Effects of Combining Selected Inhibitors of Influenza Virus Replication in Tissue Culture

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When certain compounds—mostly antimetabolites inhibitory to influenza virus replication in tissue culture—were combined in various ways, the virus inhibition was enhanced in a synergistic manner with many of the combinations studied. The selectivity of this inhibitory effect also was increased significantly in most cases. In view of these experimental results, further work seems warranted on aerosol inhalation treatment of influenza-infected mice with certain combinations of these compounds.

A NUMBER OF analogs of amino acids and at least one enzyme have been shown to inhibit the replication of one or more animal viruses in tissue culture. However, the concentrations required usually have been relatively high and the ratio between toxic and inhibitory concentrations rather low. During the course of a study of influenza virus inhibitors, it appeared worthwhile to determine whether either of these limitations could be influenced favorably by combining two or more inhibitory compounds. The results of experiments employing various combinations of such inhibitors are the subject of this report.

EXPERIMENTAL

Influenza Virus.—The Lee strain of virus was employed. It was maintained in the form of frozen chick embryo allantoic fluid stored at −60° and was titrated periodically for infectivity in 10-day chick embryos by the method previously described (1).

Compounds.—L-Canavanine was extracted from jack bean meal and isolated as the free base; lupulon was extracted from hops and purified. Both were prepared in the Department of Chemistry, Oregon State University, through the kind cooperation of Dr. B. E. Christensen. All of the other experimental compounds were obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio.

Tissue Culture Method.—The tissue culture system employed also has been described (1). About 4 cm.^{2} of chorioallantoic tissue from 10-day chick embryos was suspended in 2.0 ml. of Hanks' balanced saline solution (BSS) in 25 × 150 mm. Pyrex culture tubes. Compounds to be studied were dissolved in this solution and the pH adjusted to 7.5 by addition of 1.4% $NaHCO_3$ solution or 0.03 *N* HCl. Such solutions were sterilized by filtration through fritted glass filters. Penicillin was incorporated in a concentration of 10 units/ml. and streptomycin at 40 mcg./ml. Cultures were inoculated with sufficient virus to give a titer of at least 80 hemagglutinating units/ml. in controls after incubation. This was usually 0.1 ml. of the 10^{-2} dilution of the frozen stock and represented approximately 10^6 chick embryo ID_{50} per milliliter of culture fluid.

Cultures were incubated on a reciprocating shaker at about 90 cycles per minute at 35° for 44 to 48 hr. The virus content of culture fluids then was titrated.

Virus Titration.—Virus concentration in tissue culture fluids was measured by hemagglutinin titration, using the pattern method described by Salk (12), with a 0.5% chicken erythrocyte suspension for increased sensitivity. It is well known that agglutination of erythrocytes by influenza viruses is caused by the virus particles themselves, and that this property is more stable than the infectious property of the particles. Virus titration by the hemagglutinin method has been employed widely in studies of influenza virus inhibitors, with only occasional confirmatory titration of infectivity, because it is the most practical method where numerous titrations must be run (7, 10, 11). It is less sensitive to low virus concentrations than infectivity measurement by the dilution end point method in 10-day chick embryos, but it is capable of somewhat greater precision. A twofold reduction in virus yield usually will be significant when based upon a comparison of geometric means of hemagglutinin titration values from 12 replicate cultures and 12 controls. However, the smallest significant reduction measurable by infectivity titration, using five chick embryos per dilution, is about fourfold, and the two titrations needed require more time and materials than the 24 hemagglutinin measurements. When changes in virus concentration are measured by both methods simultaneously, the results usually agree within the limits of the experimental errors if the difference in concentration is great enough to be detected by the infectivity measurement.

With respect to the compounds employed in this study, each of them had been shown previously in this laboratory or by others to inhibit replication of influenza virus or one of the other myxoviruses in tissue culture. In each case, inhibition was observed when virus concentrations were determined by measurement of infectivity as well as when hemagglutinin titration was used (3–8). The latter method accordingly was considered adequate for tissue culture studies of various combinations of these compounds. It should be noted also that

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none of the latter had any effect on the end points of hemagglutinin titrations when present in virus erythrocyte suspensions in the same concentrations employed in tissue cultures.

