone was neither diminished nor augmented when AZT was used in combination with isoprinosine. Isoprinosine did not enhance virus yield when used alone or in combination with AZT in peripheral blood mononuclear cells, nor did it affect the growth of uninfected cells. The in vitro results indicate that this combination did not decrease the efficacy of AZT or exacerbate virus replication.

3′-Azido-3′-deoxythymidine (AZT) is a potent antiviral drug that prolongs the life of patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex (4, 14). Unfortunately, treatment with this drug is often associated with dose-related hematological abnormalities (19). Almost all available antiretroviral drugs have been studied in combination with AZT (1, 8, 10, 20, 22) with the aim of reducing the toxicity of the drugs. However, few studies have reported the interaction of AZT with compounds that are capable of restoring immune function (7).

Isoprinosine is an immunomodulating drug with modest effects on various DNA and RNA viruses in vitro and in vivo (2, 3, 15, 16). It has been reported to produce a weak anti-human immunodeficiency virus type 1 (HIV-1) activity in human peripheral blood mononuclear cells (PBMC) and H9 cells and to restore in vitro the impaired T-helper cells from patients with AIDS-related complex (5, 17, 18). However, immunomodulators, such as isoprinosine, are unlikely to be effective on their own in cases of overwhelming virus infections unless they are combined with an effective antiviral drug. In this study, we examined whether this drug caused any detrimental effects when used in combination with AZT, since patients currently treated with AZT may undergo concomitant treatment with isoprinosine to restore or enhance their immune systems. We report here the type of interaction produced by a combined modality of AZT and isoprinosine in primary human lymphocytes.

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MATERIALS AND METHODS

Compounds. Isoprinosine (inosiplex; inosine pranobex) was provided by Newport Pharmaceuticals International Inc., Newport Beach, Calif. Isoprinosine is composed of hypoxanthine riboside, *N*,*N*-dimethylamino-2-propanol, and *p*-acetamidobenzoic acid in a 1:3:3 molar ratio (molecular weight, 1,115.3). Isoprinosine is highly soluble (>20 mg/ml) in water and is chemically stable in water for more than a week. AZT was synthesized in our laboratory by the method of Lin and Prusoff (12). The purity of this material as determined by high-performance liquid chromatography and thin-layer chromatography was greater than 99%, and the material had the same retention time and melting point as an authentic sample obtained from T.-S. Lin (Yale University).

Cells. Human PBMC from healthy HIV-1-seronegative and hepatitis B virus-seronegative donors were isolated by Ficoll-Hypaque discontinuous gradient centrifugation at 1,000 × *g* for 30 min, washed twice in phosphate-buffered saline (pH 7.2), and pelleted at 300 × *g* for 10 min. Before infection, the cells were stimulated by phytohemagglutinin at a concentration of 16.7 μg/ml for 3 days in RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum, 1.5 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and 4 mM sodium bicarbonate.

Viruses. HIV-1 (strain LAV-1) was obtained from P. Feorino (Centers for Disease Control, Atlanta, Ga.). The virus was propagated in human PBMC by using RPMI 1640 medium as described previously (13) without phytohemagglutinin or an antifungal agent and supplemented with 7% (vol/vol) interleukin-2 (Advanced Biotechnologies, Inc., Silver Spring, Md.), 7 μg of DEAE-dextran (Pharmacia, Uppsala, Sweden) per ml, and 370 U of human alpha interferon (ICN, Lisle, Ill.) per ml. Virus obtained from cell-free culture supernatants was titrated and stored in aliquots at −80°C until use.

Inhibition of virus replication in human PBMC. Uninfected phytohemagglutinin-stimulated human PBMC were uniformly distributed in 25-cm^2 flasks to give a 5-ml suspension containing about 2 × 10^6 cells per ml. Suitable dilutions of

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HIV-1 were added to infect the cultures. The mean reverse transcriptase (RT) activity of the inocula was 50,000 dpm/ml, which is equivalent to about 100 50% tissue culture infective doses, determined as described previously (6). The tested drugs at twice their final concentrations in 5 ml of RPMI 1640 medium, supplemented as described above, were added to the cultures. Uninfected and untreated PBMC were grown in parallel at equivalent cell concentrations as controls. The cultures were maintained in a humidified 5% CO_2–95% air incubator at 37°C for 6 days after infection, at which point all cultures were sampled for supernatant RT activity. Previous studies indicated that maximum RT levels were obtained at that time (11).

