The Assay of Influenza Antineuraminidase Activity by an Elution Inhibition Technique

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SUMMARY

The antineuraminidase activities of rabbit and human antisera were titrated by their ability to inhibit the elution of influenza virus from fowl red cells. The method was specific, sensitive and simple, and the results correlated well with those of conventional neuraminidase inhibition tests.

INTRODUCTION

The influenza virus surface is composed of haemagglutinin and neuraminidase subunits. Following infection or vaccination, antibodies may be produced against each of these virus components and both kinds of antibody contribute to immunity to re-infection. Titration of the antihaemagglutinin activity of sera or other fluids is a simple and well-established procedure. In contrast, conventional methods for the titration of antineuraminidase activity, which depend upon measuring the enzyme activities of virus-antiserum mixtures (Ada, Lind & Laver, 1963; Webster & Laver, 1967; Aymard-Henry et al. 1973), are laborious and expensive. Consequently, several more convenient procedures have recently been devised for the assay of antibodies to neuraminidase, such as neuraminidase-specific haemagglutination inhibition (Kilbourne, Christenson & Sande, 1968; Kendal, Minuse & Davenport, 1972), single radial diffusion (Schild, Henry-Aymard & Pereira, 1972) and single radial haemolysis (Callow & Beare, 1976).

The elution of influenza virus from red blood cells is an enzymic process that is catalysed by the virus neuraminidase (Hirst, 1942; Gottschalk, 1957) and can be prevented by antineuraminidase (Brown & Laver, 1968). We have utilized this phenomenon to develop a method for the titration of antibodies to influenza virus neuraminidase. The method is simple and sensitive and may have advantages over other techniques now in use.

METHODS

Virus strains. All influenza viruses were grown in the allantoic cavity of 11-day embryonated hens' eggs and stored as infected allantoic fluids at $-70^\circ$C.

The strains used were: A/BEL/42 (HoNo) = 'BEL'; A/Singapore/1/57 (H2N2) = 'A2'; the recombinant A/BEL/42 - Singapore/1/57 (HoN2) (Schild, McCahon & Kendal, 1970) = 'BEL-A2'; X15 (HEqN2), a recombinant (Kilbourne, 1968) having the haemagglutinin of A/Equi/Prague/1/56 and a neuraminidase similar to that of A/Singapore/1/57; X15-Hong Kong (HEqN2), a recombinant kindly provided by Dr A. S. Beare, having the haemagglutinin of A/Equi/Prague/1/56 and the neuraminidase of A/Hong Kong/1/68;
XI5-Port Chalmers, a recombinant produced in this laboratory, having the haemagglutinin of A/Equin/Prague/1/56 and the neuraminidase of A/Port Chalmers/1/73; B/Lee/40.

**Red blood cell suspensions.** Blood from selected Rhode Island Red fowls was taken into Alsever's solution. The red cells were washed three times by centrifugation in phosphate buffered saline (PBS), pH 7.2, and stored as a 10 % (v/v) suspension at 4 °C for not more than 3 days. They were diluted to 0.5 % (v/v) immediately before use.

In a few experiments, blood from White Leghorn fowls or human donors was treated similarly.

**Antisera.** Hyperimmune sera against BEL, A2, BEL-A2 and B/Lee influenza viruses were obtained from New Zealand white rabbits which had received several subcutaneous injections of about 1 mg purified virus emulsified with Freund's complete adjuvant.

Twenty-three pairs of human sera, from subjects before and after immunization with a live attenuated strain of A/Hong Kong/1/68 virus, were kindly provided by Dr A. S. Beare. A further twenty-four human sera were obtained from staff of this Establishment who had recently been immunized with an inactivated A/Port Chalmers/1/73 vaccine. All sera were heated for 1 h at 56 °C.

