An Investigation of Some Factors Affecting Cross-reactivation between Influenza A Viruses

By D. McCAHON and G. C. SCHILD
National Institute for Medical Research, Mill Hill, London, N.W. 7

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SUMMARY

Some parameters of the reactivation of ultraviolet- or chemically inactivated fowl plague virus by live A2 viruses were examined. Clones of reactivated virus were obtained by two different techniques, either by isolation from plaques on chick embryo fibroblast cells or by terminal dilution in pieces of allantois-on-shell. Recombinants isolated from plaques were of one type, namely they contained only the neuraminidase antigen of the live A2 virus. In contrast, recombinants isolated in pieces of allantois-on-shell possessed a variety of A2 characteristics, and only a small proportion of these were of the type isolated by the plaque technique.

Studies in which fowl plague virus was exposed to ultraviolet or ethylene iminquinone for various time periods enabled comparisons to be made of the rate of loss of infectivity and the rate of loss of ability to be reactivated. The results of these studies were interpreted as indicating that approximately 70% of the genome of fowl plague virus could be reactivated by A2 virus, and that this portion of the genome contained the genetic information for the haemagglutinin and neuraminidase antigens, the heat stability of the haemagglutinin, lethality for the chick embryo and efficient plaque production.

INTRODUCTION

Cross-reactivation of an inactivated virus by an infective influenza A virus appears to be due to genetic recombination between the two viruses (Simpson & Hirst, 1961) and it has been frequently used to produce recombinant viruses (Kilbourne, 1963; Tumova & Pereira, 1965; Kilbourne et al. 1967; Easterday et al. 1969). However, there have been no systematic studies carried out to evaluate the usefulness of cross-reactivation in the genetic analysis of the influenza genome.

The cross-reactivation system used was that of Tumova & Pereira (1965), namely reactivation of an inactivated avian virus (FPV), which plaques efficiently in chick embryo fibroblast (CEF) cells, by live human A2 viruses, which do not plaque readily. An advantage of this system was that these two types of influenza A virus differed in a variety of biological characteristics and that their envelope antigens, haemagglutinin and neuraminidase were immunologically distinct. In the present investigation we have examined the effects of a number of variables on cross-reactivation between these two influenza viruses.

The two variables which were important were the degree of inactivation of the fowl plague parent before cross-reactivation and the method of detection of reactivated virus, either plaque assay in CEF monolayers or growth in pieces of allantois-on-shell. From these studies it was possible to measure the proportion of the influenza virus genome which could
be reactivated and to obtain some information on the genetic content of this portion of the genome.

**METHODS**

*Viruses.* The Dutch strain of fowl plague virus (FPV) (Pereira, Tumova & Law, 1965) and the human strains, A2/SINAPORE/1/57 (Lim et al. 1957) and A2/HONG KONG/1/68 (Coleman et al. 1968) were obtained from the stocks of the World Influenza Centre. A2/JAPAN/305/1957 was obtained from Dr C. E. Hoffman of Du Pont de Nemours and Company Inc, Newark, Delaware 1971, U.S.A. Stocks were grown in the allantoic cavity of 10-day-old embryonated hen's eggs. Infective allantoic fluids were clarified by low-speed centrifugation and stored at −70° in small volumes. The FPV used for all experiments was a single stock prepared after two cycles of plaque purification in chick embryo fibroblast (CEF) monolayers. This cloned FPV stock had an average plating efficiency (average of 8 parallel titrations) of 2.6 egg infectious units (EIU)/p.f.u. and the ratio of EIU to haemagglutinin units (HAU) was $10^6$ EIU/HAU (EIU = EID$_{50} \times 0.693$). A2/JAPAN/305/1957 produced small indistinct plaques in CEF monolayers at very low efficiency ($2 \times 10^5$ EIU/p.f.u.), but A2/SINAPORE/1957 and A2/HONG KONG/1/68 did not produce plaques in such cells. Single stocks of each of the A2 viruses were used throughout, of which the ratios of EIU to HAU were between $5 \times 10^4$ and $7 \times 10^4$.

*Virus infectivity titrations.* Serial three- or tenfold virus dilutions in 0.1 ml volumes into the allantoic cavity of groups of 3 to 10 embryonated eggs (10 days old). The eggs were incubated at 36° for 2 to 3 days, after which the embryos were inspected for viability and the allantoic fluids were tested for the presence of haemagglutinin. Infectivity was also measured in approximately 1 cm$^2$ pieces of allantois-on-shell (AOS) using 8 to 10 replicates/tenfold dilution, as described by Fulton & Armitage (1951) and modified by Fazekas de St Groth & White (1958). To inhibit microbial growth, antibiotics (ampicillin, 100 µg./ml. and amphotericin B, 20 µg./ml.) were incorporated in the AOS culture media. For FPV the infectivity as measured by titration in AOS cultures was approximately a third of the infectivity titres in eggs. For A2 viruses the titre in AOS was a ninth of the titres in eggs. Egg infectivity (EID$_{50}$) and lethality (ELD$_{50}$) end-points were calculated by the method of Reed & Muench (1938).

