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Combined Interferon-α_2, Rimantadine Hydrochloride, and Ribavirin Inhibition of Influenza Virus Replication In Vitro

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Recombinant DNA-produced human interferon-α_2 inhibited the replication of influenza A and B viruses in primary rhesus monkey kidney cells (RMK). Human interferon-α_2 interacted additively or synergistically with rimantadine hydrochloride or ribavirin in reducing the yield of clinical isolates of either H3N2 or H1N1 subtype influenza A viruses. The combination of human interferon-α_2 and ribavirin also inhibited the replication of an influenza B virus to a greater extent than either single agent. In addition to drug concentration, the virus inoculum and duration of culture were important variables in determining the degree of inhibition. Single drugs or combinations did not significantly inhibit the growth of uninfected RMK cells, which indicated that the observed interactions with respect to antiviral activity were not due to cell cytotoxicity.

Rimantadine hydrochloride, an analog of amantadine hydrochloride has well-documented prophylactic (4) and therapeutic (24) activity in uncomplicated influenza A virus infection after oral administration. Intermittent aerosol administration of rimantadine is associated with therapeutic effects comparable to those of oral rimantadine in experimentally induced influenza A/H1N1 virus infection (11). The nucleoside analog ribavirin has demonstrable therapeutic activity in natural influenza when administered by small-particle aerosol (14), but not when administered by the oral route (21). Prophylactic administration of intranasal human leukocyte interferon has been associated with variable protection against clinical influenza in humans (12, 22). None of these antiviral agents has marked antiviral activity or therapeutic effectiveness in established influenza, and no specific therapy of proven value currently exists for severe influenza virus infection. As an alternative to single-drug therapy, the present study was undertaken to determine the inhibitory activity of recombinant DNA-produced human interferon-α_2 (HuIFN-α_2) alone and in combination with other antiinfluenza compounds on the in vitro replication of human influenza viruses.

MATERIALS AND METHODS

Compounds. Lyophilized HuIFN-α_2 (SCH 30500; Schering Corp., Bloomfield, N.J.), with a specific activity of $10^{8.0}$ IU/mg of protein (9a, 10a) and crystalline powders of rimantadine hydrochloride (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) and ribavirin (Viratek, Inc., Covina, Calif.) were provided by the manufacturers. Compounds were dissolved in distilled water, and samples were frozen at −20°C. Before each experiment, thawed samples were further diluted in Eagle minimal essential medium containing 1% glutamine, 100 U of penicillin per ml and 50 μg of gentamicin per ml.

Viruses. A clinical isolate of influenza A/Aichi/68/H3N2, passaged approximately eight times in embryonated hen eggs, was provided by Allen Kendall, Centers for Disease Control. A/England/333/80/H1N1 was initially isolated in primary rhesus monkey kidney cell (RMK) monolayers (M. A. Bioproducts, Inc., Walkersville, Md.) and passaged twice in embryonated hen eggs. B/Lee/40, a clinical isolate that has an extensive passage history in ferrets, mice, and chicken embryo, was provided by Jerome Schulman, Mt. Sinai Hospital, New York, and passaged once in eggs before use.

Drug studies. A 0.2-ml sample containing 10 to 320 50% tissue culture infective doses ($TCID_{50}$) virus was inoculated onto four to six RMK monolayers in 16- by 125-mm screw-capped tubes, adsorbed at 36°C for 60 min, and decanted. The monolayers were washed once with Hanks balanced salt solution and then replenished with Eagle minimal essential medium containing the appropriate drug dilution. After incubation at 36°C for 24 or 48 h, the monolayers were subjected to one freeze (−70°C)-thaw cycle, and the cells and supernatants were harvested together.

Influenza A virus titers were determined by plaque assay of decimal dilutions in Madin Darby canine kidney (MDCK) monolayers (Flow Laboratories, Inc., Rockville, Md.) by previously described methods (8). B/Lee virus titers were determined by inoculation of decimal dilutions onto quadruplicate MDCK monolayers, which were maintained with Eagle minimal essential medium containing 2 μg of trypsin (Worthington Diagnostics, Freehold, N.J.) per ml and hem-adsorbed with guinea pig erythrocytes at 96 h. Infectivity titrations of the B/Lee stock virus in plain Hanks balanced salt solution and in Hanks balanced salt solution containing HuIFN-α_2 (10,000 IU/ml) gave comparable endpoint titers in this assay system. Fifty percent endpoints were determined by the Kärber formula (7).