**Assay of antineuraminidase activity**

**Neuraminidase inhibition (NI) method.** Antisera were serially diluted in saline and 0.1 ml volumes were mixed with an equal volume of test virus (XI5 or XI5-Port Chalmers) diluted to contain 25 units of neuraminidase activity. The mixtures were kept at 37 °C for 1 h followed by 4 °C overnight. Controls consisted of virus incubated with saline, normal rabbit serum or dilutions of a standard hyperimmune rabbit serum. Residual neuraminidase activities were determined by the method described by Aymard-Henry et al. (1973) except that the fetuin concentration was doubled and the time of incubation was reduced to 30 min. These modifications resulted in some increase in reproducibility at the expense of sensitivity. One unit of neuraminidase activity is defined as the amount of enzyme that releases 1 nmol of N-acetyl neuraminic acid per min under these conditions. Neuraminidase inhibition (NI) titres are expressed as the reciprocals of the serum dilutions that reduced the enzyme activity by 50 %.

For the assay of NI activity of human sera against A/Hong Kong/1/68 virus, use was made of data kindly provided by Mrs K. Callow. The sera had been reacted at a single dilution of 1/50 with XI5-Hong Kong virus and the residual neuraminidase activities measured by the method of Webster & Laver (1967) as modified by Callow & Beare (1976). The NI titres, again expressed as the reciprocals of the serum dilutions that reduced enzyme activity by 50 %, were calculated by reference to a standard curve on which percentage inhibition had been plotted against antiserum dilution.

**Elution inhibition (EI) method**

**Single plate method.** This was used in tests on hyperimmune rabbit sera. Serum samples were diluted in twofold steps in 0.25 ml volumes of PBS in the wells of a WHO haemagglutination plate, the initial serum dilution usually being 1 in 100. An equal volume of test virus, diluted in PBS to contain about 8 haemagglutinating units (HAU), was added to each well, and the reaction mixtures were kept at room temperature (about 20 °C) for 1 h. As a check on the amount of virus added, serial dilutions of the test virus were mixed with diluent instead of antiserum. Fowl red cells (0.25 ml of a 0.5 % suspension) were added to each well, and the plate was kept for a further 1 h at room temperature to confirm the occurrence of haemagglutination. The plate was then incubated at 36 °C
Rhode Island Red
red cells

White Leghorn
red cells

Dilution of virus sample

0 h
2 h
4 h
20 h
0 h
4 h

Fig. 1. Elution of X15 influenza virus from two kinds of fowl red cell. Infected allantoic fluid, diluted from 1/10 to 1/1280, was mixed with red cells from a Rhode Island Red or White Leghorn fowl. After haemagglutination at room temperature, the mixtures were incubated at 36 °C for the times indicated.

overnight in a moist atmosphere to allow elution of virus that still possessed neuraminidase activity. The elution inhibition (EI) titre of a serum was the reciprocal of the dilution that allowed partial virus elution, as shown by a pattern of red cells intermediate between the 'carpet' of complete haemagglutination and the 'button' of complete elution.

Two plate method. This was used in tests on human sera, which had lower EI titres than the hyperimmune rabbit sera. The first stage was identical to the procedure described for the single plate method, except that the initial serum dilution was 1 in 10 and about 15 HAU, instead of 8 HAU, of test virus was added. After overnight incubation of the serum-virus-red cell mixtures, samples of 0.25 ml supernatant fluid were transferred by automatic syringe from each well of the 'reaction plate' to the corresponding well of an 'indicator plate'. Red cells were added to the fluids in the indicator plate and, after 1 h at room temperature, the presence or absence of haemagglutination was noted. Haemagglutination indicated elution of virus in the reaction plate whereas lack of haemagglutination indicated failure to elute. The EI titre of a serum was the reciprocal of the dilution in the reaction plate that gave an endpoint of 50 % haemagglutination in the indicator plate.

RESULTS

Elution of influenza virus

When observed in the wells of WHO haemagglutination plates, red blood cells that have been agglutinated by influenza virus settle as a uniform 'carpet'; the subsequent elution of virus that occurs at 36 °C is revealed by conversion of the 'carpet' to a compact 'button' of single cells.

Preliminary tests showed that an important factor in influenza virus elution was the source of the red cells. Viruses eluted very poorly from the cells of White Leghorn fowls
but satisfactorily from the cells of three out of six Rhode Island Red fowls (Fig. 1). Therefore blood from selected Rhode Island Reds was used routinely. Influenza viruses also eluted from human red cells, although less rapidly than from those of the best fowls.