Plaque assays were carried out in CEF monolayers by the technique described by Porterfield (1960), with the modification that the calf-serum concentration was halved and replaced by an equal amount of 10 % skim milk.

**Serological techniques**

*Haemagglutinin-inhibition tests.* were performed in plastic trays according to the technique described by the Expert Committee on Respiratory Virus Diseases (1959), except that serum-virus mixtures were kept for 1 hr at room temperature before addition of the erythrocyte suspension.

*Sensitivity to normal horse serum inhibitor.* This test was identical to standard HAI test except that the antiserum was replaced by normal horse serum.

*Neuraminidase inhibition tests.* were done in test tubes as described by Webster & Pereira (1968) except that the antiserum and neuraminidase were incubated for 16 to 18 hr at 4° before addition of substrate.

*Heat stability of the haemagglutinin.* Allantoic fluids containing the viruses were diluted at least one in five in 0.01 M-phosphate buffered saline and the haemagglutinin titre was measured before and after heating for 1 hr at 62°. Under these conditions the haemaggluti-
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nins of FPV and A2/HONG KONG/1968 were relatively stable (< 8-fold decrease in titre) compared to the haemagglutinins of A2/SINGAPORE/1957 and A2/JAPAN/1957 which were labile (64- to 256-fold decrease in titre). At least two separate clones of each recombinant were tested in the preliminary testing, and for those recombinants which were apparently labile a few more clones (2 to 4) were examined. Only those recombinants that consistently produced clones with heat-labile haemagglutinin were classed as A2 type in their haemagglutinin stability. Viruses that produced clones with heat-stable haemagglutinin were classed as fowl plague type in their haemagglutinin stability, although some of these occasionally produced clones with heat-labile haemagglutinin.

Reactivation experiments

**Infectious centre method.** Monolayers of CEF cells in Leighton tubes (1 x 10^6 cells) were washed with phosphate-buffered saline (PBS) and infected with 0.2 ml. virus at 4°C for 1 hr, then 0.5 ml. medium was added and the cultures were incubated at 37°C for 30 min. The cells were then washed with PBS and treated with virus-neutralizing antibody (serum from hyper-immunized rabbits diluted 1/100) at 37°C for 30 min. Finally the cells were removed from the glass with trypsin-versene, washed once with medium, serially diluted in three-fold steps and 0.3 ml. volumes added to fresh CEF monolayers in Petri dishes. After allowing 2 hr for settling at 37°C the plates were drained and agar overlay added.

At high multiplication of FPV (> 6 EIU/cell) all the CEF cells in a culture were infected and produced infectious virus when assayed in eggs, but only a third of such cells produced plaques in CEF monolayers. Free unadsorbed virus, i.e. virus present in the supernatant after centrifugation to remove cells, was always less than 1% of infectious-centre titre and less than 0.1% of the input virus.

**AOS method.** Pieces of AOS (1 cm² = 3 x 10⁵ cells, approximately) were prepared from 10-day-old embryonated eggs and placed in individual wells of a plastic haemagglutinin tray containing 0.3 ml. medium. 0.1 ml. of virus was added to each well, and after 90 min. adsorption at 37°C the medium was replaced with fresh medium containing 5% receptor-destroying enzyme (*vibrio cholerae* filtrate) and incubation continued for a further 60 min. The AOS cultures were then washed once with warm medium and incubated at 37°C. After a total of 11 to 18 hr incubation the cultures were disrupted by ultrasonic vibration or by freezing and thawing and then assayed for virus yield.

**Isolation of reactivant viruses**

**Plaque isolates.** In each case, a plaque was picked and plated directly on fresh CEF monolayers from which another plaque was picked and used as inoculum to prepare a stock of the isolate in a single egg. In a few cases, principally reactivants obtained from heavily inactivated virus (30 hits/infectious particle), there was insufficient virus in the original plaque to permit plating directly on CEF monolayers. In such cases the isolate was passed once in eggs, then plated on CEF monolayers from which a plaque was picked and used as inoculum to prepare a stock of the isolate in a single egg.

**AOS isolates.** Virus was diluted in threefold steps and 0.03 ml. volumes were inoculated into the AOS system. A minimum of 10 replicates were used per dilution and generally the last positive culture selected for virus passage was at a dilution where approximately 10% of the AOS cultures were infected. These clones were then passed at limit dilution in eggs or in AOS cultures at least twice more. At least two clones of each isolate (second- and third-limit dilution passage and in some cases the fourth-limit dilution passage also) were examined for A2 and fowl plaque characteristics. In addition, most of the isolates of this type (i.e.
AOS isolates) were plated on CEF monolayers, from which a plaque was picked which was then grown at limit dilution in eggs. This plaque-derived egg-grown stock was then examined for A2 and fowl plague characteristics. All clones of each isolate gave the same results.