Drug interactions. Drug interactions were characterized by previously described methods (3, 20). The fractional yield of the compound A (YA) was defined as the virus titer in the presence of the compound divided by that obtained in the absence of the compound. If the observed yield in the presence of both compounds A and B (YAB) was less than the calculated yield for the two (YA × YB), the interaction was defined as synergistic. If YAB equaled YA × YB, the interaction was termed additive. An interaction that produced inhibition no greater than the most effective single compound was considered indifferent. Similarly, the observed yield in the presence of three compounds (YABC)

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TABLE 1. Inhibition of influenza A/England/333/80/H1N1 virus replication in RMK cell monolayers by HuIFN-α_2, rimantadine hydrochloride, and ribavirin

Compound	Concn	Expt 1			Expt 2		
		Virus titer at 48 h[a]	Fractional yield		Virus titer at 48 h[a]	Fractional yield	
			Observed	Calculated		Observed	Calculated
None		4.35 ± 0.10	1.0000		4.94 ± 0.12	1.0000	
HuIFN-α_2	2,000 IU/ml	3.38 ± 0.14	0.1072		4.05 ± 0.65	0.1288	
HuIFN-α_2	10,000 IU/ml	2.39 ± 0.20	0.0110				
Rimantadine	1.0 μg/ml	3.86 ± 0.19	0.3236		3.32 ± 0.54	0.0240	
Rimantadine-HuIFN-α_2	1.0 μg/ml–2,000 IU/ml	1.42 ± 0.15	0.0012[b]	0.0347	2.34 ± 0.27	0.0025[c]	0.0031
	1.0 μg/ml–10,000 IU/ml	1.01 ± 0.25	0.0005[b]	0.0063			
Ribavirin	1.5 μg/ml	1.13 ± 0.35	0.0006				
Ribavirin	0.3 μg/ml				4.08 ± 0.18	0.1380	
Ribavirin–HuIFN-α_2	1.5 μg/ml–2,000 IU/ml	0.36 ± 0.19	0.0001[c]	0.0001			
	1.5 μg/ml–10,000 IU/ml	0	0[d]	<0.0001			
	0.3 μg/ml–2,000 IU/ml				2.21 ± 0.30	0.0019[b]	0.0178
Rimantadine–ribavirin[e]		0	0[b]	0.0002	1.50 ± 0.37	0.0004[b]	0.0033
Rimantadine–ribavirin–HuIFN-α_2[e]		0	0	<0.0001	0.92 ± 0.04	<0.0001[c]	<0.0001

[a] Mean ± standard deviation $\log_{10}$ PFU per 0.2 ml of four to six monolayers at each concentration. The initial inoculum was approximately 320 $TCID_{50}$ per monolayer. The ribavirin concentration was 1.5 μg/ml in experiment 1 and 0.3 μg/ml in experiment 2.
[b] Synergistic interaction.
[c] Additive interaction.
[d] Additive or synergistic interaction.
[e] The concentration of rimantidine was 1.0 μg/ml in both experiments; the concentration of ribavirin was 1.5 μg/ml in experiment 1 and 0.3 μg/ml in experiment 2. The combination of rimantidine and ribavirin was used at the same concentration as the single agent within each experiment.

was compared to the lowest calculated yield derived from the yields with individual compounds (YA × YB × YC) or various combinations (e.g., YAB × YC).

Drug cytotoxicity. The effects on growth of uninfected RMK cells were determined for single drugs and combinations. Five or six screw-capped tubes were inoculated with approximately 3.8×10^4 cells. After 4 h, nonadherent cells were removed, and the attached cells were fed with Eagle minimal esential medium containing 10% fetal bovine serum and the appropriate drug dilution. After 72 h of incubation at 36°C in a 5% CO_2–95% air atmosphere, the monolayers were replenished with fresh drug-containing media. At 144 h the cells were harvested by trypsinization with 0.25% trypsin-EDTA, and the number of viable cells was determined by direct visual count (hemocytometer) in the presence of trypan blue (0.2 g/100 ml). The viability of both control and drug-exposed cells exceeded 90%.

