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Synergistic Inhibition of Human T-Cell Lymphotropic Virus Type III Replication In Vitro by Phosphonoformate and Recombinant Alpha-A Interferon

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Phosphonoformate and recombinant alpha-A interferon synergistically inhibited the replication of human T-cell lymphotropic virus type III in cultured peripheral blood lymphocytes. T-cell proliferative capability was maintained by this combination, and toxicity was minimal.

Human T-cell lymphotropic virus type III (HTLV-III) replication may be critical to the pathogenesis of the acquired immunodeficiency syndrome and related disorders. Phosphonoformate (PFA) and recombinant alpha-A interferon (rIFN-α_A) each inhibit HTLV-III replication in vitro (8, 15), probably by different mechanisms (1, 3, 6, 7a, 10–12, 15). We report here synergistic anti-HTLV-III effects of this combination.

Peripheral blood lymphocytes from healthy seronegative donors were obtained by Ficoll-Hypaque sedimentation and grown in RPMI 1640 medium containing 20% fetal calf serum and 10% interleukin-2 (Electro-Nucleonics, Inc., Columbia, Md.). Virus was obtained from supernatant fluid of HTLV-III_B-infected H9 cells (provided by R. C. Gallo) and stored at −70°C. The 50% tissue culture infective dose ($TCID_{50}$) of the virus stock was determined by titration on H9 cells (see virus yield assay below).

rIFN-α_A was obtained from Hoffmann-La Roche Inc., Nutley, N.J., and was assayed by using a cytopathic effect reduction method as previously described (8). Trisodium PFA was obtained from Astra Pharmaceuticals, Sweden.

Three separate experiments were performed. In each, peripheral blood lymphocytes were suspended in 5 ml of medium at a concentration of 4×10^5 cells per ml in 25-ml flasks, and phytohemagglutinin was added at a concentration of 20 μg/ml. Peripheral blood lymphocytes were obtained from a different donor for each experiment. After 3 h, rIFN-α_A was added where noted, and cultures were incubated overnight. PFA was then added where noted, followed by the addition of HTLV-III. Infected cell controls were included in all experiments. Triplicate (experiment 1) or duplicate (experiments 2 and 3) cultures were maintained for each drug concentration; results shown below are averages. Medium changes were performed every 3 to 4 days, at which time rIFN-α_A was added at the full initial dose. Medium with or without PFA at the noted concentrations was prepared at the start of a given experiment and used for all subsequent medium changes. Viable cell counts were obtained throughout all experiments by using trypan blue staining. Experiments 1 and 2 were carried out for 18 days, and experiment 3 was carried out for 28 days.

In experiment 1, 0, 16, and 64 U of rIFN-α_A per ml and 0, 64, and 128 μM PFA were tested alone and in all possible combinations. The same format was used in experiment 2, although it was expanded to include 0, 4, 16, 64, and 256 U of rIFN-α_A per ml and 0, 32, 64, and 128 μM PFA. Doses used in experiment 3 are shown in Table 1. The viral inoculum was 50 $TCID_{50}$s in experiments 1 and 2 and 250 $TCID_{50}$s in experiment 3.

Reverse transcriptase (RT) activity was assayed as previously described (14) every 3 to 4 days starting at day 7. Indirect immunofluorescence (IFA) for HTLV-III antigens was performed on cultured cells as previously described (16) every 3 to 4 days in experiments 1 and 2 and on day 14 in experiment 3. Virus yield assays were performed on days 10, 14, and 18 in experiment 1 by making 10 twofold dilutions of

TABLE 1. RT results and mean cell counts (experiment 3)

Drug concn[a]	RT values (counts/min per ml) (10^4) at day[b]:				Cell count[c] (no. of cells/ml) (10^6)
	6	14	21	28	
No drug	24.0	13.4	3.9	7.2	5.1
rIFN-α_A (8)	9.3	5.0	2.6	2.5	6.4
rIFN-α_A (16)	6.1	8.3	3.3	2.5	5.9
rIFN-α_A (32)	5.0	9.8	2.1	1.8	4.3
rIFN-α_A (64)	3.2	6.3	2.8	2.6	4.2
rIFN-α_A (128)	3.6	5.0	1.5	2.7	4.6
PFA (16)	6.0	4.4	1.6	4.6	3.9
PFA (32)	2.0	3.8	1.1	2.3	4.2
PFA (64)	1.0	2.8	0.7	0.4	3.9
PFA (128)	0.2	1.2	0.5	1.2	5.2
PFA (256)	0	0	0	0.1	4.6
rIFN-α_A (8), PFA (16)	3.5	4.2	0.8	2.0	5.7
rIFN-α_A (16), PFA (32)	1.0	0.6	0.3	0.3	4.9
rIFN-α_A (32), PFA (64)	0.7	0.4	0.2	0.2	6.2
rIFN-α_A (64), PFA (128)	0	0	0.3	0	4.5
rIFN-α_A (128), PFA (256)	0	0	0.1	0	2.5

[a] PFA concentrations are micromolar, and rIFN-α_A concentrations are in units per milliliter.
[b] Day in culture after virus inoculation.
[c] Cell counts from day 21.

