Immunofluorescent Studies on the Inhibition of Influenza A and B Viruses in Mammalian Cell Cultures by Amines and Ammonium Compounds*

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SUMMARY

A quantitative immunofluorescent cell-counting technique was used to investigate the virustatic effect of aminoadamantane, ammonium acetate and a number of aliphatic amines on the development of influenza virus antigens in BHK-21 cell monolayers. Influenza virus strains A2/SCOTLAND/49/57, A/NWS and B/ENGLAND/939/59 were used at high multiplicities of infection in the tests. Quantification of the activity of the antiviral compounds was provided by the direct estimation of the proportion of infected cells in which the production of influenza virus fluorescent antigens was blocked. Comparable results were obtained for A2/SCOTLAND/49/57 virus using the immunofluorescent technique and the more conventional method of measuring antiviral activity by the reduction in the ability of the virus to multiply in tissue culture in the presence of the test compounds.

INTRODUCTION

The activity of possible antiviral compounds may be assessed in vitro by the measurement of their ability to inhibit one or more of the manifestations of virus multiplication in suitable test systems (Stuart-Harris & Dickinson, 1964; Wood, 1965; Tyrrell, Bynoe & Hoorn, 1965; Oxford & Schild, 1967a). The usual methods of testing antiviral activity provide information about the inhibition of virus multiplication in large populations of cells but give no information on the function of an antiviral compound at the cellular level. The present study describes the use of specific immunofluorescent techniques for the investigation of the antiviral activity of various amines and ammonium compounds on influenza virus strains. The method has the advantage of allowing a study of the local effects of these compounds in individual cells. Furthermore, using the immunofluorescent cell-counting technique of Wheelock & Tamm (1961) antiviral activity may be quantified. The proportion of cells developing influenza antigens after infection of a given cell population with high multiplicities of virus can be estimated in the presence or absence of the test compounds. The results of the present immunofluorescent studies were compared with the antiviral activity of the compounds demonstrated by conventional techniques such as infectivity titrations and the measurement of virus haemagglutinin in tissue cultures (Schild & Sutton, 1965; Oxford & Schild, 1967b).

* A short account of this work was presented at the 5th International Congress of Chemotherapy, Vienna, 1967.
METHODS

Viruses. Neurotropic variant A/nws (Stuart-Harris, 1939) had been propagated 103 times in the mouse brain followed by four to eight allantoic passages in chick embryos. A2/scotland/49/57 virus was propagated 6 times and B/england/939/59 10 times allantoically. The latter strain was kindly provided by Dr D. Hobson, University of Liverpool.

Test compounds. The aliphatic amines and ammonium acetate were analytical grade reagents (British Drug Houses). Aminoadamantane was supplied by Dr A. Galbraith of the Geigy Pharmaceutical Company. The compounds were prepared as 1% stock solutions in deionized water and stored at -20°C. The concentrations of amines or ammonium acetate used in experiments for antiviral activity had no cytotoxic effects observable by high power microscopy when they were incubated with BHK 21 cells for 5 days at 37°C. In addition, BHK 21 cells were cultivated through 10 serial passages in growth medium containing 25 µg./ml. of aminoadamantane. The growth rate of these cells was identical to that of control cultures subcultured in normal growth medium.

Cell cultures. BHK 21 cells kindly supplied by Dr W. I. H. Shedden, University of Birmingham, were grown on coverslips in a CO₂ incubator (95% air: 5% CO₂) using Eagle's basal medium containing 10% tryptose phosphate broth and 10% inactivated calf serum.

Monolayer cultures of rhesus monkey kidney cells were grown in tubes using Eagle's basal medium containing 10% inactivated calf serum. Both monkey kidney and BHK 21 cell cultures were maintained in a medium consisting of mixture '199' with 2.2 g./l. of sodium bicarbonate.

