Peptide Mapping of 125I-labelled Influenza Virus Proteins. Matrix Proteins as Markers in Recombination

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SUMMARY

We have examined the matrix proteins of A/Okuda/57, A/Finland/4/74 and A/New Jersey/8/76 viruses and several recombinant strains by radioiodination of the purified polypeptides followed by tryptic peptide mapping. The method is rapid and requires only small amounts of material. Reproducible differences were detected between the matrix proteins of the above parents and allowed the origin of the matrix proteins of the recombinant viruses to be determined. The possible use of matrix protein identity as a marker in recombination work is discussed.

INTRODUCTION

Structural characterization of influenza virus strains so far has largely had to depend on analysis of the envelope glycoproteins, haemagglutinin and neuraminidase. The internal ‘P’ proteins, nucleoproteins and matrix proteins of group A influenza viruses seem to offer very little opportunity for distinction between strains as their respective molecular weights are constant (Skehel & Schild, 1971; Brand, 1972) and as the matrix protein (Schild, 1972) and ribonucleoprotein (Lief, Fabiyi & Henle, 1958) show little or no antigenic variation between strains.

We have used chemical techniques to examine the smallest internal component, the matrix protein, of some influenza viruses which are of particular interest for preparing recombinant strains as potential live, attenuated influenza vaccines. Matrix protein composition provides a marker for identification of recombinants and its possible relevance to biological properties of the virus is discussed.

METHODS

Parental viruses and recombinants. Parental virus strains, A/Okuda/57 (H2N2) and A/Finland/4/74 (H3N2), and the preparation and the identification of recombinants have been described by McCahon, Beare & Stealey (1976). A/New Jersey/8/76 (Hsw1N1) was obtained from Dr G. C. Schild (National Institute for Biological Standards and Control). Recombinants were investigated clinically by Dr A. S. Beare at the MRC Common Cold Unit, Salisbury.

Measurement of virus growth and infectivity. The virus yield in allantoic fluid was measured as haemagglutination units (HAU) with 0.5% chick red blood cells.

Examination of the virus growth in allantois-on-shell (AOS) cultures (Fazekas de St Groth & White, 1958) was carried out by two methods: (1) Virus stocks were assayed for infectivity at 36, 40 and 42 °C in AOS cultures to give a rough estimate of the degree of...
temperature sensitivity of a strain (> 40 °C or < 40 °C). (2) The infectivity titre produced in AOS cultures was measured in the pooled medium containing chick red blood cells and chorioallantoic membrane from eight of those cultures which were calculated to have been infected with approx. 10 AOS ID_{50} of virus in stage (1). Virus adsorbed to the red blood cells was eluted by incubation for 1 h at 37 °C, and the pool was sonicated for 30 s in a Megason ultrasonic bath before titration in AOS cultures.

Virus growth and purification. Viruses were grown for 48 h at 36 °C in the allantoic cavity of 10-day-old embryonated fowl eggs and purified by density gradient centrifugation essentially as described by Skehel & Schild (1971). Virus suspensions were adjusted to a final protein concentration of 10 mg/ml in phosphate buffered saline (PBS), pH 7-2.

Removal of envelope components. Haemagglutinin and neuraminidase components were removed from the viruses by digestion with bromelain as described by Brand & Skehel (1972). The spikeless bromelain-treated particles were separated from the released subunits and residual enzyme by sedimenting the particles followed by centrifugation on 20 to 60 % (w/v) sucrose density gradients (in PBS) for 16 h at 25000 rev/min (Beckman SW27 rotor). The bromelain-treated virus bands were collected, diluted with PBS and sedimented by centrifugation for 40 min at 60000 rev/min (Beckman T60 Ti rotor), after which the bromelain-treated particles were again adjusted to a final protein concentration of 10 mg/ml in PBS.