Toxicity for Tissue Cells.—The method employed to determine toxicity of compounds and mixtures for chick embryo cells was similar to that described by Lum and Smith (2). Finely minced suspensions of chorioallantoic membranes from 10-day chick embryos were washed in sterile BSS, centrifuged, and resuspended in BSS to give an approximately 50% tissue suspension, which was added in 0.1-ml. volumes to sterile 16 × 150 mm. Pyrex tubes. The fragments were distributed as uniformly as possible over the lower third of the tubes, and the latter held in a horizontal position for 2 hr. at 35° to cause the fragments to adhere to the glass. BSS containing the desired concentrations of the compounds to be tested in sterile solution at pH 7.4 to 7.5 then was introduced into the tubes in a volume of 0.9 ml. Cultures were incubated at 35° on a roller drum operating at 0.2 r.p.m. for 44 to 46 hr. All tubes then were examined microscopically for evidence of cellular outgrowth. The lowest concentrations of compounds giving complete suppression of cell growth were considered the minimum toxic concentrations.

RESULTS

Combinations of Two Inhibitors.—Combinations of nine compounds known to be individually inhibitory to the Lee influenza virus in tissue culture at concentrations nontoxic to the tissue were selected for studies of their combined inhibitory activity. Those selected, most of which are antimetabolites, included L-canavanine, DL-β-phenylserine, DL-*p*-fluorophenylalanine, DL-*m*-tyrosine, DL-alanyl-DL-serine, galactosamine, benzimidazole, lupulon, and the enzyme ribonuclease. These compounds were chosen from a large group examined for inhibition of influenza virus replication because most of them appeared to interfere with new virus formation by somewhat different mechanisms and showed some measurable degree of selectivity in this respect. None had direct effect on the virus itself.

Because it was not feasible to study all possible pairs, 15 two-component mixtures were selected somewhat arbitrarily. In eight of them, canavanine was combined individually with each of eight other compounds; five more consisted of β-phenylserine combined with each of five others; combinations of benzimidazole with *m*-tyrosine, and *p*-fluorophenylalanine with ribonuclease also were studied.

In each experiment, at least four replicate tissue cultures were employed for each inhibitor concentration, and repetition of the experiment provided eight or 12 such replicates.

In the case of the combinations of canavanine with lupulon and with galactosamine, the combined inhibitory effect was no greater than that of one of the components alone. For five other pairs of inhibitors, the combined effect was at least additive. These included the combinations of canavanine with alanyl serine, with *p*-fluorophenylalanine, and with ribonuclease, and β-phenylserine with galactosamine and with *p*-fluorophenylalanine.

Another group of seven pairs of compounds gave inhibitory effects considered definitely synergistic. These were combinations producing a distinctly more than additive effect or in which the addition of an ineffective concentration of one compound to a marginally active level of another resulted in a marked degree of inhibition. Such effects were shown by the combinations of canavanine with benzimidazole, with β-phenylserine, and with *m*-tyrosine; β-phenylserine with benzimidazole and with *m*-tyrosine; benzimidazole with *m*-tyrosine; and *p*-fluorophenylalanine with ribonuclease. These combinations were studied thoroughly; the results obtained with a representative pair, canavanine and *m*-tyrosine, are shown in Fig. 1. Each point on each curve was calculated as the geometric mean of the hemagglutinin titers of eight separate cultures in two experiments. It may be noted that *m*-tyrosine, at a concentration of 62.5 mcg./ml., did not cause a significant reduction in virus yield; but when combined with a borderline concentration of canavanine, 12.5 mcg./ml., a marked reduction in virus titer resulted.[1] This was considered an acceptable criterion for synergism. Curves similar to those in Fig. 1 were obtained with the other synergistic pairs of compounds.

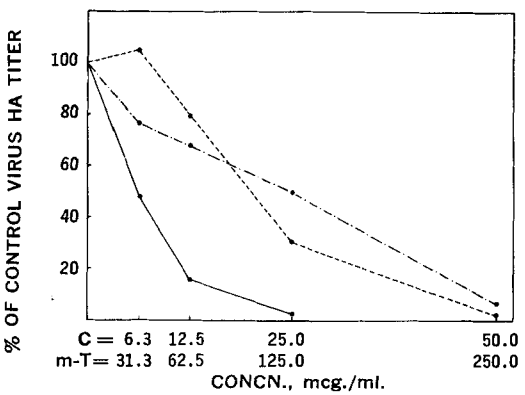

Fig. 1.—Inhibition of replication of Lee influenza virus in tissue culture by L-canavanine and DL-*m*-tyrosine. Key: · · · · ·, canavanine; – – – –, *m*-tyrosine; ——, canavanine + *m*-tyrosine.

Among the two-component mixtures studied, the most potent in terms of minimum concentrations producing significant virus inhibition was the combination of canavanine and *p*-fluorophenylalanine. This mixture was active at a concentration of 6.3 mcg. of each compound per milliliter, which was only one-eighth the concentration of each required when used alone.