*Inactivation of the plaque-producing virus (FPV)*

**Bayer A-139:** this ethylene iminoquinone, kindly provided by Dr C. Scholtissek, was used to treat FPV as described by Scholtissek & Rott (1964).

**Ultraviolet irradiation:** allantoic fluid was dialysed overnight against Gey’s A (Porterfield, 1960) solution without phenol red, then diluted threefold in ice-cold distilled water at 4° and irradiated immediately in 5 ml. volumes in Petri dishes of 10 cm. diameter. Immediately after irradiation the tonicity of the samples was brought back to normal by the addition of 10 × concentrated Gey’s A solution and they were stored as small samples at -70°. Titration on control preparations which were not irradiated demonstrated that this short exposure to hypotonic conditions did not affect the infectivity of the virus.

In both cases the rate of inactivation of infectivity followed first-order kinetics down to about 0.01 % survival. A single large stock of virus of known titre was inactivated in each case and samples were stored after various times of inactivation at -60° until used. The degree of inactivation of the virus is described as the average number of hits/infectious particle, \( r = \log_e \frac{V_0}{V_t} \), where \( V_0/V_t \) is the reciprocal of the surviving infectivity (Luria, 1947).

**Calculation of the multiplicity of infection.** The multiplicities are generally expressed as input multiplicities of EIU added/cell. However, with animal viruses, the input multiplicity is not usually the same as the number of particles successfully infecting a cell (called here the effective multiplicity). Therefore for the multiplicity reactivation experiments in which it was important to know the effective multiplicity this was calculated using the Poisson formula \( n = -\log_e P[0] \), where \( n \) is the effective multiplicity and \( P[0] \) the proportion of uninfected cells) from the proportion of infectious centres produced in a parallel culture inoculated with live virus. In such calculations the infectious centres were expressed as a proportion of the cells capable of producing plaques measured in the same experiment.

**RESULTS**

**Multiplicity reactivation of inactivated plaque-producing virus (FPV)**

To confirm that the inactivation of virus by ultraviolet light or A-139 was due to damage to the RNA rather than to structural proteins of the virus, the ability of inactivated virus to undergo multiplicity reactivation was investigated. If damage had occurred to the proteins of the virus multiplicity reactivation would not be expected to occur. Multiplicity reactivation was readily demonstrated in infectious-centre experiments (Fig. 1). At input multiplicities of greater than 1 EIU/cell (infectivity measured before inactivation), multiplicity reaction occurred with both u.v.-\((r = 6.6)\) and A-139-\((r = 8.0)\) inactivated viruses showing that such viruses could enter cells and take part in genetic interactions. A measure of the efficiency of the reactivation can be obtained by calculating the number of multiply infected cells and comparing it with the number of infectious centres observed, as has been done for bacteriophage (Luria, 1947). This calculation was made for the experiment in Fig. 1 involving the u.v.-inactivated virus (see Table 1), and it appears that reactivation occurred in 21 to 73 % of the multiply infected cells.
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Fig. 1. Multiplicity reactivation in chick embryo fibroblasts of FPV inactivated by ultraviolet light or by ethylene iminoquinone (A-139). CEF cells were infected with varying multiplicities of u.v.- or A-139-inactivated FPV and assayed for infectious centres on CEF monolayers. ●, u.v.-inactivated virus (6.6 hits/infectious particle). ○, A-139-inactivated virus (8 hits/infectious particle).

Table 1. Efficiency of multiplicity reactivation of u.v.-inactivated FPV in CEF cells*

<table>
<thead>
<tr>
<th>Input† multiplicity (EIU/cell)</th>
<th>'Effective'‡ multiplicity (calculated)</th>
<th>Multiply‡ infected cells (calculated)</th>
<th>Infectious centres (observed)</th>
<th>Reactivation§ ratio (observed/calculated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.07</td>
<td>1.1 × 10⁵</td>
<td>8.0 × 10⁴</td>
<td>0.73</td>
</tr>
<tr>
<td>0.3</td>
<td>0.2</td>
<td>8.3 × 10⁵</td>
<td>3.7 × 10⁴</td>
<td>0.45</td>
</tr>
<tr>
<td>0.9</td>
<td>0.6</td>
<td>5.7 × 10⁴</td>
<td>1.8 × 10⁴</td>
<td>0.32</td>
</tr>
<tr>
<td>2.7</td>
<td>1.9</td>
<td>2.5 × 10⁵</td>
<td>5.3 × 10⁴</td>
<td>0.21</td>
</tr>
<tr>
<td>8.1</td>
<td>5.7</td>
<td>4.2 × 10⁵</td>
<td>1.3 × 10⁵</td>
<td>0.31</td>
</tr>
</tbody>
</table>