RT activity assay. Samples (6 ml) of supernatant from each culture were clarified from cells at 300 × *g* for 10 min. Virus particles were then pelleted from 5-ml samples at 40,000 rpm for 30 min by using a Beckman 70.1 Ti rotor and suspended in 200 μl of virus-disrupting buffer (50 mM Tris chloride [pH 7.8], 800 mM NaCl, 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5% Triton X-100).

The RT assay (RTA) was performed by a modification of the method of Spira et al. (21) in 96-well microtiter plates. The radioactive cocktail (180 μl), containing 50 mM Tris chloride (pH 7.8), 9 mM $MgCl_2$, 5 mM dithiothreitol, 4.7 μg of $(rA)_n \cdot (dT)_{12-18}$ per ml, 140 μM dATP, and 0.22 μM [^{3}H]TTP (specific activity, 78.0 Ci/mmol; equivalent to 17,300 cpm/pmol; New England Nuclear Corp. Research Products, Boston, Mass.), was added to each well. The sample (20 μl) was added to the reaction mixture, which was then incubated at 37°C for 2 h. The reaction was terminated by the addition of 100 μl of 10% trichloroacetic acid containing 0.45 mM sodium PP_i. The acid-insoluble nucleic acids which precipitated were collected on glass filters by using a Skatron semiautomatic harvester (setting 9). The filters were washed with 5% trichloroacetic acid and 70% ethanol, dried, and placed in scintillation vials. Four milliliters of scintillation fluid (Econofluor; New England Nuclear Corp. Research Products) was added, and the amount of radioactivity in each sample was determined by using a Packard Tri-Carb liquid scintillation analyzer (model 2,000CA). The results were expressed in disintegrations per minute per milliliter of original clarified supernatant.

p24 HIV-1 EIA. Cell-free culture supernatants were adjusted to 0.5% Triton X-100 prior to determination of the HIV-1 p24 levels using a commercial Abbott p24 antigen enzyme immunoassay (EIA) kit. A standard curve was generated over the range of 30 to 1,000 pg of p24 per ml by using partially purified inactivated viral lysate (calibrated against purified p24). The lower limit of sensitivity for this assay was 30 pg of p24 per ml.

PBMC proliferation assay. The drugs were evaluated for their potential toxic effects on uninfected phytohemagglutinin-stimulated human PBMC. Flasks were seeded so that the final concentration was 3×10^5 cells per ml. The cells were cultured with and without drug for 6 days, at which time aliquots were counted for cell viability, as assessed by a hemacytometer and the trypan blue dye-exclusion method.

Statistical evaluation. The statistical analyses presented in Table 1 were performed on the data for the combination of AZT and isoprinosine at a variable ratio. The means of the combination data were compared with data for the most potent agent alone (i.e., AZT) at an equivalent concentration by using a Student *t* test [$(\bar{x} - \mu)/s_{\bar{x}}$].

TABLE 1. Effect of AZT and isoprinosine alone and in combination against HIV-1 (LAV) in human PBMC[a]

Treatment and ratio	Concn (μM)	% Inhibition	
		RTA[b]	EIA[c]
AZT	0.002	45.4	59.5
	0.004	77.0	74.5
	0.008	89.2	88.2
	0.016	91.7	91.3
Isoprinosine	10	42.7	29.3
	20	21.9	27.5
	40	42.3	40.5
	80	10.3	14.5
	160	36.2	37.3
	320	38.7	47.1
AZT-isoprinosine			
1:10^3	0.001/1	56.7	51.7
	0.002/2	77.2	78.1
	0.004/4	68.6	77.4
	0.008/8	73.0	78.4
	0.016/16	88.4	89.4
	0.032/32	93.9	95.2
1:10^4	0.001/10	41.1	43.2
	0.002/20	51.8	56.1
	0.004/40	71.1	72.9
	0.008/80	87.3	89.7
	0.016/160	88.5	87.7
	0.032/320	92.7	97.2
Variable	0.008/10	82.6[d]	68.9[d]
	0.008/20	85.2[d]	81.6[d]
	0.008/40	76.1[d]	82.1[d]
	0.008/80	56.1[d]	84.3[d]
	0.008/160	77.5[d]	85.8[d]
	0.008/320	77.6[d]	100.0[d]
Variable	0.016/10	75.4[d]	95.2[d]
	0.016/20	84.8[d]	88.6[d]
	0.016/40	84.4[d]	90.5[d]
	0.016/80	82.6[d]	91.4[d]
	0.016/160	88.0[d]	91.6[d]
	0.016/320	87.5[d]	95.8[d]

[a] The amount of virus or virus products present in the supernatant 6 days after infection was determined by an RTA and EIA. See Materials and Methods.