RESULTS

Inhibitory activity of HuIFN-α_2. Initial studies determined that exposure of primary RMK monolayers to HuIFN-α_2 (500 to 4,000 IU/ml) from 24 h pre-inoculation to 24 h post-inoculation was associated with 1.16 to 2.13 $\log_{10}$ reductions in A/Aichi/68/H3N2 yield compared with nonexposed monolayers. In a separate experiment the mean 24-h virus yield of monolayers inoculated with approximately 10 $TCID_{50}$ of A/Aichi was reduced by 3.25 $\log_{10}$, when the monolayers were both preincubated and maintained with HuIFN-α_2 (10,000 IU/ml) 24 h pre-inoculation to 24 h post-inoculation, as compared with 1.70 $\log_{10}$ when the HuIFN-α_2 was added after virus adsorption (1 to 24 h post-inoculation). All subsequent experiments utilized postadsorption addition of HuIFN-α_2 to model the therapeutic situation and to enable comparison with earlier studies (5, 9, 15).

HuIFN-α_2 inhibition of A/England (Table 1) and B/Lee (Fig. 1) yield was also concentration dependent. As noted in Table 1, the fractional yield of A/England decreased 10-fold with a 5-fold increase in HuIFN-α_2 concentration. In contrast, HuIFN-α_2 (10,000 IU/ml) did not inhibit A/Aichi yield at 48 h, despite a low initial virus inoculum (Table 2). Other experiments determined that the mean A/Aichi yield was reduced by 1.5 $\log_{10}$ at 24 h after a low inoculum, but when the inoculum was increased 10-fold, the 24-h yield was minimally reduced (Table 2). HuIFN-α_2 (10,000 IU/ml) exposure was also associated with reduced fractional yields of B/Lee, which depended slightly on the initial virus inoculum (Table 3). An inoculum effect was not unique for HuIFN-α_2, since the inhibitory effects of rimantadine, ribavirin, and various combinations were found to be dependent on virus inoculum and age of culture (Table 2).

Inhibitory activity of combinations. For each combination of two antiviral drugs (HuIFN-α_2–rimantadine, HuIFN-α_2–ribavirin, or rimantadine–ribavirin), the observed fractional

TABLE 2. Effect of inoculum and duration of culture on inhibition of influenza A/Aichi/68/H3N2 virus replication in RMK cell monolayers by HuIFN-α_2, rimantadine, and ribavirin

Compound[a]	Fractional virus yield (inoculum/monolayer) compared with control after[b]:		
	24 h (~32 $TCID_{50}$)	24 h (~320 $TCID_{50}$)	48 h (~10 $TCID_{50}$)
HuIFN-α_2	0.0912	0.8128	1.3490
Rimantadine	0.2455	0.3090	1.2883
Rimantadine–HuIFN-α_2	0.0052[c]	0.0523[c]	0.0132[c]
Ribavirin	0.0001	0.1097	0.3631
Ribavirin–HuIFN-α_2	<0.0001[d]	0.0661[d]	0.0933[c]
Rimantadine–ribavirin	0[c]	0.0151[d]	0.0145[c]
Rimantadine–ribavirin–HuIFN-α_2	0	0.0050[e]	0.0023[d]

[a] Concentrations were as follows: HuIFN-α_2, 10,000 IU/ml; rimantadine, 1.0 μg/ml; ribavirin, 1.5 μg/ml.
[b] Mean $\log_{10}$ PFU per 0.2 ml of four to six pooled monolayers at each concentration. The virus yields in control monolayers were 4.93, 5.36, and 5.31 $\log_{10}$ PFU per 0.2 ml, respectively.
[c] Synergistic interaction.
[d] Additive or synergistic interaction.
[e] Additive interaction.

TABLE 3. Inhibition of influenza B/Lee/40 virus in RMK monolayers by HuIFN-α_2, rimantadine hydrochloride, and ribavirin

Compound[a]	Fractional yield (inoculum/monolayer) in 48-h titer compared with control[b]	
	Expt 1 (~10 $TCID_{50}$)	Expt 2 (~320 $TCID_{50}$)
HuIFN-α_2	0.003	0.010
Rimantadine	0.316	1.000
Ribavirin	0.001	0.001
HuIFN-α_2–rimantadine	0.006[c]	0.006[c]
HuIFN-α_2–ribavirin	<0.001[d]	<0.001[d]
Rimantadine–ribavirin	0.001[c]	0.001[c]
HuIFN-α_2–rimantadine–ribavirin	<0.001[c]	<0.001[c]