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TABLE 2. Effects of rIFN-α_A and PFA, singly or in combination, on HTLV-III yield (experiment 1)

Drug concn		$TCID_{50}$ (ml) of HTLV-III on day[a]:		
rIFN-α_A (U/ml)	PFA (μM)	10	14	18
0	0	2,500	640	640
16	0	500	640	ND
64	0	0	640	ND
0	64	750	2,500	ND
16	64	<20	320	160
64	64	0	40	80
0	128	<20	640	ND
16	128	0	<20	<20
64	128	0	0	<20

[a] Values are the average of triplicate (duplicate on day 18) data. Values of <20 indicate that one value was positive at the lowest dilution, whereas the others were negative. ND, Not done.

cell-free culture supernatant fluids in 96-well microtiter plates (Costar, Cambridge, Mass.) and then adding an equal volume of medium containing uninfected H9 cells at a concentration of 4×10^5 cells per ml. Antibodies against alpha interferon (National Institutes of Health standard preparation) were added in doses sufficient to neutralize residual interferon in the supernatant fluids. On day 11, cytopathic effects were evaluated, and IFA was performed. Cytopathic effects consisted of giant cells and syncytia as previously described (13). Six separate titrations were done from culture supernatants at each drug dosage. The $TCID_{50}$ was calculated by noting the dilution at which 50% of the wells showed evidence of infection.

The fractional iso-inhibitory concentration method (2) and the multiple drug effect analysis (4, 5) were used to calculate combined drug effects. In using the former method, the concentration of each agent which has a given effect in combination is divided by the concentration of that agent having the same effect alone. If the sum of the resulting fractions is greater than 1, the combination is deemed antagonistic; if less than 1, the combination is synergistic; and if equal to 1, the combination has additive effects. Data from all three experiments were analyzed by using this method. RT data from experiment 3 were also analyzed by using the multiple drug effect analysis (4). This method involves plotting dose-effect curves for each agent and for the combination by using a median effect equation and then determining a combination index by using a second equation. (Further details of these calculations are available from us.)

The antiviral effects of rIFN-α_A were similar in all three experiments, demonstrating dose-dependent reduction of HTLV-III replication by RT, IFA, and virus yield assays. The antiviral effect of interferon diminished over time in all experiments. The antiviral effect of interferon was most transient in experiment 3, perhaps because of the higher viral inoculum used (Table 1). Virus yield assays showed a pronounced but transient effect of interferon alone (Table 2). Variable dose-related antiproliferative effects of rIFN-α_A were observed. In experiment 1, 20 and 30% reductions in cell counts occurred at 16 and 64 U/ml, respectively. Less pronounced reductions in cell counts occurred at these doses in experiment 2, and no reductions were seen at doses as high as 128 U/ml in experiment 3 (Table 1).

The antiviral effect of PFA alone was more persistent than that of rIFN-α_A (Table 1) and did not appear to be altered by increased viral inoculum. Results from experiments 1 and 2 were similar to those shown for experiment 3. In experiment

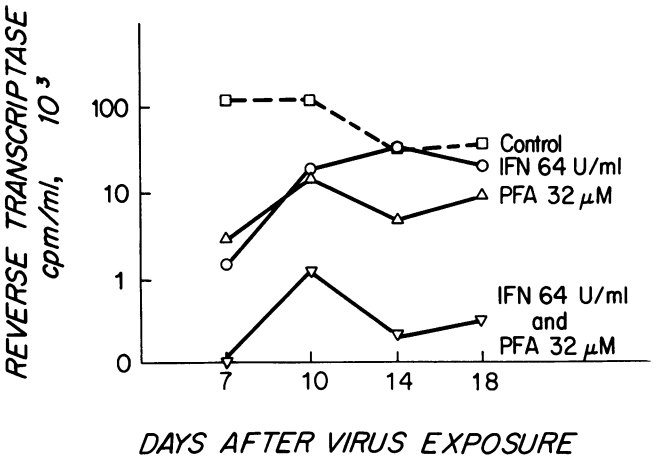

FIG. 1. Effect of rIFN-α_A (64 U/ml) and PFA (32 μM), alone and in combination, on RT activity (experiment 2).

2, the observed effects of PFA were more pronounced, with 64 μM being fully inhibitory. IFA results paralleled those of RT. Virus yield results paralleled RT results on day 10 but not on day 14 (Table 1). No consistent antiproliferative effects of PFA alone were seen in these experiments.

In all experiments, a persistent antiviral effect of combinations was observed in excess of that induced by either agent alone. In experiments 1 and 3, 128 μM PFA was incompletely suppressive of viral replication alone but became completely suppressive in the presence of 16 or 64 U of rIFN-α_A per ml. In experiment 2, a similar result was seen at 32 μM PFA (Fig. 1).

Synergy calculations using the fractional iso-inhibitory concentration method with 90 and 99% inhibition of RT activity as the endpoint showed most combinations to be synergistic in all three experiments. RT data from experiment 3 (Table 1) were also evaluated by using the multiple drug effect equation (4), and synergy was again found consistently at doses causing high degrees of RT inhibition (Table 3).

The interactive effects of the combination on RT activity were paralleled by IFA results (data not shown) and by virus yield assays (Table 2). The antiproliferative effects of the combination did not exceed those of the interferon dose used, with the single exception of the combination of PFA at 256 μM and rIFN-α_A at 128U/ml. In fact, numbers of T4 helper cells (17) were actually increased in treated cultures, when compared with untreated controls (data not shown).

These experiments demonstrate a synergistic anti-HTLV-III interaction for the combination of rIFN-α_A and PFA at in vitro concentrations that are achievable in vivo (7, 9).

TABLE 3. Combination indices of mixtures of rIFN-α_A and PFA (experiment 3)

% RT inhibition	Combination index[a] on day:				
	6	9	14	17	21
50	1.59	0.73	1.32	0.64	0.11
75	0.84	0.56	0.48	0.33	0.14
90	0.73	0.48	0.20	0.17	0.20
95	0.77	0.44	0.11	0.11	0.26

[a] Combination indices of <1.00 indicate synergism. Calculations were made by using multiple drug effect analyses (4, 5).

Combination antiviral therapy may prove useful in the therapy of acquired immunodeficiency syndrome and other HTLV-III-associated disorders.

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