[b] Values for the dpm for the blank, uninfected control, and infected control (untreated) in the original supernatant were 664 ± 71 (mean ± standard deviation), 2,725, and 81,480 ± 18,375, respectively.

[c] The EIA was performed on supernatant by using a commercial Abbott antigen kit. Samples were treated with Triton X-100 and stored at −70°C prior to testing. The pg of HIV p24/ml of infected control supernatant was 64.24 ± 4.50.

[d] Not significantly more effective than AZT alone; $P > 0.05$ (Student *t* test).

RESULTS

AZT was effective in reducing the replication of HIV-1 in PBMC at concentrations close to 1 nM, whereas isoprinosine was essentially inactive or produced only slight inhibition when tested up to 320 μM (Table 1). Because of the variability of the data, especially at low-effect values, the no-effect dose for isoprinosine could not be determined accurately.

The interaction of AZT (range, 1 to 32 nM) and isoprinosine (10 to 320 μM) at 1:10^3 or 1:10^4 molar ratios on the replication of HIV-1 (strain LAV) in human PBMC was determined by RTA of disrupted virus obtained from supernatants of cells that were exposed to virus and the drugs

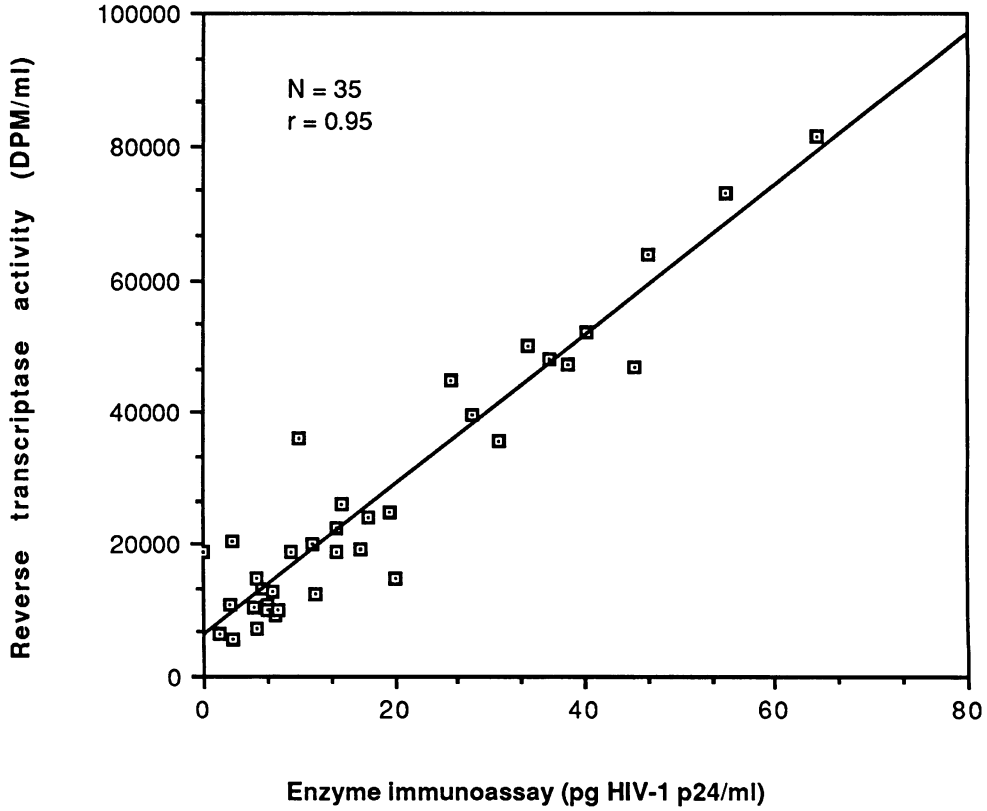

FIG. 1. Correlation between RT and EIA data.