Virus titrations. Viruses were titrated by allantoic inoculation of 10-day-old chick embryos. The allantoic fluids were harvested after 72 hr and tested for influenza virus haemagglutinin by the W.H.O. (1953) standard method. In certain experiments influenza virus strains were assayed using end-point infectivity titrations in BHK 21 or rhesus monkey kidney cells, evidence of virus multiplication being obtained by haemadsorption with chick erythrocytes.

Infection of coverslip cultures with viruses. For 1 hr before virus infection coverslip cultures in Petri dishes were incubated at 37°C in maintenance medium containing the appropriate concentration of amine. The cells were then infected with influenza virus diluted in maintenance medium containing the amine and the cultures again incubated for 24 hr at 37°C in a CO₂ incubator.

Immunofluorescence technique. Twenty-four hours after infection with influenza virus the coverslip cultures were washed in phosphate-buffered saline (PBS) and fixed in acetone at room temperature for 3 min. The coverslips were then freed from acetone by incubation at 37°C for 45 min. and washed in PBS before fluorescent staining. Horse anti-human serum globulin conjugated with fluorescein isothiocyanate (Progressive Laboratories, Baltimore, lot no. 3125) was used in the indirect fluorescent technique. The preparations were viewed with a Gillet & Sibert Conference Microscope under illumination from an iodine-quartz source using a G.S. 30/065 primary filter. Photomicrographs were taken on Ilford FP 3 film at a standard exposure of 2 min.

The two antisera used in the fluorescein staining test were from children aged 6 years. In experiments with A2/scotland/49/57 virus the serum used had a haemag-
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glutination-inhibition antibody titre of 1/720 and complement-fixation antibody titre of 1/256 with influenza A soluble antigen. For influenza B/ENGLAND/939/59 the serum used had a haemagglutination-inhibition titre of 1/1280 and a complement-fixation antibody titre of 1/256 with soluble influenza B antigen. In addition, two human sera with no detectable haemagglutination-inhibiting antibodies for the two test viruses at a serum dilution of 1/8 were used as controls.

The specificity of the immunofluorescence was tested in three ways. Uninfected BHK 21 cells were stained using the same procedure as for infected cells; the intermediate serum in the staining procedure was omitted; human sera with no detectable influenza A or B complement-fixing or haemagglutination-inhibiting antibodies were used as the intermediate sera. No antigen was visualized by immunofluorescence in any of these controls.

Cell counts. The number of fluorescing cells was counted in at least 30 randomly selected fields for each test using the 40 x objective. A field of a confluent cell sheet at this magnification contained approximately 100 cells.

RESULTS

A preliminary study was undertaken of the growth of influenza viruses in BHK 21 cells. On first passage in BHK-21 cell cultures all three virus strains produced cytopathic changes which developed within 24 hr of the addition of large virus inocula (10^7 EID 50/ml.). The cytopathic changes included the rounding up of cells and their detachment from the glass, the cell monolayers being completely destroyed after 48 hr incubation. Influenza A/NWS virus was propagated serially three times in BHK 21 cell cultures and produced similar progressive cytopathic changes each time. In contrast, attempts to propagate influenza A2/SCOTLAND/49/57 and B/ENGLAND/939/59 viruses serially in BHK 21 cells were unsuccessful; only small amounts of infective virus (about 3 EID 50/ml.) were detected in the cultures after the first passage and no virus could be detected in two subsequent passages. These two viruses also failed to induce cytopathic changes in BHK 21 cell cultures after the first passage.

Time of appearance of fluorescing influenza antigens in BHK 21 cells

To detect the time of first appearance of influenza antigens, BHK 21 cells were infected with 1000 EID 50 per cell of influenza A/NWS, A2/SCOTLAND/49/57 or B/ENGLAND/939/59 viruses and the cells harvested every 60 min. for the first 8 hr and stained by the indirect fluorescence technique. Influenza A2/SCOTLAND/49/57 virus antigen was first seen as weakly fluorescing spots in the nucleus in approximately 1% of cells 3 hr after infection. After 4 hr nuclear fluorescence was visible in 90% of cells while cytoplasmic fluorescence was first noted 5 hr after infection. The intensity of nuclear and cytoplasmic fluorescence then increased until 18 hr after infection when the cell monolayer had been partly destroyed by virus cytopathic effects, although 100% of the remaining cells continued to fluoresce brightly.

