A Quantitative, Single-radial-diffusion Test for Immunological Studies with Influenza Virus

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Antibodies for influenza haemagglutinin and neuraminidase are assayed routinely in haemagglutination-inhibition (Hirst, 1942) and neuraminidase-inhibition (Ada, Lind & Laver, 1963) tests which involve reaction systems of three components. More recently, influenza antibodies have been detected by gel immuno-double-diffusion tests with influenza viruses disrupted by detergents (Styk & Hanna, 1966; Schild & Pereira, 1969). Although the latter method has the advantage that it detects antibodies for each of the major antigens of the virus in a single test system (Schild et al. 1972a; Schild, 1972) the quantitation of antibody is not readily achieved. In the present report we describe a quantitative, single-radial-diffusion test which is a sensitive, rapid and convenient method for the estimation of antibodies to the haemagglutinin and neuraminidase antigens of the influenza virus. A unique feature of this test is that only one component, the antibody, diffuses during the test; the other component, the antigen in the form of intact influenza virus particles, does not diffuse. The method does not depend on the formation of immune precipitates. In contrast, in the radial-immunodiffusion method described by Mancini, Carbonara & Heremans (1965) for the assay of serum protein antigens, both antibody and antigen are in diffusible form. The test we describe has many potential applications in immunological, diagnostic and epidemiological studies with influenza and other viruses.

For our own studies, influenza viruses were cultivated in embryonated eggs and purified and concentrated using a continuous-flow centrifuge (Skehel & Schild, 1971), or by conventional methods on sucrose density gradients (Schild & Pereira, 1969). Virus concentrates contained approximately 4 x 10^6 haemagglutinin units (H.A.U.)/ml and 10 to 15 mg. protein/ml. Before use in single-radial-diffusion tests, virus concentrates were treated by ultrasound (Burndept Ultrasonic cleaning bath, 80 kHz) for 3 to 5 sec. to break up aggregates of virus. This did not disrupt virus particles.

Suspensions of purified, intact influenza virus were prepared in agarose gels. An agarose with a low (37°) setting temperature (Indubiose A-37 from l’Industrie Biologique Francaise) was employed routinely and avoided the exposure of the virus antigen to the relatively higher temperatures (45°+) necessary to maintain other types of agar or agarose in a molten state. Agarose gels (1.5%, w/v, Indubiose A-37 in 0.15 M-NaCl + 0.01 M-phosphate buffer, final pH 7.1 + 0.1% sodium azide) were melted at 100° and cooled to 42° before the addition of virus to a final concentration of 4 x 10^4 H.A.U./ml. (0.1 mg. protein/ml.). The agarose and virus were mixed by vigorous shaking and uniform, 1.5 mm., layers of gel were made as described by Mancini et al. (1965) using photographic glass-plates. Alternatively, plastic immunoplates (Hyland Laboratories, California, U.S.A.) with 2.5 ml. agarose per plate were used to give a gel thickness of 2.5 mm. Circular wells (2.0 mm. diameter) were cut in the agarose to accommodate the antisera. Volumes of 3 μl. of sera, diluted where appropriate, were added to the wells and the plates kept in a moist chamber at room temperature.

The presence in test sera of antibodies to the haemagglutinin or neuraminidase of the virus was detected by the appearance of zones of opalescence surrounding the wells. The zones were readily visible within 3 to 4 hr after setting up the tests and reached maximum size at 7 to 8 hr. For convenience, tests were read after leaving overnight for 16 to 18 hr. Accurate
Figs. 1, 2. Single-radial-diffusion reactions of anti-haemagglutinin and anti-neuraminidase sera in 1.5% A-37 agarose gels containing a suspension of $4 \times 10^4$ H.A.U./ml. of purified, intact A/HONG KONG/1/68 (H₃N₂) virus (X-31 strain). Volumes of 3 μl. of antisera were added to the wells.

Fig. 1. Reactions of antisera to purified H₃ haemagglutinin and purified N₂ neuraminidase. Wells 1, 2, 4, 8 and 12 contain samples of different rabbit sera against H₃ haemagglutinin, and wells 5, 13 and 14 contain rabbit sera against purified N₂ neuraminidase. Well 10 contains mixtures of anti-H₃ and anti-N₂ sera and wells 3, 6, 7 and 9 contain rabbit antisera against purified A/HONG KONG/1/68 virus (anti H₃ + anti N₂). Well 12 contains potent antiserum to influenza A ribonucleoprotein. Antisera against haemagglutinin gave intense opalescent zones with distinct margins. Anti-neuraminidase sera gave less intense zones with diffuse margins. Zones with intense central areas and less dense haloes were observed when sera contained both antibodies.

measurements of zone diameters were made under dark-ground illumination using an eyepiece micrometer scale (×8 magnification, Matchless Machines Ltd., U.K.) or directly from photographic enlargements (×3 magnification). Once formed, the zones were stable for several weeks and their diameters were constant in size when the plates were stored by immersion for several weeks in phosphate buffered saline.

