Segregation of Antigenic and Biological Characteristics during Influenza Virus Recombination

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Genetic recombination between different strains of influenza A virus takes place readily under laboratory conditions (reviewed by Kilbourne, 1963) and this fact has recently found practical application in techniques for 'tailor making' antigenic 'hybrid' strains of influenza, the antigenic composition of which is pre-determined by the appropriate choice of parent strains (Tumova & Pereira, 1965; Kilbourne et al. 1967; Webster, 1970; McCahon & Schild, 1971). Recombination has also been suggested as a method for the production of strains with high growth capacity in the embryonated egg for use in the preparation of inactivated influenza vaccines (Kilbourne & Murphy, 1960) and recently Kilbourne (1969) has reported the isolation of a recombinant virus (X-31) using A0/PR8/34 virus and A2/HONG KONG/68 as parent viruses. X-31 was considered as appropriate for vaccine production since it exhibited the high yielding growth characteristics of A0/PR8 but was antigenically identical to the current epidemic virus A2/HONG KONG/68.

In this report we describe the segregation by recombination of four properties of the influenza virus, namely, antigenic characteristics of the envelope proteins (haemagglutinin and neuraminidase antigens), growth capacity in the embryonated egg and mouse pathogenicity. Such studies form a useful basis for future attempts to use recombination for the preparation of strains of virus suitable for use in both inactivated and attenuated vaccines.

The recombination system that was used involved the partial inactivation by u.v. light of one of the parent viruses (A0 parent – high growth capacity in eggs) prior to recombination in the embryonated egg with the live parent virus (A2 parent – low growth capacity). In this system the amount of parental viruses in the yield was small and so it was possible to isolate recombinants without the use of selection pressures, such as specific antisera, which are normally necessary in other systems (Kilbourne et al. 1967; Webster, 1970) to remove the large excess of parental viruses. In the course of this work a variety of recombinants were isolated including some, which like X-31, might be considered as potentially suitable for the production of inactivated virus vaccines.

The parent viruses used in this study were A0/PR8/34 and A2/ENGLAND/939/69 (kindly provided by Dr A. S. Beare, MRC Common Cold Research Unit, Harvard Hospital, Salisbury). A0/PR8/34 was a mouse pathogenic strain which produces high yields of virus after 48 hr incubation in the allantoic cavity. A2/ENGLAND/939/69 was a recent isolate of the current epidemic strain A2/HONG KONG/68 which is not pathogenic in mice and which produces low yields of virus in eggs. Both the envelope antigens, haemagglutinin and neuraminidase, of the two parent viruses were distinct so that their identification in recombinant viruses was readily achieved.

The two viruses were recombined in the embryonated egg and clones were isolated at limit dilution as illustrated in Fig. 1. Prior to recombination the A0 parent virus was diluted 1 in 33 and inactivated to 1 % survival of its original infectivity by u.v. light as described by Tumova & Pereira, 1965. This material was then diluted and 0.1 ml. (10^7 EID50) was inoculated into the allantoic sac of a 10-day-old embryonated egg. The egg was then incubated with 0.1 ml. of neat allantoic fluid stock of the A2 parent virus (10^8 EID50) and incu-
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**A0 / PR 8/34**
(10⁶ EID₅₀ - partially inactivated with u.v. light - high growth potential)

**A₂/ENGLAND/939/69**
(10⁸ EID₅₀ - live virus - low growth potential)

Allantoic sac of 10 d.o. egg
15 hr at 35°C

Isolation of clones at limit dilution

AOS cultures (13 isolates)

10 d.o. eggs (21 isolates)

Preliminary examination

Presumptive Parental Parental recombinants A₀ virus A₂ virus

Cloned by two serial limit dilution passages in eggs or AOS cultures
3 or more clones of each isolate examined for A₀ and A₂ characteristics

Recombinant Parental Recombinant viruses A₀ virus viruses

* One presumptive recombinant proved to be a mixture of three distinct recombinants on further cloning

![Flowchart](chart.png)

Fig. 1. Production and isolation of influenza virus recombinants.