With influenza A/NWS virus, stainable antigen was detected in the nucleus of 10% of cells 5 hr after infection and increased in brightness until at 8 hr all cells were fluorescing brightly in the nucleus and a small proportion (5%) showed weak cytoplasmic fluorescence. By 18 hr all cells remaining on the glass had brightly fluorescing nuclei.

Influenza virus B/ENGLAND/939/59 antigen was first detected as fluorescent spots in
the nucleus 4 hr after infection. After 5 hr incubation, 80% of cells had bright nuclear fluorescence and also cytoplasmic fluorescence, particularly around the nuclear membrane. At 8 hr, fluorescence was brighter in the cytoplasm than in the nuclei and steadily increased in intensity until at 18 hr all cells showed fluorescence of maximal brightness.

**Relationship between virus inoculum and proportion of cells fluorescing at 24 hr**

BHK 21 cell monolayers on coverslips were infected with serial tenfold dilutions of the three test strains of influenza virus and incubated in the CO₂ incubator. Twenty-four hours after infection the number of fluorescing cells on each coverslip was counted. The relationship between the virus inoculum and proportion of fluorescing cells was linear for all three virus strains when plotted on a logarithmic scale (Fig. 1).

**Table 1. Antiviral effect of amines and ammonium acetate on influenza viruses**

<table>
<thead>
<tr>
<th>Compound tested</th>
<th>Concentration (µg./ml.)</th>
<th>Log TCD₅₀/ml. reduction in end-point infectivity titre*</th>
<th>% BHK 21 cells fluorescing 24 hr after infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Aminoadamantane</td>
<td>25</td>
<td>5.5</td>
<td>12.0</td>
</tr>
<tr>
<td>Cells subcultured 10 times with aminoadamantane</td>
<td>25</td>
<td>Not tested</td>
<td>0.6</td>
</tr>
<tr>
<td>Ammonium acetate</td>
<td>200</td>
<td>4.0</td>
<td>16.6</td>
</tr>
<tr>
<td>Methylamine</td>
<td>150</td>
<td>2.0</td>
<td>39.3</td>
</tr>
<tr>
<td>Ethylamine</td>
<td>100</td>
<td>3.0</td>
<td>23.2</td>
</tr>
<tr>
<td>n-Propylamine</td>
<td>100</td>
<td>3.5</td>
<td>19.2</td>
</tr>
<tr>
<td>n-Butylamine</td>
<td>120</td>
<td>3.5</td>
<td>38.6</td>
</tr>
</tbody>
</table>

* Compared to control cultures inoculated with virus only. Titrations in rhesus monkey kidney cultures and end-points determined by haemadsorption.

**Effect of amines on cell infection and the subsequent development of influenza virus antigens**

The effects were studied of several amines and ammonium acetate on the development of influenza virus fluorescent antigens (Table 1). At 24 hr 100% of cells in control cultures infected initially with 1000 EID₅₀ per cell of influenza A/2/SCOTLAND/49/57 virus showed immunofluorescence. The percentage of fluorescing cells was only 12% in BHK 21 cultures infected initially with the same dose of virus but maintained in medium containing 25 µg./ml. of aminoadamantane. However, in the small proportion of cells fluorescing in monolayers treated with aminoadamantane the intensity of cytoplasmic and nuclear fluorescence was similar to that in control cultures. This experiment was repeated with BHK 21 cells which had been serially subcultivated 10 times in the presence of 25 µg./ml. of aminoadamantane when the proportion of cells showing fluorescent antigens was reduced to 0.6%. This indicated that resistance to the antiviral activity of aminoadamantane did not develop in cells cultivated in the presence of the compound and also that 25 µg./ml. of aminoadamantane did not
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inhibit multiplication of BHK 21 cells over a prolonged period. Aminoadamantane slightly inhibited the development of antigen demonstrable by fluorescence in BHK 21 cells infected with A/NWS virus (80-7 % of cells showed fluorescence in drug-treated monolayers). However, there was no significant inhibition of the development of antigen by influenza B/ENGLAND/939/59. In studies on cell cultures maintained with non-toxic concentrations of ammonium acetate (200 μg./ml.) the degree of inhibition of antigen production with the three influenza virus strains was similar to that found with aminoadamantane.