[a] Concentrations were as in Table 2.
[b] Values are the means of six pooled monolayers at each concentration. The virus control yield was 3.25 $\log_{10}$ $TCID_{50}$ per 0.2 ml in experiment 1 and 4.5 $\log_{10}$ $TCID_{50}$ per 0.2 ml in experiment 2.
[c] Indifferent interaction.
[d] Subadditive interaction.

yields for each influenza A virus were less than the calculated yields (Tables 1 and 2). This observation was also true for A/Aichi (Table 2) and B/Lee (Table 3) under various conditions of initial virus inoculum and age of culture. An indifferent or antagonistic interaction was not observed for either influenza A virus strain. In certain experiments with A/Aichi or A/England, the potent inhibitory effect of ribavirin alone precluded a clear distinction between an additive or synergistic interaction with the other compounds. In experiments in which the observed degree of inhibition by two drugs was not complete, the addition of the third agent was associated with further reductions in yield that indicated at least an additive and, possibly for A/Aichi (Table 2), a synergistic interaction.

The yield of B/Lee was also reduced by HuIFN-α_2 or ribavirin (Table 3 and Fig. 1). Rimantadine (1 μg/ml) was

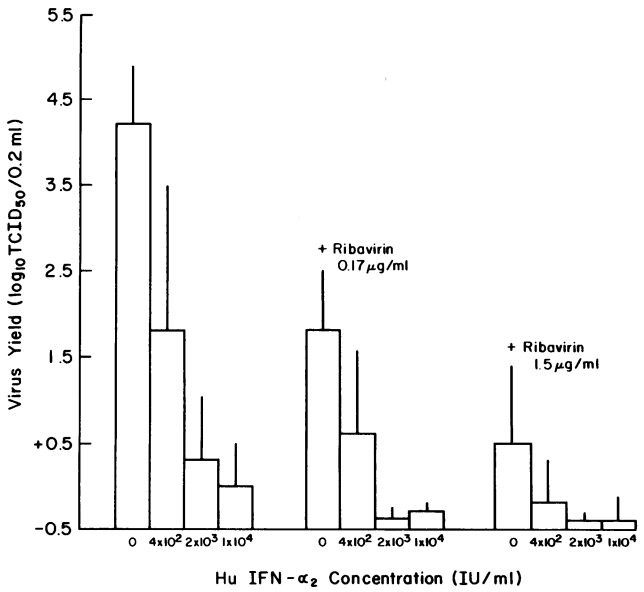

FIG. 1. Inhibition of influenza B/Lee/40 virus growth in RMK cell monolayers by various concentrations of IFN-α_2 and ribavirin. The drug interactions were characterized as additive or subadditive in all instances.

associated with no or minimal reductions in virus yield compared with no treatment, and the addition of rimantadine to either HuIFN-α_2 or ribavirin was not associated with further reductions in virus yield as compared with either single drug (Table 3). The marked yield reduction associated with ribavirin (1.5 μg/ml) prevented a clear characterization of its interaction with HuIFN-α_2, although the observed yields with the combination were lower than with ribavirin alone. Other experiments with ninefold lower ribavirin concentrations and various concentrations of HuIFN-α_2 found greater yield inhibition by the combinations than by single agents (Fig. 1). The interactions were characterized as additive or subadditive in all instances.

Drug cytotoxicity. The growth of uninfected secondary RMK cells was not significantly (Student's *t* test) reduced by drug exposure (Table 4). The number of viable cells per monolayer approximately doubled during the first 72 h and increased approximately 10-fold by 144 h in both control and drug-exposed monolayers.

DISCUSSION

This study utilized a yield reduction assay to determine the inhibitory activity of HuIFN-α_2 alone and in combination with other antiinfluenza agents against representative human influenza viruses. Primary RMK cell monolayers were used because of their availability and susceptibility to productive infection with influenza viruses and because of earlier work demonstrating that HuIFN-α_2 was protective against experimental vaccinia virus infections of rhesus monkeys (18, 19). HuIFN-α_2 reduced the yield of all three influenza viruses tested. The timing of HuIFN-α_2 addition, the size of the virus inoculum, the duration of culture, and the HuIFN-α_2 concentration influenced the magnitude of the observed effect. Earlier studies found that human leukocyte interferon inhibited the replication (0.5 $\log_{10}$ yield reductions) of H3N2 subtype influenza A viruses in human embryonic kidney cell monolayers pretreated with concentrations of 10 IU/ml and lower (16, 17). The results of the current study are not directly comparable because of the use of a different cell culture system and postinoculation addition of HuIFN-α_2.