* The experimental details are described under Fig. 1.
† The input multiplicities were calculated from the average egg titre of the uninactivated virus and the total number of cells in a culture.
‡ Calculated from number of infectious centres producing plaques obtained with live virus as described in the text.
§ This is the ratio of experimentally determined 'reactivant' infectious centres (col. 4) to the calculated number of multiply infected cells (col. 3).
Optimum multiplicities for cross-reactivation

This was examined in an experiment in which the multiplicity of each parent (live A2/SINGAPORE and u.v.-inactivated FPV) was varied independently. The results are shown in Table 2. Multiplicity reactivation was seen with the inactivated virus at input multiplicities greater than 1 and was found to increase with increasing multiplicity. The live virus showed interference in plaque production by the inactivated virus at input multiplicities greater than 1. Therefore, for studying cross-reactivation uncomplicated by multiplicity reactivation of the inactivated virus or by interference by the live virus the optimum multiplicities were about 1 for the inactivated virus and about 3 for the live virus.

Table 2. Optimum multiplicities for cross-reactivation of u.v.-inactivated FPV by live A2/SINGAPORE virus*

<table>
<thead>
<tr>
<th>Live A2 input multiplicity (EIU/cell)</th>
<th>Inactivated FPV† input multiplicity (EIU/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
</tr>
<tr>
<td>74</td>
<td>3.3%§</td>
</tr>
<tr>
<td>7.4</td>
<td>0.3%</td>
</tr>
<tr>
<td>0.74</td>
<td>0.1%</td>
</tr>
<tr>
<td>0</td>
<td>0.5%</td>
</tr>
<tr>
<td>7.2%</td>
<td>0.5%</td>
</tr>
<tr>
<td>7.0%</td>
<td>0.4%</td>
</tr>
<tr>
<td>7.0%</td>
<td>0.1%</td>
</tr>
</tbody>
</table>

* CEF monolayers were infected at 4°C, then medium was added and the cultures were incubated at 37°C for 30 min. The cultures were then treated with neutralizing antibody to both parent viruses and then the cells were trypsinized off and assayed on CEF monolayers.
† The input multiplicities were calculated from the average egg titre of the virus stocks (titre of the un-inactivated virus in the case of FPV) and the total number of cells in a culture.
‡ U.v.-inactivated FPV, r = 8.8 hits/infectious particle.
§ The number of infectious centres which produced plaques is expressed as a percentage of the total number of cells in each suspension.

Effect of method and degree of inactivation of FPV on cross-reactivation

FPV inactivated by exposure to A-139 and u.v. light for various times was reactivated in AOS cultures by A2/SINGAPORE and the yield was then assayed in CEF monolayers (Fig. 2a and 2b). There was considerable variation in the yields obtained from AOS cultures, but the amount of cross-reactivation was apparently independent of the method of inactivation. Cross-reactivation decreased at a slower rate than the inactivation of FPV infectivity, therefore the proportion of reactivants in the yield increased so that when the infectivity of FPV was reduced to between 0.1 and 0.01% of the original infectivity almost 100% of the yield was due to reactivation. When the infectivity of FPV was reduced to below 0.01% of the original infectivity very little cross-reactivation could be detected.

Detection of poor plaque-producing reactivants

Plaque assay on CEF monolayers would be expected to demonstrate the presence only of reactivants which resembled FPV in their plaquing efficiency. However, the use of pieces of AOS in which both parents can grow would be expected to demonstrate the presence of reactivants, irrespective of their plaque-producing abilities. Therefore, in addition to assaying the yields from the experiment illustrated in Fig. 2b (u.v.-inactivated FPV × A2/SINGAPORE in AOS) for plaque-forming virus the yields were also assayed in AOS cultures (Fig. 2c). The A2 virus was detectable in AOS cultures but, under the conditions of this reactivation experiment, very little A2 virus was produced (1.3 × 10⁵EID₅₀/AOS culture). There was apparently more reactivation detectable in AOS than by the plaque technique (Fig. 2c) and
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this could not be explained by the small amount of A2 virus present which would decrease only slightly the slope of the reactivation curve.

The existence of poor or non-plaque-producing reactivants in the yield from mixedly infected cultures was confirmed by examination of clones obtained at limit-dilution in AOS cultures from another reactivation experiment in which u.v.-inactivated FPV was reactivated by live A2/JAPAN/1957 (see next section).