In addition to enhancing virus inhibition by combining certain compounds, it seemed possible that the selectivity of this inhibition, compared to the toxic action upon the host cells, might be increased. This was measured as the ratio of the minimum concentrations of a particular combination of compounds suppressing growth of chick chorioallantoic

[1] The reduction in virus yield caused by adding *m*-tyrosine at this concentration to canavanine as shown was found to be significantly greater than that produced by canavanine alone. Student's *t* test (9), applied to the logs of the virus titers of the eight replicate cultures in each experimental group, showed the difference in means to be significant with a value of $P < 0.05$.

TABLE I.—TOXIC AND VIRUS INHIBITORY CONCENTRATIONS OF SINGLE COMPOUNDS AND COMBINATIONS

Compd. or Combination	MTC,[a] mg./ml.	VIC,[b] mg./ml.	MTC/VIC
L-Canavanine	0.200	0.050	4
Benzimidazole	1.200	0.600	2
DL-β-Phenylserine	1.200	0.200	6
DL-*m*-Tyrosine	1.000	0.125	8
DL-*p*-Fluorophenylalanine	1.000	0.050	20
Ribonuclease	25.600	0.800	32
L-Canavanine +	0.200	0.013	16
DL-*m*-tyrosine	1.000	0.063	
DL-β-Phenylserine +	2.000	0.063	32
DL-*p*-fluorophenylalanine	0.200	0.006	
L-Canavanine +	0.800	0.006	128
DL-*p*-fluorophenylalanine	0.800	0.006	
DL-*p*-Fluorophenylalanine +	0.800	0.006	128
ribonuclease	12.800	0.100	
L-Canavanine +	0.200	0.006	
benzimidazole +	1.200	0.038	32
DL-β-phenylserine +	1.200	0.038	
DL-*m*-tyrosine	1.000	0.031	
L-Canavanine +	0.200	0.006	
benzimidazole +	1.200	0.038	32
DL-β-phenylserine +	1.200	0.032	
DL-*p*-fluorophenylalanine	0.200	0.006	
L-Canavanine +	0.200	0.006	
DL-β-phenylserine +	1.200	0.038	32
DL-*m*-tyrosine +	0.500	0.016	
DL-*p*-fluorophenylalanine	0.200	0.006	
L-Canavanine +	0.200	0.0063	
benzimidazole +	1.200	0.0375	32
DL-β-phenylserine +	1.200	0.0375	
ribonuclease	6.400	0.2000	

[a] The minimum concentration suppressing growth of chick chorioallantoic cells in culture. [b] The minimum concentration reducing the virus titer in tissue culture fluid to 25% or less of the control.

cells in culture to the minimum concentrations causing significant reduction in virus yield in similar cultures. The results obtained with individual compounds and several different combinations are shown in Table I. For the four pairs of compounds listed, this ratio is higher than for the individual compounds in each pair, indicating that virus inhibition was enhanced to a greater extent than tissue toxicity. This was true also for combinations of β-phenylserine with *m*-tyrosine and with *p*-fluorophenylalanine and for ribonuclease with canavanine. The combinations of canavanine with *p*-fluorophenylalanine and the latter compound with ribonuclease were found to have ratios of 128, the highest observed for any combinations studied. It should be noted that this ratio also is dependent upon the concentration of virus used to inoculate the cultures, which was relatively high in these experiments. At lower levels of virus concentration, the MTC/VIC ratios would all have been considerably higher than those shown in Table I.

Combinations of Three or Four Inhibitors.—To determine whether further advantage could result from combining more than two compounds, three mixtures containing three components and four containing four components were studied. Among the three-component mixtures, synergism and increased selectivity were observed but not in greater degree than among the pairs of compounds.

One of the four component mixtures studied was the combination of canavanine, β-phenylserine, *m*-tyrosine, and *p*-fluorophenylalanine. The combined results of two experiments with this mixture are shown in Fig. 2. Again each point on each curve is the geometric mean value from eight replicate cultures. It is readily apparent that canavanine alone at a concentration of 6.3 mcg./ml. did not reduce the virus yield; but when the same concentration was added to the other three components at concentrations permitting the formation of about 60% as much virus as in control cultures,