In contrast to the study of Richman et al. (17), which reported that multiplicities of infection ranging from 0.01 to 1.0 were associated with similar levels of interferon sensitivity, we found that the observed yield reductions were substantially influenced by inoculum size. The fact that an inoculum effect was evident for all three antiviral agents tested against A/Aichi, but less so for B/Lee (Table 3), suggested that the growth characteristics of the A/Aichi

TABLE 4. Growth of RMK cells in the presence of HuIFN-α_2, ribavirin, and rimantadine hydrochloride

Compound[a]	% Increase at 144 h[b]
Growth medium	1,225 ± 425
HuIFN-α_2	1,025 ± 274
Ribavirin	967 ± 167
Rimantadine	967 ± 175
HuIFN-α_2–ribavirin	917 ± 200
HuIFN-α_2–rimantadine	892 ± 142
HuIFN-α_2–ribavirin–rimantadine	967 ± 142

[a] Concentrations were as in Table 2.
[b] The values are listed as mean ± standard deviation percent increase compared with baseline (0 h) values. The mean cell counts of control monolayers were 3.8×10^4 (0 h), 7.2×10^4 (72 h), and 53×10^4 (144 h).

strain were important variables in this observation. A similar inoculum effect was found in earlier studies of the inhibition of influenza A virus replication in MDCK cell monolayers by rimantadine and ribavirin (9).

Our results confirm earlier studies by Lavrov et al. (15), which reported greater inhibition of influenza A/WSN virus replication in chicken embryo cells with combinations of amantadine and low concentrations of crude chicken interferon than with single agents. In contrast to this study, we used highly purified HuIFN-α_2 and clinical isolates of contemporary influenza A viruses. Our results also confirm earlier in vitro studies in MDCK (9) and chicken embryo fibroblast (5) cell cultures, which showed that rimantadine and ribavirin combinations have enhanced antiviral activity against influenza A viruses. More importantly, we found that under certain in vitro conditions the combination of all three antiviral agents could be shown to have greater inhibitory activity than the combination of any two agents. In addition, these studies show that combinations of HuIFN-α_2 and ribavirin have greater inhibitory activity than single agents against influenza B virus.

Comparison between the activity of individual antiinfluenza agents or particular combinations are not warranted within the context of the current study, in part because we only evaluated one concentration for several agents tested. For example, in experiments with A/Aichi the observed inhibition with combinations of rimantadine plus HuIFN-α_2 or of rimantadine plus ribavirin was greater than that observed with ribavirin plus HuIFN-α_2 under some test conditions, but not others (Table 2). A different pattern of inhibition occurred with A/England (Table 1). The limitations of in vitro susceptibility testing of influenza viruses are illustrated by the findings of Browne et al. (2), who showed that rimantadine had greater plaque inhibitory activity than ribavirin against human influenza A viruses, whereas the opposite was true in multiple cycle growth yield reduction experiments.

The in vivo significance of our observations remains to be determined. In experimental murine influenza, ribavirin in combination with amantadine or rimantadine is associated with greater therapeutic activity than either single agent (23; F. G. Hayden and B. A. Anderson, Clin. Res. **29**:868A, 1981). In humans an endogenous interferon response is detectable in respiratory secretions and blood during uncomplicated influenza (6, 13, 16, 17), but interferon was reportedly absent from the lung tissue of persons dying with influenza virus pneumonia (1). The drug concentrations used in our studies are within the range observed in nasal wash samples after topical administration of HuIFN-α_2 (9a, 10a), amantadine hydrochloride (10), or ribavirin (J. Connor, unpublished observations) to the respiratory tract of humans. The combined use of exogenous HuIFN-α_2 with rimantadine or ribavirin (or both) may prove more effective than any single agent in treating serious influenza virus infection.

In summary, recombinant DNA produced HuIFN-α_2 inhibited the replication of clinical isolates of influenza A and B viruses in vitro. HuIFN-α_2 interacted additively or synergistically with rimantadine hydrochloride or ribavirin in reducing the yield of either H3N2 or H1N1 subtype influenza A viruses. The combination of HuIFN-α_2 and ribavirin also inhibited the replication of an influenza B virus to a greater extent than either single agent. Studies of the growth of uninfected RMK cells indicated that observed interactions with respect to antiviral activity were not due to cell cytotoxicity.

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