Fig. 2. Cross-reactivation in AOS cultures by A2/SINGAPORE of FPV inactivated by ultraviolet light or ethylene iminoquinone (A-139). One set of AOS cultures were infected with FPV (1·0 EIU/cell) and live A2 virus (1·3 EIU/cell). ••••, reactivation curve. A replicate set of cultures were infected with FPV only. ○○○○, inactivation curve. After 90 min. incubation at 37°C the cultures were treated with cholera filtrate and then incubated further for a total of 11 hr (A-139-inactivated FPV, a) or 18 hr (u.v.-inactivated FPV, b). Each point in the figure represents the average virus yield of 2 to 4 replicate cultures. (a), reactivation of A-139-inactivated FPV assayed in CEF plates; (b), reactivation of u.v.-inactivated FPV assayed in CEF plates; (c), reactivation of u.v.-inactivated FPV assayed in AOS cultures.

Isolation and characterization of reactivants

A total of 75 reactivant clones were examined to determine whether the type of reactivant was affected by the method or degree of inactivation, the strain of A2 virus used, the type of cell in which reactivation occurred or the type of cell in which reactivants were detected. In addition, 26 clones obtained from experiments involving live FPV and live A2 virus were examined. The majority of clones examined (86 out of 101) were derived as plaques on CEF monolayers, and they possessed the plaque-producing capacity of FPV. A few clones (15) were isolated at limit-dilution in AOS cultures and they possessed various degrees of efficiency of plaque production in CEF cells. For convenience, these two types of isolate will be described separately and according to the method of inactivation before cross-reactivation. A summary of the production and characterization of all the clones examined is shown in Table 3.
Table 3. Summary of production and characterization of isolates obtained by reactivation and recombination

<table>
<thead>
<tr>
<th>Type of isolate</th>
<th>Method of inactivation</th>
<th>Degree of* inactivation (average no. of hits/ infectious particle)</th>
<th>Parental viruses</th>
<th>Cell system used for reactivation</th>
<th>Number of clones examined</th>
<th>Haemagglutinin</th>
<th>Neuraminidase antigen</th>
<th>Non-lethality for eggs</th>
<th>A2 plaque production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque from CEF monolayer</td>
<td>U.v.</td>
<td>8.8 to 39.4</td>
<td>Inactivated FPV × live</td>
<td>CEF</td>
<td>35</td>
<td>0/35</td>
<td>0/35</td>
<td>0/29</td>
<td>27/31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17</td>
<td>Inactivated FPV × live</td>
<td>CEF</td>
<td>5</td>
<td>0/5</td>
<td>0/5</td>
<td>—</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 and 9</td>
<td>Inactivated A2/HONG KONG/1968 × live FPV</td>
<td>AOS</td>
<td>5</td>
<td>0/5</td>
<td>—</td>
<td>—</td>
<td>4/4</td>
</tr>
<tr>
<td>A-139</td>
<td>1 to 4</td>
<td></td>
<td>Inactivated FPV × live</td>
<td>AOS</td>
<td>15</td>
<td>0/15</td>
<td>0/15</td>
<td>0/14</td>
<td>4/12</td>
</tr>
<tr>
<td>None</td>
<td>4 and 9</td>
<td></td>
<td>Live FPV × live A2/ SINGAPORE/1957</td>
<td>CEF</td>
<td>15</td>
<td>0/15</td>
<td>0/15</td>
<td>0/9</td>
<td>5/15</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td></td>
<td>Live FPV × live A2/ HONG KONG/1968</td>
<td>Eggs</td>
<td>11</td>
<td>0/11</td>
<td>0/8</td>
<td>—</td>
<td>11/11</td>
</tr>
<tr>
<td>Limit dilution in AOS</td>
<td>U.v.</td>
<td>4.4</td>
<td>Inactivated FPV × live</td>
<td>AOS</td>
<td>15</td>
<td>15/15</td>
<td>—</td>
<td>8/15</td>
<td>14/15</td>
</tr>
</tbody>
</table>

* The degree of inactivation is expressed as the average number of hits per infectious particle, calculated from the survival of infectivity measured on another occasion.

† Egg-grown stocks of the cloned isolates were examined for antigenic character by haemagglutination-inhibition tests (haemagglutinin) and neuraminidase-inhibition tests (neuraminidase), for egg lethality by comparing the egg-killing titre (ELD50) with the egg infectivity titre (EID50) for sensitivity to γ inhibitor by testing for haemagglutination in the presence of normal horse serum; for lability at 62°C by measuring the haemagglutinin titre before and after heating a 1 in 5 dilution at 62°C for 1 hr; and for plaque production by comparing the plaque titre with the egg infectivity titre (EIU/p.f.u.).

‡ The A2 viruses used did not plaque or were very inefficient at plaque production in CEF cells (approximately 10^2 EIU/p.f.u.) compared to FPV (approximately 3 EIU/p.f.u.).