Separation of virus proteins by SDS polyacrylamide gel electrophoresis. The bromelain-treated particles were disrupted by the addition of a reducing mixture consisting of SDS (2 %), 2-mercaptoethanol (4 %) and urea (8 M), and boiled for 2 min. Material was applied to 8-5 % SDS-polyacrylamide gels at a level of 60 μg of virus protein in a total vol. of 0·2 ml/gel. The preparation of the gels and the conditions for their subsequent electrophoresis were essentially as described by Skehel & Schild (1971). The protein in the gels was stained with Coomassie brilliant blue (Raymond A. Lamb, North Acton, London).

Extraction of matrix protein. The stained matrix protein bands of between 2 and 6 gels were cut out, pooled and ground in a hand homogenizer using 0·05 M-phosphate buffer, pH 7·2. When the consistency of the gel had reached a fine slurry, 10 % SDS (w/v) was added to give a final concentration of 0·1 %. The gel was then centrifuged for 5 min at 1000 g at room temperature and the supernatant removed. The gel was washed twice more with 2·5 vol. of 0·05 M-phosphate buffer, pH 7·2, containing 0·1 % SDS and the supernatant fractions containing the blue stain were pooled.

Iodination of matrix protein. The eluted matrix protein was further processed and iodinated essentially as described by Bray & Brownlee (1973) but with several modifications. Our procedure was as follows. The extracted protein and stain were precipitated by the addition of 2 M-potassium chloride to a final concentration of 0·2 M and the samples were left on ice for 15 min. The precipitates were sedimented by centrifugation at 2000 g for 10 min, drained and most of the stain was removed by washing once with acetone containing 0·1 M-HCl and once with acetone alone. The samples were dried in a vacuum and then suspended in 0·05 M-sodium phosphate buffer, pH 7·2, at approx. 50 μl/original gel band. 125-I-sodium iodide with a specific activity of 11 to 17 mCi/mg of iodine (obtained from the Radiochemical Centre, Amersham) was added in an amount adjusted for the quantity of extracted protein. For protein pooled from four gels, approx. 1 mCi (1·5 to 2 μl) of 125-I-sodium iodide was added. To start the iodination reaction, chloramine T solution at 5 mg/ml was added to a final concentration of 1 mg/ml. The sample was held in a warm bath (40 °C) for 30 min and then the reaction was stopped by the addition of 2 vol. of sodium metabisulphite solution (5 mg/ml).
Peptide mapping of matrix proteins

Separation of labelled matrix protein. To dilute the remaining free $^{125}$I-sodium iodide, potassium iodide solution (50 mg/ml) was added to a final concentration of 5 mg/ml. The reaction mixtures were put on ice for 10 min and then mixed with at least an equal vol. of bovine serum albumin (2 mg/ml) as carrier protein. The protein was precipitated by the addition of 100% trichloroacetic acid to a final concentration of 10%. After leaving on ice for a further 15 min the precipitates were collected by centrifugation (2000 g for 30 min), washed in acetone containing 0.1 M-HCl and then in acetone alone, and dried in a vacuum.

Trypsinization. The dried samples, without subjection to performic acid oxidation, were taken up usually in 1 to 2 ml of 0.1 M-ammonium bicarbonate and dialysed overnight against at least 100 vol. of 0.1 M-ammonium bicarbonate in order to remove any remaining unbound iodine. Trypsin solution (Sigma, type I from bovine pancreas) at 1 mg/ml in 0.05 M-tris chloride, pH 7.4, was added to a final concentration of 40 to 50 μg/ml, and the mixtures were incubated with shaking for 16 h at 37°C before being centrifuged briefly (2000 g for 10 min) to sediment any undissolved material. The supernatant fractions were lyophilized twice to remove all traces of ammonium bicarbonate. The dry samples were finally taken up in 50 to 100 μl pyridine-acetate buffer (pH 3.5), centrifuged briefly (2000 g for 10 min) to remove insoluble material and assayed for radioactivity in an ICN gamma-set model GS500 gamma counter.