...bated at 35°C for 15 hr. The limit dilution technique used both in the isolation and subsequent cloning of isolates was as follows: the virus preparation was treated in a Burndent Ultrasonic cleaner (input 80 kcyc./sec.) for 30 sec., diluted in 2-fold or 3-fold steps and then inoculated into 10-day-old embryonated eggs or allantois-on-shell cultures (AOS cultures) using a minimum of eight replicate cultures for each dilution. These AOS cultures were prepared and incubated according to the method described by Fazekas de St Groth & White, (1958) except that the medium used was Leibovitz L15 (Leibovitz, 1963) to which had been added Ampicillin (250 µg./ml) and Amphotericin B (25 µg./ml) and that the plates were individually sealed with ‘Sellotape’ and placed in polythene bags after the virus had been added. The limit dilution clones were isolated from cultures in which the virus dilution used infected only approximately 10% of the replicates as indicated by positive haemagglutination when tested with fowl erythrocytes. At least three clones of each isolate (usually from first and second limit dilution steps) were examined for A₀ and A₂ characteristics in parallel with limit dilution clones from eggs which received either of the parent viruses (Table 1). Unfortunately the importance of the use of ultrasonic treatment to disrupt virus aggregates prior to limit dilution isolation was not realized in the early stages of this work and it was not used in the isolation of some of the original isolates although it was used in the subsequent cloning of such isolates. Egg isolate number 64 was one of these original isolates for which ultrasonic treatment was not used and therefore aggregation seems the most likely explanation as to why it gave three distinct recombinants (64b, 64c, and 64d) on
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Table 1. Immunological and biological characteristics of isolates*

<table>
<thead>
<tr>
<th>Viruses infecting</th>
<th>Number of isolates examined</th>
<th>Antigenic composition†</th>
<th>Growth capacity in 10-day-old embryonated eggs§</th>
<th>Mouse virulence‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inactivated A0 (10⁷ EID 50)</td>
<td>6</td>
<td>A0</td>
<td>High</td>
<td>Virulent</td>
</tr>
<tr>
<td>Live A2 (10⁷ EID 50)</td>
<td>6</td>
<td>A0</td>
<td>High</td>
<td>Avirulent</td>
</tr>
<tr>
<td>Inactivated A0 (10⁷ EID 50) × Live A2 (10⁷ EID 50)</td>
<td>6</td>
<td>A0</td>
<td>High</td>
<td>Avirulent</td>
</tr>
</tbody>
</table>

* These isolates were obtained at limit dilution from the eggs of the experiment described in the text. The isolates derived from the mixedly infected egg (10⁷ EID 50 of inactivated A0 virus + 10⁷ of live A2 virus) were cloned twice more at limit dilution in eggs or AOS cultures and at least two clones were examined for A0 and A2 characteristics.

† This is based on haemagglutination and neuraminidase inhibition tests.


‡ Mouse virulent = extensive pulmonary consolidation after intranasal inoculation of 1₀ to 1 H.A.U. (Horsfall, 1939).

Table 2. Recombinants with characteristics of potential vaccine strains

<table>
<thead>
<tr>
<th>Strain designation</th>
<th>Antigenic composition</th>
<th>Growth capacity in 10-day-old embryonated eggs (H.A.U./₀.₂₅ ml.)</th>
<th>Mouse lung virulence (lung lesion score)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinants</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>A2/HK</td>
<td>2₅₆₀–₅₁₂₀</td>
<td>2·₅–₃·₅†</td>
</tr>
<tr>
<td>7</td>
<td>A2/HK</td>
<td>2₅₁₂₀–₅₁₂₀</td>
<td>0·₀–₆</td>
</tr>
<tr>
<td>6₄c</td>
<td>A2/HK</td>
<td>6₄₀–₁₂₈₀</td>
<td>2·₁–₂·₂</td>
</tr>
<tr>
<td>6₄d</td>
<td>A2/HK</td>
<td>6₄₀–₁₂₈₀</td>
<td>0·₀–₆</td>
</tr>
<tr>
<td>Parental viruses</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A₀/P.R 8/³⁴</td>
<td>A₀</td>
<td>2₅₁₂₀</td>
<td>2·₅–₃·₆†</td>
</tr>
<tr>
<td>A₀/ENGLAND 939/₆⁹</td>
<td>A₂/HK</td>
<td>3₂–₁₂₈</td>
<td>0·₀–₈</td>
</tr>
</tbody>
</table>

* Inoculum virus was diluted to contain 2₅ H.A.U./₀.₂₅ ml. Lung lesion scores in Palles mice (5 to 7 weeks of age) were assessed six days after inoculation using the inoculation method and scoring system described by Horsfall (1939).