Fig. 1. Relationship between virus inoculum and proportion of BHK 21 cells fluorescing at 24 hr. O, Influenza A2/SCOTLAND/49/57; ●, influenza B/ENGLAND/939/59. The curve for influenza A/NWS was identical to that for influenza B/ENGLAND/939/59 virus

All the primary aliphatic amines tested inhibited antigen production by A2/SCOTLAND/49/57 virus but differences in activity were found; n-propylamine was the most active of these compounds and methylamine the least active. For A/NWS virus the aliphatic amines were more active than aminoadamantane. Two of the amines tested, n-propylamine and n-butylamine, significantly inhibited the development of antigen by influenza B/ENGLAND/939/59 virus, differing in this respect from ammonium acetate and aminoadamantane.

We were interested to learn if the range and degree of antiviral activity of the amines were comparable in different test systems. Therefore, the compounds were also tested for the ability to reduce the infectivity of influenza A2/SCOTLAND/49/57 virus in rhesus monkey kidney tissue cultures. In this test serial tenfold dilutions of virus were inoculated into two sets of tissue culture tubes, one of which contained the test compound in the maintenance medium and the other normal maintenance medium. The difference in the virus infectivity titre in the two sets of tubes may be taken as a measure of the ability of the test compound to prevent progressive virus multiplication in cultures inoculated with different virus doses. The results obtained by both
methods were compared (Table 1). By both techniques aminoadamantane and ammonium acetate had a relatively high degree of antiviral activity against influenza A2/SCOTLAND/49/57 while the aliphatic amines tested were less active.

Effect of the time of addition of aminoadamantane on the development of influenza A2/SCOTLAND/49/57 virus antigens

BHK 21 cell monolayers on coverslips were infected with varying concentrations of A2/SCOTLAND/49/57 virus. Aminoadamantane (25 μg./ml.) incorporated in the maintenance medium, was added to the cells 0, 15, 30, 60 and 120 min. after infection and the cultures incubated with the compound for 24 hr (Table 2). With a large virus inoculum (1000 EID50 per cell) antiviral activity was demonstrable only if aminoadamantane was added to the cultures within 30 min. of infection. However, with smaller virus inocula (10 or 100 EID50 per cell) aminoadamantane added progressively later after virus infection was still active.

Table 2. Inhibition of influenza A2/SCOTLAND/49/57 virus fluorescence in BHK 21 cells by aminoadamantane added at different times after infection

<table>
<thead>
<tr>
<th>Virus inoculum (EID 50 per cell)</th>
<th>% cells fluorescing in control cultures—no aminoadamantane</th>
<th>% cells fluorescing when 25 μg./ml. aminoadamantane added after infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>100</td>
<td>9.1  28.1  52.8  100  100</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>1.8  Not tested  2.8  42.2  90.9</td>
</tr>
<tr>
<td>10</td>
<td>20.3</td>
<td>0.15  0.1  Not tested  1.3  4.8</td>
</tr>
</tbody>
</table>