Tryptic peptide mapping by two-dimensional high voltage paper electrophoresis. Approximately $2 \times 10^6$ ct/min of labelled matrix protein digest were applied in 1 μl to Whatman 3MM paper (46 × 57 cm sheets). Electrophoresis was carried out in the first dimension in pH 6.5 buffer (pyridine 10% v/v acetic acid 0.3% v/v) for 45 min at 3 kV in a glass tank containing white spirit as coolant. The paper was then removed, dried and subjected to electrophoresis in the second dimension using pH 1.8 buffer (acetic acid 8%, v/v, formic acid 2%, v/v) for 45 min at 3 kV. One-dimensional peptide separations for rapid evaluation of matrix protein samples were carried out by electrophoresis at pH 1.8 for 45 min at 3 kV.

Autoradiography. The paper electropherograms were completely dried, exposed to X-ray film (12 in. × 15 in. Kodirex, Kodak) for 24 h and developed using Phenisol (Ilford) developer.

RESULTS

Isolation and characterization of recombinant viruses

A/Okuda/57 has been chosen as an attenuated parent virus which grows relatively well for live vaccine recombination work in our laboratory. Recombinants produced between this virus and A/Finland/4/74 or A/New Jersey/8/76 have been selected for the envelope antigens of the recent parent and the good growth characteristics of the A/Okuda/57 parent. The results with four such recombinant strains are summarized in Table 1. Recombinants in both groups grew in eggs to a titre similar to that of the A/Okuda/57 parent, although the increase over the A/Finland/4/74 parent was marginal for WRL 100. The recombinant viruses also had infectivity titres in AOS cultures near or above that of the A/Okuda/57 parent and distinctly higher than that of the wild type strains. However, the A/Finland/4/74 recombinants had the temperature sensitivity of the wild-type parent and not that of A/Okuda/57.

In studies in volunteers the three recombinant virus strains bearing the A/Finland/4/74 envelope antigens were less virulent than A/Finland itself and showed a spectrum of attenuation from the poorly-attenuated WRL 100, through WRL 105 to the 'over-attenuated' WRL 94 (McCahon et al. 1976). The relatively low degree of virulence of the
Table I. Biological and clinical properties of parental and recombinant virus strains

<table>
<thead>
<tr>
<th>Influenza A virus strain</th>
<th>Parental WRL code</th>
<th>Envelope antigens</th>
<th>HA yield in eggs*</th>
<th>Growth on AOS</th>
<th>Temperature sensitivity †</th>
<th>Clinical characteristics</th>
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<td></td>
<td>Parents</td>
<td>Haemagglutinin</td>
<td>Neuraminidase</td>
<td>Expt. 1</td>
<td>Expt. 2</td>
<td>T °C</td>
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<td>646</td>
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<td></td>
<td></td>
<td>Hsw1</td>
<td>124</td>
<td>—</td>
<td>&gt; 40</td>
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* Geometric mean titre of haemagglutination units per 0.25 ml allantoic fluid from between 20 and 50 eggs.
† Temperature sensitivity–inhibition of growth by temperatures above or below 40 °C.
‡ Infectivity titre of harvest from 8 AOS cultures.

A/New Jersey/8/76 parent (Beare & Craig, 1976) made it difficult to detect any reduction in virulence in the recombinant WRL 124 (A. S. Beare, personal communication).

Isolation and characterization of matrix protein

Matrix protein preparations of A/Finland/4/74, A/Okuda/57 and recombinant WRL 105 were isolated by extraction from SDS-polyacrylamide gels. Because of the differences in migration of the haemagglutinin and neuraminidase components with these strains, it was necessary to remove the envelope glycoproteins by bromelain treatment to prevent their co-migration with the internal proteins. Fig. 1 shows the stained bands of internal proteins P1, P2, nucleoprotein and matrix protein in the bromelain-treated viruses. The gels show the clear resolution of matrix protein from the nearest protein band—the nucleoprotein.

