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Antiviral Effect of Cyclic Amines on a 1969 Isolate of A_2 Influenza

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Cyclooctylamine and amantadine inhibit the growth of 1969 isolates of A_2 influenza virus to a significant degree. There was slightly more inhibition of the virus by the cyclooctylamine (COA) than the amantadine; however, the dose of COA used was greater than the dose of amantadine. There was no significant difference between flasks treated 3 or 4 hr and those treated 2 hr. However, there was a curious relationship of more plaques in the flasks exposed to the two drugs for the longer intervals. Other experiments done with slight modifications in technique support the antiviral effect demonstrated in this experiment when the cell system is pretreated prior to virus infection. In two experiments, pretreating the cells for 2 hr with COA at 100 μg/ml but removing the drug solution and washing the cells prior to virus inoculation revealed no differences in plaque counts between controls and treated cells. This would indicate that the antiviral effect required the presence of the drug during the early stages of penetration of the cells by the virus particles.

The laboratory and clinical evaluation of amantadine hydrochloride (1-adamantane; reference 1) have indicated its usefulness as an antiviral agent specifically of use against A_2 strains of influenza virus. Each new variant of the A_2 family should be examined for in vitro and in vivo (experimental) efficacy. We also examined another similar antiviral agent (cyclooctylamine hydrochloride; reference 2) for in vitro activity against an A_2 strain isolated in Loma Linda in January of 1969.

MATERIALS AND METHODS

Virus. The A_2/LL/1/69 strain isolated in Loma Linda during the winter influenza epidemic was propagated in the chorioallantoic cavity of 10-day-old chick embryos and harvested after 24 hr of incubation at 37 C. This was used as virus stock in all experiments. It was diluted 10-fold in Hepes Balanced Salt Solution (HBS) and inoculated onto rabbit embryo kidney (ERK) monolayers. After 24 hr, guinea pig red blood cells were added (as described below), and hemadsorption plaque counts were made. It was found that a 10^{-3} dilution of virus stock gave an optimal number of hemadsorption plaques for counting. Hepes was obtained from Calbiochem (Los Angeles, Calif.) and 2.38 g was mixed with the following: dextrose, 1 g; NaCl, 8 g; KCl, 0.4 g; $CaCl_2$, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; water, 1 liter. The solution was adjusted to *p*H 7.2 with NaOH and autoclaved at 10 lb/in^2 for 10 min.

Cell cultures. Twenty 8-day-old primary ERK cell cultures were trypsinized with 0.25% trypsin solution. The cells were diluted 1:300 (v:v), and 5-ml volumes were introduced into 30-ml plastic Falcon screw-top tissue culture flasks. Growth and maintenance medium consisted of TC 199 supplemented with 10% fetal calf serum, 20 units of penicillin per ml, and 50 μg of streptomycin per ml.

Experimental drugs. Cyclooctylamine (COA) was obtained from Smith Kline and French Laboratories (courtesy of Jerome A. Gold) and was dissolved in HBS. Control flasks were treated exactly the same as experimental flasks, except that HBS only was used in place of the COA solution.

Amantadine obtained from the Stine Laboratory of E. I. du Pont de Nemours & Co. (courtesy of T. R. Wood) was also examined in this cell system and was dissolved in HBS.

Cytotoxicity. Various concentrations of COA solution were used on 8-day-old ERK monolayers for varying periods of time up to 24 hr. The highest concentration tried that caused no cytotoxic effects after 24 hr was 100 μg/ml. Both unstained as well as Giemsa-stained cells were examined microscopically.

Cytotoxicity of amantadine was also determined for the ERK cells; the 10 μg/ml dosage was well below the concentration associated with cytotoxic morphological changes.

Inoculation. The medium was decanted and cells were washed twice with 5 ml of HBS prior to inoculation. Cells were treated for 2 to 4 hr with 1.8 ml of

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COA solution, amantadine solution, or HBS. A 10^{-2} concentration of virus was then added directly to the flask in 0.2-ml volumes to make a final volume of 2 ml and a virus concentration of 10^{-3}. This mixture was agitated gently for 1 min to assure thorough mixing and allowed to remain on the cells for 1 hr. This was decanted, and the cells were washed twice with HBS. The flasks were then covered with medium and returned to the incubator at 37 C for 24 hr.

Testing for antiviral effects. After 24 hr of incubation, both treated and control flasks were washed twice with cold HBS, and a 0.5% suspension of guinea pig red blood cells was added in 2-ml volumes per flask. After 1 hr at 4 C, nonabsorbed red cells were washed off with three washings of cold HBS. Ten per cent Formalin in HBS was then added, and the guinea pig red blood cells were fixed to the infected cells. In some instances, Giemsa staining was carried out to facilitate the counting of uninfected as well as infected cells. It was also easier to observe the presence of cytotoxicity as evidenced by cytoplasmic or nuclear vacuolization. At the concentrations of both drugs reported here, no such cytopathic effects were noted. Hemadsorption plaques were counted in 100 fields per flask at 100× magnification by an orderly scanning of the flasks. Plaque counts of control flasks were compared with those of flasks treated with COA solution and amantadine solution.

TABLE 1. *Action of cyclic amines on A_2/LL/1/69 in rabbit embryo cell monolayers*

Agent tested	Reaction time	Plaque count
	hr	
Control	3	606[a]
Cyclooctylamine	2	208
	3	249
	4	296
Amantadine	2	329
	3	365
	4	384

[a] Average of duplicate determinations.

RESULTS

Significant differences in plaque counts between control flasks and treated flasks were observed. Six flasks were treated with COA solution (100 μg/ml) and six flasks with amantadine (10 μg/ml). Two flasks were used as controls. The average number of plaques per 100 fields per flask of the control flask was 606. The average for COA-treated flasks was 251; the average for amantadine-treated flasks was 359 (Table 1).

The probability of significance as calculated by the *t* test between control groups and comparable 3-hr drug-treated groups was greater than 0.99 for both COA- and amantadine-treated flasks. Although specific 2- and 4-hr controls are not included, comparisons with the 3-hr controls also gave significant differences for both drug-treated groups.

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