In a further experiment the effect of high serum protein concentration on the inhibitory action of aminoadamantane was investigated. BHK 21 cells were incubated with mixture '199' alone, or mixture '199' containing 20% inactivated calf serum. Aminoadamantane (25 μg./ml.) was added to a proportion of the coverslips and the cultures were infected with 1000 EID50 per cell of influenza A2/SCOTLAND/49/57 virus. Twenty-four hr after infection antigen was detected in all cells in control cultures (not treated with aminoadamantane), although the presence of 20% calf serum delayed the development of antigens and virus cytopathic effects by approximately 12 hr. The percentage of cells fluorescing in monolayers treated with 25 μg./ml. aminoadamantane was 5% both in the presence and absence of 20% calf serum. Thus, high serum concentration did not inhibit the antiviral activity of aminoadamantane in these cells.

DISCUSSION

The BHK 21 continuous line of hamster kidney cells has the advantage of providing a standard, readily cultivated, cloned cell population for immunofluorescent studies with influenza viruses. Progressive multiplication of influenza A/NWS virus in BHK 21 cells with the production of antigens detectable by fluorescence and cytopathic change was described by Fraser (1967). However, influenza A/MEL and A/WSE viruses produced abortive infection in the same cell line. We have confirmed the progressive growth of influenza A/NWS in BHK 21 cells and quantified it by the immunofluorescent cell counting technique.
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The antiviral activity of aminoadamantane and other amines (Jensen & Liu, 1963; Schild & Sutton, 1965; Oxford & Schild, 1967b) had previously been quantified by the inhibitory effects of these compounds on production of infective virus, cytopathic effects and virus haemagglutinin. These amines are virustatic and have no direct virucidal effect on influenza viruses. The present study suggests that aminoadamantane, and to some extent the other amines tested, inhibit the formation of influenza A virus antigens detectable by immunofluorescence early after infection of BHK 21 cells. Even with a sensitive virus strain, influenza A2/SCOTLAND/49/57, and the maximal non-toxic dose of aminoadamantane the suppression of the development of intracellular antigen was not complete: a small proportion of cells showed fluorescence of equivalent intensity to that in control cultures. It is not at present clear whether this indicates the presence of drug-resistant virus particles in the inoculum or whether a proportion of cells is resistant to the protective action of aminoadamantane. Nor is it known whether aminoadamantane acts by blocking virus penetration or by inhibiting some subsequent stage. Our results may only be taken as evidence that aminoadamantane acts at a stage in the virus growth cycle preceding the formation of influenza virus antigen in the cell.

Ammonium acetate and the active aliphatic amines appeared to act similarly to aminoadamantane in blocking the production of antigens by influenza A viruses. Using the immunofluorescent cell counting technique differences in sensitivity to aminoadamantane were detected between the test strains of virus; A2/SCOTLAND/49/57 was a highly sensitive strain, A/NWS was of intermediate sensitivity and B/ENGLAND/939/59 was insensitive. This emphasizes the importance of using a number of different influenza virus strains for the routine testing of compounds for antiviral activity. The relative sensitivities of the virus strains used in the present study correlated well with those measured in tissue culture by haemadsorption tests (Schild & Sutton, 1965).

The use of BHK 21 cells with an immunofluorescent cell-counting technique forms a relatively sensitive and rapid method for the investigation of the properties and mode of action of certain antiviral compounds. It may also prove useful for the rapid preliminary screening of compounds for virustatic activity. However, immunofluorescence provides information only for antiviral compounds which act at a relatively early stage in the virus growth cycle and inhibit virus antigen formation. Qualitative immunofluorescence techniques have been used successfully to investigate the inhibition of Sendai virus (paramyxovirus type 3) by n-isobutylbiguanide hydrochloride (Kashiwazaki & Ishida, 1965) and to study the effect of various inhibitors of DNA viruses on the formation of adenovirus or SV40 virus antigens (Rapp et al. 1965). In addition, the results of a recent study (Denk & Kovac, 1967) suggest that the immunofluorescent technique may be useful in investigating antiviral activity in experimental animals.

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