A possible contaminant of the purity of extracted matrix protein was that of residual bromelain-cleaved HA2 polypeptide (BHA2) from the haemagglutinin. This possibility was examined as follows. In preliminary experiments it was found that under mild proteolytic conditions, Finland-type glycoproteins were more resistant to removal than were the Okuda-type glycoproteins. Also, the HA2 polypeptide bands of Finland haemagglutinin separated well from matrix protein (see Fig. 1a) on 8.5% polyacrylamide gels. After proteolysis, any remaining BHA2 still just resolved from matrix protein. However, when A/Finland/4/74 or any other influenza viruses were subjected to our extensive bromelain treatment (Methods), no BHA2 was detected after purification of the bromelain-treated particles. Further confirmation of this was obtained by analysis of bromelain-treated viruses on polyacrylamide gels containing 10% or 12.5% acrylamide. Under these conditions, improved separation of BHA2 and matrix protein would have shown any contamination, but this was not found.
Peptide mapping of matrix proteins

The tryptic peptide maps of A/Okuda/57 and A/Finland/4/74 (Fig. 2) appear generally similar but there are some clearly discernible differences. One intensely labelled spot in the Okuda fingerprint is absent from that of the Finland parent and other less obvious differences also exist. Recombinant WRL 105, which was selected on the basis of its clinical properties as a live influenza virus vaccine, was examined to determine the origin of its matrix protein. It shows a fingerprint (Fig. 2) identical to A/Okuda/57, indicating that, during recombination, the RNA segment coding for the matrix protein component was derived from the A/Okuda/57 parent. The fingerprint patterns of the matrix proteins for these viruses have been obtained with separate batches of purified virus, thus proving the reproducibility of the system. Differences in the matrix proteins of A/Finland/4/74 and A/Okuda/57 could also be seen in one-dimensional maps from high-voltage electrophoresis performed at pH 1.8. However, differences were better defined in the two-dimensional maps which used no more labelled material than the one-dimensional maps but required about double the exposure time for autoradiography.

Peptide maps of recombinants WRL 94 and WRL 100

Two other recombinant viruses from A/Okuda/57–A/Finland/4/74 cross which possess the envelope antigens of A/Finland/4/74 but not its virulent character were examined to
Fig. 2. Matrix protein fingerprints of A/Okuda/57, recombinant WRL 105 and A/Finland/4/74. The $^{125}$I-labelled tryptic peptides of matrix proteins were separated by high-voltage paper electrophoresis and subjected to autoradiography as described in Methods. Arrows on A/Okuda/57 and WRL 105 fingerprint patterns indicate the major peptide difference from the A/Finland/4/74 fingerprint.
Peptide mapping of matrix proteins

Fig. 3. Matrix protein fingerprints of recombinants WRL 94 and WRL 100.

determine the origin of their matrix protein components. The maps, shown in Fig. 3, clearly indicate that as for WRL 105, the matrix proteins of WRL 94 and WRL 100 were derived from the A/Okuda/57 parent.

Peptide maps of A/New Jersey/8/76, A/Okuda/57 and recombinant WRL 124

Similar analyses of the matrix proteins of A/New Jersey/8/76 and strain WRL 124, obtained by recombination between this parent and A/Okuda/57, were carried out. Again, a clear cut difference existed between A/Okuda/57 and the recently isolated virus (Fig. 4). However, the recombinant contained the A/New Jersey/8/76 matrix protein as well as the envelope antigens of this parent. The apparent similarity of the A/New Jersey/8/76 and A/Finland/4/74 matrix protein fingerprints (Fig. 2 and 4) may be due to identical matrix proteins in these viruses, but was not further investigated at this stage.

DISCUSSION

We have examined the matrix proteins of the attenuated A/Okuda/57 and recent wild type A/Finland/4/74 and A/New Jersey/8/76 influenza viruses, and have found reproducible, clear-cut differences in their peptide fingerprints indicating variation in polypeptide sequences. Our labelling procedure detects only tyrosine-containing peptides and other differences may also exist in those sequences of the protein which lack tyrosine. Our findings complement those of Laver & Downie (1976) who detected differences by one-dimensional electrophoresis at pH 6.5 between A/Okuda/57 and a human virus isolated in 1973. The peptide difference which we have shown can also be detected by one-dimensional analysis but the cationic peptide differences are best revealed by electrophoresis at pH 1.8, unlike the major anionic peptide difference revealed by Laver & Downie (1976). Structural analyses of this type
Fig. 4. Matrix protein fingerprints of A/Okuda/57, recombinant WRL 124 and A/New Jersey/8/76. The arrow on the A/Okuda/57 peptide maps shows the major peptide difference from A/New Jersey/8/76 and recombinant WRL 124. The low amount of A/New Jersey/8/76 matrix protein available for ¹²⁵I-iodine labelling, because of its low growth, was consistently found to produce fingerprints of reduced spot intensity.
Peptide mapping of matrix proteins