The rate of elution also depended upon the strain of influenza virus. However, most strains of virus eluted completely if the agglutinated cells were held at 36 °C for 16 h.

### Inhibition of virus elution by hyperimmune rabbit sera

The virus used in any type of test for antibodies to influenza virus neuraminidase should possess a heterologous haemagglutinin that does not react with the sera under examination. Accordingly, the elution inhibiting activity of a serum pool from rabbits hyperimmunized with A2 (H2N2) virus was measured against X15 (HEn1N2) virus. The serum was titrated against a range of dilutions of test virus, using the 'single plate method'. The appearance of this chessboard titration, both before and after incubation at 36 °C, is shown in Fig. 2. Low dilutions of antiserum caused some neuraminidase-specific inhibition of haemagglutination (Fig. 2a), but inhibition of virus elution occurred at much higher dilutions (Fig. 2b). The endpoints between wells showing elution of virus ('button' formation) and continued haemagglutination (retention of 'carpet') were clear and they did not alter with more prolonged incubation.

The results of elution inhibition (EI) titrations on a variety of rabbit antisera, using a constant dilution of X15 virus, were compared with those of conventional neuraminidase inhibition (NI) titrations (Table 1). The titres obtained by EI tests were about ten times higher than those from NI tests; a greater sensitivity of the EI test was to be expected, since a smaller amount of virus was used. Normal rabbit serum had no EI activity. The low EI titres associated with hyperimmune BEL and B/Lee antisera may have been caused by small amounts of anti-chick antibodies, produced by immunization with egg-grown virus; the EI titre of BEL antiserum, unlike that of A2 antiserum, was independent of the concentration of X15 virus, suggesting that the antiserum was reacting with the red cells rather than the virus.
Assay of antineuraminidase activity

Table 1. Comparison of neuraminidase inhibition (NI) and elution inhibition (EI) assays for antineuraminidase activity

<table>
<thead>
<tr>
<th>Rabbit hyperimmune serum against influenza strain</th>
<th>NI titre*</th>
<th>EI titre†</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2 (H₂N₂)</td>
<td>5300</td>
<td>70000</td>
</tr>
<tr>
<td>BEL-A2 (H₀N₂)</td>
<td>3600</td>
<td>30000</td>
</tr>
<tr>
<td>BEL (H₀N₀)</td>
<td>&lt;50</td>
<td>120</td>
</tr>
<tr>
<td>B/JLee</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Normal serum</td>
<td>&lt;50</td>
<td>&lt;20</td>
</tr>
</tbody>
</table>

* Against X₁₅ (HE₁₁N₂) allantoic fluid diluted 1/12.
† Against X₁₅ allantoic fluid diluted 1/100.

Fig. 3. Three stages of an elution inhibition test against X₁₅-Port Chalmers virus. R₁ and R₂: hyperimmune rabbit serum at initial dilutions of 1/1000 and 1/4000 respectively plus test virus diluted 1/50. 52, 52A, 95 and 95A: human sera at initial dilution of 1/10 plus test virus diluted 1/50. V: test virus at initial dilution of 1/50 plus PBS. (a) Reaction plate 1 h after addition of red cells. (b) Reaction plate after further 16 h at 36 °C. (c) Indicator plate 1 h after addition of red cells to fluids transferred from the reaction plate.

Thus, the results obtained with rabbit antiserum indicated that the EI test was a specific and sensitive method for the detection of antibodies to influenza virus neuraminidase.