alone and in combination. An HIV-1 p24 EIA was also performed on Triton X-100-treated supernatant to confirm the activity. Isoprinosine, which had a low and variable antiviral activity in this system (e.g., 39 to 47% inhibition at 320 μM, depending on the virus product detection method), did not augment the virus yield when used alone or in combination with AZT. In addition, the antiviral activity of AZT was not markedly diminished when AZT was used in combination with isoprinosine (Table 1). When the concentration of AZT was kept constant (at 8 and 16 nM) and that of isoprinosine was varied (10 to 320 μM), similar results were obtained ($P > 0.05$; Student t test). There was a good correlation between the results obtained by the RTA and p24 HIV-1 EIA; the correlation coefficient for the 35 datum points was 0.95 (Fig. 1). At only one point (combination of AZT-isoprinosine of 0.008/320 μM) was there a marked difference in the RTA and EIA data; the EIA showed a complete inhibition of p24 production at these concentrations. AZT and isoprinosine alone (up to 100 and 640 μM, respectively) and isoprinosine in combination with AZT did not affect the growth of uninfected PBMC (Table 2).

DISCUSSION

Pompidou and co-workers (18) recently showed that isoprinosine at 200 μg/ml, or about 180 μM, produced a 48% inhibition of virus replication as measured by an RTA on supernatant obtained from isoprinosine-treated PBMC. However, in H9 cells, isoprinosine had no effect on virus replication (18). In a similar PBMC system, our results with this drug alone were highly variable and complete inhibition of virus yield was not achieved even when isoprinosine was tested at 320 μM (357 μg/ml). However, isoprinosine had significant antiviral activity against a Friend retrovirus at concentrations close to 100 μM, suggesting that this drug had weak antiretroviral properties (unpublished results). Isoprinosine did not enhance HIV-1 yield when used alone or in combination with AZT in PBMC. Cell culture results also indicated that isoprinosine was well tolerated up to 640 μM and produced no inhibition of cell growth when combined with AZT in uninfected human PBMC. The in vitro studies in PBMC suggested that this immunomodulator was not toxic, did not appear to have an intrinsic anti-HIV-1 activity, and neither enhanced nor diminished the antiviral properties of AZT. The RTA and p24 EIA used to determine

TABLE 2. Effect of isoprinosine alone and in combination with AZT on growth of uninfected mitogen-stimulated human PBMC

Treatment	Concn (μM)	Mean no. of cells (10^6)/ml[a]	% Inhibition
None	0	3.5	0
Isoprinosine	160	3.0	14
	320	2.7	23
	640	3.5	0
AZT	1	3.5	0
	10	3.5	0
	100	3.0	14
AZT-isoprinosine	0.008/80	3.0	14
	0.016/160	2.8	20
	0.032/320	2.6	26

[a] The cells were counted 6 days after addition of drug by the trypan blue exclusion method.

the antiviral activities of the drugs alone and in combination produced almost identical results, with a high correlation coefficient.

Although no serious toxicity has been encountered with the chronic use of isoprinosine in humans over extended periods (3, 5), no study has yet been performed to determine the toxicity and efficacy of the combination of AZT and isoprinosine in patients with AIDS-related complex and AIDS. It could be argued that since *p*-acetamidobenzoic acid, a major component of isoprinosine, is known to be metabolized to the glucuronide form (2), the competition of this drug with AZT, which is also metabolized primarily by glucuronidation in the liver (9), may produce an elevation of the concentration of AZT in serum. Unless the drug levels are carefully monitored, this potential benefit could also lead to increased bone marrow suppression, resulting in more-rapid or severe anemia in patients treated with the combined modality. The fact that mice do not glucuronidate AZT as effectively as primates (about 2 versus 80%) (20) suggests that additional metabolic studies in a nonhuman primate model for AIDS may be warranted prior to human studies. In addition, biochemical studies aimed at determining the degree of competition for the liver enzymes responsible for glucuronidation should be performed with AZT and isoprinosine. Finally, the effect of AZT on the immunopotentiating activity of isoprinosine should be addressed in future studies.

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