† Range of values in tests with at least three clones of each recombinant or parent virus strain.

Subsequent cloning (see Fig. 1) although the possibilities of a heterozygote and/or recombination during isolation cannot be excluded. No evidence of aggregation or heterozygosis was ever seen when ultrasonics were used prior to limit dilution.

Eleven out of 36 isolates derived from the mixedly infected egg were recombinant on the basis of the four markers examined and eight different types of recombinant were found. The markers used were stable since the properties of all clones derived from any particular cloned isolate or from the two parental viruses, were identical. There was no evidence of covariation between any of the four markers used, suggesting that they reflect different primary gene functions. However, in the case of growth capacity in eggs, the occurrence of recombinants with intermediate growth capacity suggests that this marker may be dependent on more than one primary gene function. Since there are two possible alternatives
Fig. 2. Growth capacity of parent and recombinant viruses at various temperatures in AOS cultures. AOS cultures (approximately 3 x 10^6 cells) were infected with 2 H.A.U. of virus and incubated with shaking in water baths at the temperatures shown for 24 hr. The haemagglutinin produced was measured after the cultures were frozen and thawed and treated with ultrasonic vibration. Each point represents the average value for two replicate cultures. O—O, Ao parent virus; •—•, A2 parent virus; □—□, recombinant virus; ■—■, recombinant virus 64d.

(Ao or A2) for each of the four markers then theoretically there are 2^4 possible arrangements of these four markers. Two of these arrangements will be represented by the parent viruses and six of the other possibilities have been obtained (see Table 1 - for the purpose of this calculation we have ignored those recombinants with intermediate growth capacity in eggs). Of the eight remaining theoretically possible recombinants six would have low growth capacity in eggs and this could explain why they have not been detected in the yield since it contains principally virus of high growth capacity (32 out of 36 isolates).

Those isolates which had the characteristics of potential vaccine strains were further cloned and these additional clones were examined to further check the stability of the antigenic and biological characters of these recombinants. All clones of any isolate had the same properties and the characteristics of the four different types of recombinant vaccine strains are shown in Table 2.

Since the difference between recombinants 6 and 64c and 7 and 64d was based on only a small difference in growth capacity in eggs, this property was studied further by comparing their growth at various temperatures in AOS cultures with that of the parent viruses (Fig. 2) and this confirmed that these viruses differed from their parent viruses and each other in this property. The results for recombinants 6 and 64c are not shown on the figure since they closely resembled 7 and 64d respectively.

In some studies when only the type of recombinant with the characteristics of potential vaccine strains was desired, selection pressure was used to accelerate the process of isolation of recombinants. A sample of the allantoic fluid from the mixedly infected egg (see Fig. 1) was incubated overnight at 4 ° with 1/100 rabbit immune serum prepared against Ao/PR8/34 and then clones were isolated from this mixture at limit dilution in eggs. This treat-
ment with antibody increased the probability of isolating the required recombinants since
in the absence of antibody treatment 25 out of the 36 isolates (see Fig. 1) were parental Ao
virus whereas after antibody treatment all the clones isolated (9 out of 9) possessed A2
haemagglutinin and two clones had the properties of potential vaccine strains.

We conclude from the present studies that the properties of the influenza virus which were
investigated (i.e. haemagglutinin and neuraminidase antigens, growth capacity in eggs and
mouse pathogenicity) can be segregated by recombination and that by the use of the tech-
niques described it is possible to prepare rapidly and reproducibly vaccine strains with high
growth capacity for use against the new epidemic influenza A virus that might arise. In
addition, other recombinants such as antigenic ‘hybrids’ can be readily isolated.

Recombinant viruses with high growth capacity in eggs will be of value for the preparation
of vaccines containing inactivated virus particles or for possible future vaccines containing
isolated virus subunits. In addition it seems worthwhile to investigate whether recombination
can also be applied to the production of attenuated live virus vaccine strains specially since
the present methods of attenuation are both unpredictable and unreliable (Tyrrell & Beare,
1969). However, little is known of the genetic determinants of virulence and growth charac-
teristics in the influenza virus and their transference during recombination. A recombination
system such as that described in the present study provides the basis for a systematic
approach to this problem and studies to investigate the value of recombination in the prepa-
ration of attenuated influenza vaccine strains are currently in progress. (A. S. Beare, per-
sonal communication).

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