Differentiation of the Haemagglutinin Genes of Variant Influenza Viruses by RNA-RNA Hybridization

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SUMMARY

The genetic compositions of four antigenic variants of A/Memphis/1/71 (H3N2) influenza virus, which were selected by growth in the presence of monoclonal antibodies against the haemagglutinin, were compared. The results indicate that the mutant haemagglutinin genes can be differentiated by polyacrylamide gel electrophoresis of the double stranded RNA hybrids formed between virion RNA and transcripts isolated from infected cells.

Mixed infections with influenza viruses frequently lead to the production of recombinants, probably as a consequence of the segmented nature of the virus genomes. This characteristic property is of obvious value in genetic analyses (e.g. Ritchey et al. 1976; Scholtissek et al. 1976; Almond et al. 1977) and has also been exploited to advantage in the preparation of diagnostic reagents (e.g. Schulman & Kilbourne, 1969) and in the production of vaccines (Kilbourne, 1969). In many of these applications, analyses of the genetic composition of the recombinants are required and several suitable methods are detailed in the publications cited above. We have also described a procedure involving the formation of RNA–RNA hybrids and their analysis by polyacrylamide gel electrophoresis, which may be of particular value in the characterization of recombinants prepared as candidate live vaccine strains (Hay et al. 1977). This is a report of attempts to estimate the sensitivity of this procedure by comparative analysis of influenza virus RNA molecules with defined nucleotide sequence differences. Several recent publications have described the isolation of antigenic variants of influenza viruses obtained following virus growth in the presence of monoclonal antibodies specific for the virus haemagglutinins (Gerhard & Webster, 1978; Laver et al. 1979). Both the frequency of isolation of these variants, their antigenic properties and the demonstrated amino acid substitutions in their haemagglutinins suggest that single base changes in the haemagglutinin gene are involved in their generation. The genetic compositions of four such variants derived from A/Memphis/1/71 (H3N2) have therefore been determined to ascertain whether the mutant RNAs can be differentiated.

The procedures used in the production of monoclonal antibodies against influenza viruses and their application in the selection of antigenic variants have been described (Gerhard & Webster, 1978; Laver et al. 1979). Briefly, monoclonal antibodies specific for different antigenic determinants on the haemagglutinin of A/Memphis/1/71 were mixed with cloned parental virus and inoculated into embryonated eggs. The viruses that grew in the presence of undiluted monoclonal antibody were cloned twice at limiting dilution in embryonated eggs. The viruses used were grown from the same cloned stocks of variant viruses used in the amino acid sequence studies (Laver et al. 1979).