—, not applicable. The A2 parent virus used did not possess this characteristic.
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Plaque isolates

Reactivants obtained after ultraviolet inactivation

Ultraviolet-inactivated FPV × live A2/SINGAPORE/1957 in CEF cells. In this experiment FPV, after varying degrees of u.v.-inactivation, was reactivated by live A2 virus. In addition, live FPV was recombined with A2 virus. This was an infectious-centre experiment and therefore each plaque was the result of a separate reactivation event. The plaque isolates were examined for A2 and fowl plague characteristics, and the results are shown in condensed form in Table 3, divided into those obtained from inactivated FPV (row 1) and those obtained from live FPV (row 5) and also in more detail in table 4. The clones were either indistinguishable from parental FPV by the tests used or were recombinant for the neuraminidase antigen of the A2 virus. The proportion of isolates with A2 neuraminidase antigen increased from 33% when live FPV was used to 100% when FPV had been inactivated to between 0.1% and 0.01% of its original infectivity (see Table 4). With more heavily inactivated virus reactivation was difficult to detect, and of the three isolates obtained only one grew sufficiently well to be characterized.

Table 4. Characterization of plaque-isolates obtained from reactivation of u.v.-inactivated FPV by live A2/SINGAPORE/1957 in CEF cells*

<table>
<thead>
<tr>
<th>Degree of inactivation of FPV (average no. of hits/p.f.u.)†</th>
<th>No. of clones examined‡</th>
<th>Fraction of clones with A2 neuraminidase antigens§</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15/15</td>
<td>5/15</td>
</tr>
<tr>
<td>8.8</td>
<td>12/13</td>
<td>7/11</td>
</tr>
<tr>
<td>17.5</td>
<td>12/12</td>
<td>9/9</td>
</tr>
<tr>
<td>26.2</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td>39.4</td>
<td>1/3</td>
<td>1/1</td>
</tr>
</tbody>
</table>

* CEF monolayers were infected with live or u.v.-inactivated FPV (1.2 EIU/cell) and live A2 virus (2.0 EIU/cell). After adsorption at 4°C the cultures were incubated with antibody at 37°C. Finally the cells were removed with trypsin-versene and assayed for infectious centres on CEF monolayers. Plaques from the plates were picked and after plaque purification stocks were prepared of each isolate in a single egg.
† FPV was u.v.-inactivated and the degree of inactivation is expressed as the average no. of hits (r), calculated from the survival of plaque-forming ability measured on another occasion.
‡ This is expressed as a fraction of the number of plaques picked.
§ Egg-grown stocks of the clones were examined by the neuraminidase-inhibition test.

Ultraviolet-inactivated FPV × live A2/HONG KONG/1968 in CEF cells. This was an infectious centre experiment similar to the one described above, except that only heavily inactivated FPV (average of 39 hits/p.f.u.) was used. Only five plaques were picked and all five were recombinants possessing the neuraminidase antigen of the A2 virus (see row 2 of Table 3).

Ultraviolet-inactivated A2/HONG KONG/1968 × live FPV in AOS cultures. In this experiment the A2 virus was inactivated with u.v. light (average of 4 and 9 hits/EIU) and then reactivated with live FPV in AOS cultures (approximately 3 EIU/cell of each virus) to see if this affected the type of reactivant obtained. The yields from such cultures were then assayed on CEF monolayers in the presence of an antiserum which inhibited plaque production and plaque size of the parental live FPV that was present by at least 99.9% at the dilution used. Five medium-sized clear plaques were picked from amidst a background of very small indistinct plaques. One isolate produced insufficient neuraminidase for testing but the remaining four were recombinant only for the neuraminidase antigen of the A2 virus (see row 3 of Table 3).
Reactivants obtained after A-139 inactivation

Only one cross-reactivation experiment was performed with A-139-inactivated FPV, namely, reactivation with A2/SINGAPORE in pieces of AOS illustrated in Fig. 2a. Fifteen plaque isolates were obtained, principally from cultures receiving slightly inactivated FPV (0.25 to 1 hr exposure A-139, 1 to 4 hits/p.f.u.) and live A2 virus. The clones were either indistinguishable from parental FPV by the tests used or were recombinants possessing only the neuraminidase antigen of the A2 virus (see row 4 of Table 3).

Recombinants obtained from live FPV and live A2 virus

These recombinants were obtained for comparison with those obtained by reactivation and some have already been described as part of a reactivation experiment between FPV and A2/SINGAPORE/1957 in CEF cells (see row 5 of Table 3). In addition, live FPV was recombined with live A2/HONG KONG/1968 in 10-day-old eggs (10^5, 10^6, and 10^7 EIU/egg of each virus). The yields from such eggs after 18 hr incubation at 36° were assayed on CEF monolayer in the presence of an antiserum which inhibited plaque production and plaque size of the parental live FPV that was present by at least 99.9% at the dilution used. Eleven medium-sized clear plaques were picked from amidst a background of very small indistinct plaques and after cloning examined for A2 and fowl plague characteristics. All were recombinants possessing only the neuraminidase antigen of the A2 virus (row 6 of Table 3).