Elution inhibition tests on human sera

Human sera contained considerably less neuraminidase antibody than hyperimmune rabbit sera, and non-specific effects of the lower serum dilutions gave rise to some initial difficulties. Serum frequently caused slipping of agglutinated red cells, with the formation of a 'pseudo-button' similar to the button of cells resulting from virus elution. The effect was enhanced by prolonged incubation at 36 °C and often prevented an accurate assessment of the elution endpoint. To overcome this difficulty, the occurrence of elution was detected indirectly by use of the 'two plate method' of assay. Fig. 3 shows the successive stages of such an assay for antibody to A/Port Chalmers/1/73 neuraminidase, using a hyperimmune rabbit serum and two pairs of human sera. 'Pseudo-button' formation was caused by low dilutions of the human sera even before incubation at 36 °C (Fig. 3a). This effect obscured the elution endpoints of the human sera after overnight incubation, although not those of the hyperimmune rabbit serum (Fig. 3b). Readable endpoints were produced by all sera when
supernatant fluids were transferred from the reaction plate to an indicator plate and were then tested for the presence of haemagglutinin (Fig. 3c).

Forty-six human sera from subjects taking part in a trial of an attenuated A/Hong Kong/1/68 vaccine were assayed for EI activity against X15-Hong Kong virus. The results were compared with those of conventional NI assays (Fig. 4). The NI titres in this experiment were calculated from the inhibition produced by a single (1/50) dilution of each serum and should, therefore, be regarded as only approximate. There was a significant correlation between the results of the two types of assay.

The two methods were compared more critically using sera from 24 subjects who had been immunized with inactivated A/Port Chalmers/1/73 virus. The test virus was X15-Port Chalmers. NI titres were derived from the results of two titrations, each employing four dilutions of antiserum, and EI titres were the mean results of duplicate or triplicate titrations. Fig. 5 shows that there was a good correlation between the results of EI and NI tests.

The reproducibility of elution inhibition titrations is shown in Table 2. Four pairs of human sera, taken before and after immunization with A/Port Chalmers/1/73 vaccine, were titrated for EI activity against X15-Port Chalmers virus on six or more occasions. The replicate results from all but one of the sera fell within a twofold range of values.
Assay of antineuraminidase activity

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Fig. 5. Elution inhibition and neuraminidase inhibition titres of 24 human sera measured against influenza A/Port Chalmers/1/73 neuraminidase.

Table 2. Reproducibility of elution inhibition titrations on human sera

<table>
<thead>
<tr>
<th>Serum</th>
<th>Number of titrations</th>
<th>Elution inhibition titre against X15-Port Chalmers virus</th>
</tr>
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<tbody>
<tr>
<td>10</td>
<td>6</td>
<td>337</td>
</tr>
<tr>
<td>10A</td>
<td>6</td>
<td>1133</td>
</tr>
<tr>
<td>15</td>
<td>6</td>
<td>887</td>
</tr>
<tr>
<td>15A</td>
<td>6</td>
<td>1027</td>
</tr>
<tr>
<td>29</td>
<td>7</td>
<td>56</td>
</tr>
<tr>
<td>29A</td>
<td>8</td>
<td>268</td>
</tr>
<tr>
<td>51</td>
<td>6</td>
<td>25</td>
</tr>
<tr>
<td>51A</td>
<td>6</td>
<td>123</td>
</tr>
</tbody>
</table>

DISCUSSION

Antibodies directed against the virus neuraminidase probably play a significant part in resistance to influenza infection and a convenient assay for antineuraminidase activity is now needed. The elution inhibition technique has several advantages. Most importantly, the results of elution inhibition (EI) tests appear to correlate well with those of the conventional, but more laborious, neuraminidase inhibition (NI) tests. The assay is sensitive, because only a small amount of test virus is used. It is also simple to perform and uses no expensive reagents. We have titrated antibodies to the neuraminidases of influenza viruses A/Singapore/1/57, A/Hong Kong/1/68 and A/Port Chalmers/1/73 and the method should
be equally applicable to any virus that can be recombined with a non-reactive haemagglutinin such as HEqI.

The method has some drawbacks. A titration requires nearly 24 h for completion. The accuracy is no greater than that of a haemagglutination inhibition titration, replicate titrations producing values scattered over about a twofold range. Also, EI titres are not expressed in absolute units, so that it may be necessary to exchange standard antisera if results are to be compared between laboratories. Some of those criticisms apply equally to other methods for measuring antineuraminidase activity. Overall, the simplicity of the elution inhibition techniques appears to justify its further trial for the assay of antibodies to influenza virus neuraminidase.

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REFERENCES


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