The antigenic relationships of the haemagglutinins of the purified viruses from which virion RNAs were extracted for this study are presented in Table 1. They are precisely the same as those of the originally characterized isolates (Laver et al. 1979). Comparative amino acid sequence data are available for the haemagglutinins of three of the four variants and the single amino acid changes detected from the sequence of the glycoprotein of the
Fig. 1. Analysis of homologous and heterologous double-stranded RNAs. Monolayers of chick embryo fibroblasts (5 x 10^7 cells/culture) were incubated in tris-Gey's medium containing cycloheximide (100 μg/ml) for 30 min before, during and after infection with either A/Memphis/1/71 or the variants listed in Table 1. The virus inoculum (1 ml/culture; approx. 3000 HA units) was concentrated from allantoic fluid by precipitation with polyethylene glycol. 3H-uridine (200 μCi/culture) was added at 3 h after infection and at 4 h cells were lysed in 0.5% SDS, 10 mM-sodium acetate, pH 5.0, extracted twice with phenol and the RNA precipitated in 70% ethanol and washed with 70% ethanol, 30 mM-NaCl. Each complementary RNA preparation, dissolved in water, was divided into five aliquots, to each of which was added approx. 10 μg virion RNA extracted in the same way from purified preparations of each virus. The RNA samples (0.05 ml) were denatured by incubating for 30 min at 45°C following the addition of 9 vol. of dimethyl sulphoxide; 30 mM-NaCl, 10 mM-tris-HCl, pH 7.5, and 1.5 mM-EDTA were added, the concentration of dimethyl sulphoxide reduced to 63% and the RNA annealed at 37°C for 14 h. The RNA was precipitated in 75% ethanol, washed and re-dissolved in 100 mM-NaCl, 10 mM-sodium acetate, pH 4.5, 1 mM-ZnSO4 and incubated with nuclease S1 (1000 units/ml/mg RNA) at 37°C for 3 h. The RNA was re-precipitated with ethanol, washed and dissolved in 7 M-urea, 0.5 mM-EDTA, 10 mM-tris-acetate, pH 7.8 and the dsRNAs separated by electrophoresis at 70 V for 60 h in polyacrylamide gels containing 7.5% acrylamide, 0.2% N,N'-methylene-bisacrylamide, 0.4% N,N',N'-tetramethylthylethylenediamine, 6 M-urea, 0.1% SDS, 10 mM-EDTA, 40 mM-tris-acetate, pH 7.8, and 1 mg/ml ammonium persulphate, and detected by fluorography. The samples are arranged in groups of five according to the complementary RNA used and those containing homologous virion RNA and complementary RNA are indicated by *. The direction of electrophoresis was from top to bottom. See Table 1 for designations 1 to 5.
Table 1. Discrimination between antigenic variants of *A/Memphis/1/71* in haemagglutination-inhibition tests with monoclonal antibodies to the haemagglutinin*

<table>
<thead>
<tr>
<th>Variants of A/Memphis/1/71 selected with the following monoclones:</th>
<th>HI antibody titres with the following monoclonal antibodies</th>
<th>Amino acid substitutions in HA molecule†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variants no.</td>
<td>H14/A2</td>
<td>H14/A20</td>
</tr>
<tr>
<td>(1) Wild type</td>
<td>0</td>
<td>200</td>
</tr>
<tr>
<td>(2) H14/A2</td>
<td>1</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>(3) H14/A20</td>
<td>2</td>
<td>200</td>
</tr>
<tr>
<td>(4) H14/A21</td>
<td>1</td>
<td>200</td>
</tr>
<tr>
<td>(5) H14/A21</td>
<td>2</td>
<td>200</td>
</tr>
</tbody>
</table>

* Haemagglutination inhibition tests were done in the micro assay (Palmer et al. 1975) with RDE treated ascitic fluids from hybridoma-bearing mice using the same virus preparations as in the RNA–RNA hybridizations. Figures are the reciprocals of the dilutions inhibiting 4 agglutinating doses of virus.
† From Laver et al. 1979.
largest RNA, the haemagglutinin gene, have a different electrophoretic mobility from the homologous hybrids and this is in contrast to the constancy in mobility of the hybrids containing the other virus RNAs. It should be noted also, however, that the heterologous hybrids formed with the largest complementary RNA of variant H14/A20 V-2 (No. 3 in Fig. 1; Table 1) have different mobilities from the homologue which indicates that the nucleotide sequence of this gene, in addition to that of the haemagglutinin gene, has changed in this variant. In all cases the heterologous hybrids have either the same or a lower rate of migration than the completely ds molecules and this has been a common observation which was also made by Ito & Joklik (1972) in their analyses of reovirus mutants.

The conclusion from these experiments together with the limited sequence information available is that single base changes can be detected by this analytical procedure. Moreover, although it is clear that under the conditions of electrophoresis used not all heterologous hybrids are distinguished, the results give confidence that by the use of virion RNA from appropriately related viruses all mutations could be detected. It is also possible that by employing different and more extensive nuclease digestions the sites of mutations could be defined.

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National Institute for Medical Research
Mill Hill, London NW7 1AA, U.K.

* Permanent address: St Jude Children’s Research Hospital, 332 North Lauderdale, Memphis, Tennessee 38101, U.S.A.

REFERENCES


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