AOS limit-dilution isolates

AOS limit-dilution clones were only examined from one experiment. In this experiment pieces of AOS were infected with u.v.-inactivated FPV (average of 4.4 hits/p.f.u.) and live A2/JAPAN/1957 at approximately 3 EIU/cell of each virus. The yields were then assayed in CEF monolayers and AOS cultures (see Table 5), and in the yield from the mixedly infected AOS there was clearly more infectious virus detectable in the AOS titration than in the

Table 5. Detection of poor plaque-producing reactivants in AOS cultures*

<table>
<thead>
<tr>
<th>Virus</th>
<th>Infectivity/AOS culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CEF monolayers (p.f.u.)</td>
</tr>
<tr>
<td>Inactivated FPV</td>
<td>1.6 x 10^5</td>
</tr>
<tr>
<td>Inactivated FPV + live A2/JAPAN/1957</td>
<td>1.2 x 10^5</td>
</tr>
<tr>
<td>Live A2/JAPAN/1957</td>
<td>&lt; 10</td>
</tr>
</tbody>
</table>

* AOS cultures were infected with u.v.-inactivated FPV (average of 4.4 hits/infectious particle) and/or live A2/JAPAN/1957 (approximately 3 EIU/cell of each virus). After 12 hr incubation at 37° the yields were assayed in AOS cultures and in CEF monolayers.

plaque assay. This increase could not be explained by the greater sensitivity of the AOS method or by the presence of A2 virus, although some A2 virus was produced (4 x 10^9 AOS-ID50/AOS) and was detectable only in the AOS titration. Fifteen clones were obtained at limit dilution in AOS cultures (see Methods) from the yield from the mixedly infected AOS culture, and these results are shown in the summary in Table 3 (row 7) and also in more detail in Table 6.

The majority of the isolates (10 out of 15 isolates) were recombinants which differed from those isolated by the plaque technique in that they possessed a variety of A2 charac-
teristics and had plaquing efficiencies intermediate between those of the two parent viruses. These recombinants could be further subdivided into three types, on the basis of the tests used, and these are shown in Table 6 (rows 1 to 3) in order of their frequency of occurrence. Of the remaining five isolates, two were recombinants of the type isolated by the plaque technique (i.e. fowl plague in all characters except neuraminidase antigen) and three were identical to parent virus (row 5 of Table 6).

Table 6. Characterization of limit-dilution isolates obtained from reactivation of u.v.-inactivated FPV by A2/JAPAN/1957 in AOS cultures*

<table>
<thead>
<tr>
<th>No. of isolates</th>
<th>Neuraminidase antigen</th>
<th>Haemagglutinin antigen</th>
<th>Labile haemagglutinin</th>
<th>Non-lethality for eggs</th>
<th>A2 plaque† production</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>$-(2 \times 10^3$ to $8 \times 10^3)$</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>--</td>
<td>+</td>
<td>$-(2 \times 10^3$ to $3 \times 10^6)$</td>
</tr>
<tr>
<td>1</td>
<td>--</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>$-(2 \times 10^6$ to $3 \times 10^9)$</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>$-(2 \times 10^5$ to $3 \times 10^9)$</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>$+(3 \times 10^5$ to $8 \times 10^9)$</td>
</tr>
</tbody>
</table>

* These isolates were obtained at limit dilution in AOS cultures from the yield of an AOS culture infected with u.v.-inactivated FPV and live A2/JAPAN/1957 (described in Table 5). At least two clones of each isolate (second and third limit-dilution passage in eggs) were examined for A2 and fowl plague characteristics as described in footnote § of Table 4: + indicates A2-like characteristics, − indicates fowl plague-like characteristics.

† In these experiments FPV had a plaquing efficiency of $2 \times 10^3$ EIU/p.f.u. compared to A2/JAPAN/1957 which had a plaquing efficiency of $2 \times 10^9$ EIU/p.f.u. Many of the isolates had plaquing efficiencies intermediate between that of the two parent viruses, and these are shown in brackets.

DISCUSSION

Using the system of cross-reactivation described in this paper a variety of types of recombinant influenza A viruses were produced. These findings suggest that cross-reactivation could be of value in the qualitative analysis of the influenza genome and as a method of producing recombinants for particular experimental or practical uses. For both these purposes cross-reactivation was superior to recombination between live viruses, since a major problem in studies of the latter type is the detection and isolation of recombinants in the presence of a large excess of parental viruses. In this cross-reactivation system the recombinants were a major proportion of the yield from mixedly infected cells since the fast-growing parent (FPV) was inactivated and the other parent (live A2 virus) grew slowly. Therefore recombinants could be isolated after a single growth cycle and without resort to selection procedures such as the use of specific antiserum.