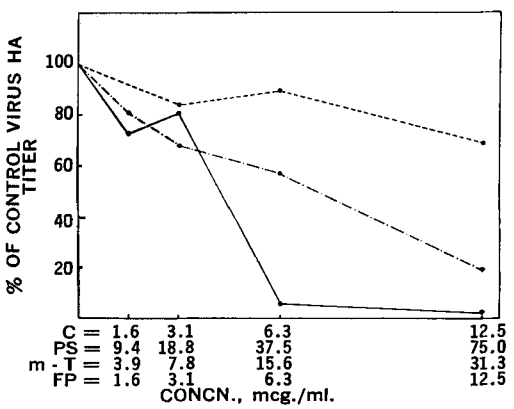

Fig. 2.—Inhibition of replication of Lee influenza virus in tissue culture by a combination of L-canavanine, DL-β-phenylserine, DL-*m*-tyrosine, and DL-*p*-fluorophenylalanine. Key: – – – –, canavanine; · · · · ·, β-phenylserine + *m*-tyrosine + *p*-fluorophenylalanine; ——, canavanine + β-phenylserine + *m*-tyrosine + *p*-fluorophenylalanine.

the virus titer was reduced to less than 10% of the mean control value. This appears to be a clearly synergistic effect.

The addition of a marginally active concentration of *p*-fluorophenylalanine to a mixture of canavanine, benzimidazole, and β-phenylserine produced inhibitory effects which were essentially additive.

In the case of a third four-component mixture, when a noninhibitory concentration of *m*-tyrosine was combined with a mixture of canavanine, benzimidazole, and β-phenylserine, which permitted the development of 60% as much virus as in control cultures, the inhibition of virus replication was enhanced in a synergistic manner.

The fourth combination of four compounds contained ribonuclease, canavanine, benzimidazole, and β-phenylserine. Concentrations of the enzyme which were quite ineffective in themselves, when added to mixtures of the other three at concentrations which were likewise inactive, resulted in marked inhibition of the virus.

The selectivity ratio for all of the four-component mixtures was 32, as shown in Table I. This was a

satisfactory value but indicated that in this respect no further advantage had been gained over the better two-component mixtures.

Earlier experiments conducted in this laboratory or by others showed that none of the nine compounds employed in this study had direct inactivating or virucidal effect at minimum concentrations showing marked inhibition of virus replication in tissue culture (3–8). To extend these observations, two of the most active four-component mixtures also were examined for any possible inactivating effect on the virus. None was observed during 24-hr. exposure of the virus at 35° to the minimum concentrations showing strong inhibition of virus replication.

All of the compounds in Table I were tested individually in influenza infected mice, as were the mixtures shown and several others not in the table. Compounds were injected intraperitoneally as sterile solutions at intervals of 12 to 14 hr. for 3 days. Mice were infected intranasally with about 10 mouse LD_{50} of Lee influenza virus within 2 or 3 hr. after the first injection. After 3 days, the animals were sacrificed, and the lungs of each experimental group were removed, pooled, ground in a tissue blender to a 5% suspension, and titrated for virus content. None of the single compounds ever caused a reduction in the virus content of the lungs. The mixture containing canavanine, benzimidazole, β-phenylserine, and *m*-tyrosine reduced the virus content of the lungs of treated mice six to twelvefold in several experiments. The doses required were large and slightly toxic, however, and the results were not regularly reproducible.

DISCUSSION

Among the various combinations of compounds individually inhibitory to influenza virus replication, only those containing one or more of the amino acid analogs, benzimidazole, or ribonuclease showed combined inhibitory effects that were either additive or synergistic.

The inhibition of virus development by canavanine and β-phenylserine has been shown to be due to competitive antagonism of arginine (3) and phenylalanine (4), respectively. Similarly, *m*-tyrosine and *p*-fluorophenylalanine apparently interfere directly with a specific phase of protein metabolism, while benzimidazole and ribonuclease probably do so indirectly through an effect on nucleic acid metabolism. It seems likely that the synergistic effect noted with certain combinations of compounds may be due to interference with two or more different reactions directly or indirectly essential to a single biosynthetic process, *i.e.*, synthesis of virus protein.

Although the experiments with these combined inhibitors in influenza infected mice treated by intraperitoneal injection did not appear promising, they seem to indicate that the chief problem may be that of bringing effective concentrations of the compounds in contact with the infected cells of the experimental animal.

In view of the considerable degree of selectivity of the virus inhibition shown by certain combinations of the compounds studied, such as that of canavanine and *p*-fluorophenylalanine and the four-component mixtures in Table I, it appears that further experiments in animals infected with a respiratory virus should be carried out, employing a method of administration more closely resembling the conditions in the infected tissue cultures. One such experimental approach might be the treatment of mice infected with minimal doses of one of the influenza viruses by inhalation of an aerosol containing the desired virus inhibitors. If the results were encouraging, the activity against one or more of the adenoviruses, parainfluenza viruses, or rhinoviruses could be explored in tissue culture. A nontoxic preparation inhibitory to viruses of the human upper respiratory tract by aerosol inhalation could have worthwhile applications.

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