appear to be one of the few ways in which matrix proteins of different strains can be distinguished. Schild (1973) reported from immuno-double diffusion studies that there were no antigenic differences between the matrix proteins of the influenza A viruses. However, recent serological investigations using radio-immunoassay by J. Lecomte & J. S. Oxford, 1976 (personal communication) indicate some antigenic variation in this protein.

Examination of recombinant viruses derived from A/Okuda/57 and A/Finland/4/74 showed the matrix protein of the virus segregated independently from the haemagglutinin and neuraminidase. Although only three recombinant viruses from this cross were examined, each possessed matrix protein derived from the A/Okuda/57 parent and envelope antigens of the A/Finland/4/74 parent. In agreement with these findings and those of Laver & Downie (1976), the accompanying paper by Dimmock et al. (1977) describing a different labelling and analytical technique also showed the matrix protein to segregate independently.

The matrix protein is the most abundant protein in the virion (Compans et al. 1970; Schulze, 1970; Skehel & Schild, 1971) but is of unknown function. It has been suggested that it fulfills a general capsid function within and around the ribonucleoprotein component of the virus (Schulze, 1973), and is important in virus assembly. It is therefore of great importance to discover if the matrix protein provides a marker for a biological property of the virus such as growth potential, temperature sensitivity or virulence. In one of our recombination systems, the two parents A/Okuda/57 and A/Finland/4/74 differed in their virulence but little in their growth. It was therefore of interest to see if the origin of the matrix protein in the recombinants correlated with the biological properties of either parent. Fingerprinting showed that the three recombinants examined possessed the matrix protein of the Okuda parent, and clinically they had, to different extents, the avirulent character of A/Okuda/57. Their infectivity in AOS was similar to or greater than that of A/Okuda/57, whereas in temperature sensitivity they resembled the A/Finland/4/74 parent. Since only those recombinants growing to high titre were selected for clinical investigation, it might be thought that the matrix protein correlates with this property, but this is not supported by the findings of the Okuda–New Jersey recombination system. Here, the good-growing recombinant, WRL 124, did not possess the matrix protein of the A/Okuda/57 parent. Unfortunately this latter system does not contribute much information concerning a possible correlation of virulence with matrix protein type since the wild-type parent virus, A/New Jersey/8/76, was itself relatively avirulent. In any event, the phenomenon of virulance may be, and probably is, a multi-genic function (Rott, Orlich & Scholtissek, 1976).

The radiolabelled fingerprinting technique which we have described has several advantages for comparing the structure of virus proteins. It uses relatively small amounts of virus proteins, labelling can be achieved to high specific activity incorporation of $^{125}$I and, as a consequence of this, exposure times of the paper electropherograms to the X-ray film are reduced to hours rather than days. The capacity of this technique to reveal differences in matrix proteins will be very useful in examining a range of human influenza strains in order to find how much and how often this protein changes and whether such changes occur at the time of the major ‘shifts’ or ‘drifts’ in the envelope glycoprotein antigens.

We intend to investigate similarly the nucleoproteins of the parental and recombinant influenza viruses. Extension of the technique to the ‘P’ proteins may not be practical because the large size of these proteins would be expected to produce very complex maps. Such detailed examinations in several recombination systems may reveal relationships between structural changes and important biological properties of the virus. Hence valuable new genetic markers might be produced.
We thank Mr M. Merret and Dr D. Stone for co-operation and assistance with high-voltage electrophoresis and Miss F. Dudgeon and Miss C. Parsons for technical assistance. We are very grateful to Dr A. S. Beare for providing excellent facilities for clinical examination of our recombinant viruses at the MRC Common Cold Unit, Salisbury.

REFERENCES


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