The type of recombinant obtained by cross-reactivation was a function of the method of isolation of the recombinants (i.e. plaquing in CEF cells or limit dilution in AOS cultures) rather than any of the other factors investigated. The plaque technique was convenient for the isolation of clones, but it selected for one class of recombinant – those with only the neuraminidase antigen of the A2 virus. The less convenient limit-dilution technique, however, showed that a variety of recombinants could be obtained by cross-reactivation, and only a small proportion of these (2 out of 12 clones) were of the type normally isolated by
the plaque technique. In view of the suggestion that the neuraminidase is involved in the release of virus from cells (Webster & Laver, 1967) and hence in plaque production, it is interesting that in these studies the ability of a recombinant to produce plaques as efficiently as FPV was related to the possession of fowl plague haemagglutinin and chick embryo lethality rather than possession of fowl plague neuraminidase. However, the ability to produce plaques appeared to be dependent on other factors in addition to the possession of fowl plague haemagglutinin and chick embryo lethality since 6 of the 12 recombinants obtained at limit dilution in AOS cultures resembled A2 in all the markers examined except plaquing efficiencies, which were similar to that of FPV (1/10 to 1/40 of FPV plaquing efficiency — see row 1 of Table 6).

A minimum estimate of the proportion of the fowl plague capable of being reactivated by A2 viruses can be made from a comparison of the slopes of the inactivation and reactivation curves (Fig. 2) if a number of assumptions are made: (1) that infectivity is a function of the whole virus genome, and (2) that the rate of inactivation of a function of the genome by

\[
\begin{align*}
(a) & \text{AOS isolate} \\
\text{Unknown functions} & \text{Neuraminidase antigen + haemagglutinin antigen and heat stability + chick-embryo lethality + FPV-like plaque production + unknown functions.} \\
\text{Unreactivable region} & \text{30%} \\
\text{Reactivable region} & \text{70%} \\
(b) & \text{Plaque isolate} \\
\text{Unknown functions + haemagglutinin antigen,} & \text{Neuraminidase antigen + unknown functions} \\
\text{heat stability and } \gamma\text{-inhibitor sensitivity +} & \text{chick embryo lethality + FPV-like plaque production} \\
\text{Unreactivable region} & \text{50%} \\
\text{Reactivable region} & \text{50%} \\
\end{align*}
\]

Fig. 3. Diagrammatic representation of the functions of the reactivable and unreactivable regions of the fowl plague genome

ultraviolet light (or Bayer A-139) is directly proportional to the proportion of the genome coding for that function. If we accept these assumptions then the slope of the inactivation curve reflects the size of the whole genome, whilst the slope of the reactivation curve reflects the size of the unreactivable region of the genome. By comparing the two slopes it is possible to obtain a minimum estimate of the proportion of the genome that is reactivable. From this comparison plus the analysis of the properties of the recombinants obtained in a variety of cross-reactivation experiments several conclusions can be made: (1) Approximately 30% of the fowl plague genome is unreactivable by A2 virus when AOS cultures are used to analyse the yield from cross-reactivation experiments. Therefore, at least 70% of the fowl plague genome can be replaced by the genome of the live A2 virus and a diagrammatic
Cross-reactivation with influenza viruses

representation of the genetic composition of such a reactivant (i.e. 30% fowl plague, 70% A2) is shown in Fig. 3a. The 70% of the fowl plague which is reactivable contains at least the genetic information for the neuraminidase and haemagglutinin antigens, heat stability of the haemagglutinin, lethality for the chick embryo and efficient (FPV-like) plaque production in CEF cells. (2) Approximately 50% of the fowl plague genome is apparently unreactivable by A2 virus when the plaque technique is used to assay the yield from cross-reactivation experiments. Therefore, up to 50% of the fowl plague genome can be replaced by the A2 virus genome before the efficiency of plaque production is affected, and a diagrammatic representation of the genetic composition of such a reactivant is shown in Fig. 3b. The 50% of the fowl plague genome which is reactivable contains at least the genetic information for the neuraminidase antigen but not the genetic information for the haemagglutinin antigen, heat stability of the haemagglutinin, γ-inhibitor sensitivity of the haemagglutinin, lethality for the chick embryo or efficient plaque production in CEF cells. (3) By inference from the above conclusions, 20% of the fowl plague genome contains at least the information coding for the haemagglutinin antigen, heat stability and γ-inhibitor sensitivity, lethality for the chick embryo and efficient plaque production in CEF cells.

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REFERENCES


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