Fig. 1 shows the reactions of various antisera in agarose gels containing X-31 virus (Kilbourne et al. 1971), a high-yielding recombinant influenza A virus antigenically identical to A/HONG KONG/1/68 (H₃N₂) (WHO, 1971). Rabbit antiserum prepared against purified H₃ haemagglutinin derived from A/HONG KONG/1/68 (H₃N₂) virus (Schild et al. 1972b) gave intense and sharply defined zones of opalescence, up to 10 mm. diameter. In contrast, antibody prepared against purified N₂ neuraminidase derived from A/HONG KONG/1/68 virus (Schild, in preparation) gave less intense zones of up to 13 mm. diameter with less well defined margins. The difference in appearance of the zones produced by anti-neuraminidase and anti-haemagglutinin antibodies was consistently observed with a number of different strains of influenza virus and with different sources of antisera (rabbit, guinea pig, mouse, ferret). Rabbit antisera against preparations of whole influenza virus (A/HONG KONG/1/68) which contained antibodies to both surface antigens (H₃ and N₂) of the virus gave zones with a dense central area (anti-haemagglutinin antibody) and a less dense ‘halo’ (anti-neuraminidase antibody). Zones of similar appearance appeared when mixtures of anti-haemagglutinin and anti-neuraminidase sera were used (see Fig. 1).
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Fig. 2. Effect on zone size of dilution of anti-haemagglutinin serum. Wells 1, 2, 3, 4, 5, 6 and 7 contain 1:1, 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64 dilutions of rabbit antiserum against purified H3 haemagglutinin (HI titre 1:1500).

Potent antiserum to influenza A ribonucleoprotein antigen (Schild & Pereira, 1969) or to influenza A internal matrix protein antigen, a recently characterized, type-specific internal antigen of the influenza virus (Schild, 1972), failed to give visible zones in gels containing intact X-31 virus. In addition, sera known to contain non-specific inhibitors of haemagglutination for influenza X-31 virus failed to give opalescent zones: these tests included the sera of man, rabbit, horse, guinea pig, swine, mouse and chicken.

In the present studies with intact influenza virus particles only antibodies to the virus envelope proteins gave opalescent zones. However, in gels containing detergent-disrupted influenza virus, antibodies to influenza A ribonucleoprotein and internal matrix protein (Schild, 1972) gave opalescent zones which allowed the quantitation of these antibodies (Schild, in preparation).

To determine the relationship between zone size and antibody concentration, a series of dilutions of anti-haemagglutinin and anti-neuraminidase sera were tested against X-31 virus. A representative experiment with dilutions of anti-haemagglutinin (H3) serum is shown in Fig. 2. The linear relationship between zone area and antibody concentration is shown by the plot of annulus area of the opalescent zone (omitting the diameter of the well) against relative antibody concentration (Fig. 3). Thus zone areas give a direct measure of the relative concentrations of either anti-haemagglutinin or anti-neuraminidase antibodies.

The relationship between the levels of antibody to influenza envelope antigens measured by zone area and by conventional techniques (haemagglutination-inhibition (HI), virus neutralization and neuraminidase-inhibition) is under investigation. However, in tests with immune rabbit and guinea-pig sera, the single-radial-diffusion test was slightly less sensitive than the HI test (WHO Expert Committee on Influenza, 1953). Sera with HI titres of $\frac{1}{80}$ gave small but distinct zones whilst sera with lower HI titres of $\frac{1}{40}$ or less failed to give visible reactions. For anti-neuraminidase (rabbit) sera the radial-diffusion methods was about as sensitive as neuraminidase-inhibition tests (Schild & Newman, 1969a). Sera with NI 50 titres of $\frac{1}{16}$ gave small zones of 2.1 mm. diameter.
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Fig. 3. Dose–response curves for anti-haemagglutinin and anti-neuraminidase serum. The plots of annulus area (excluding area of the well) against relative antibody concentration give a straight line for each type of antiserum. ●●●, Rabbit antiserum in well tested against purified N2 neuraminidase (NI50 titre 1:300) in gel; ▲▲▲, rabbit antiserum in well tested against purified H3 haemagglutinin (HI titre 1:1000) in gel.

Table 1. Specificity of single-radial-diffusion reactions of anti-haemagglutinin and anti-neuraminidase sera

<table>
<thead>
<tr>
<th>Immune rabbit sera in wells</th>
<th>Anti-BEL</th>
<th>Anti-SING</th>
<th>Anti-HONG KONG</th>
<th>Anti-SING</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain of intact virus in gels</td>
<td>haemagglutinin</td>
<td>haemagglutinin</td>
<td>haemagglutinin</td>
<td>neuraminidase</td>
</tr>
<tr>
<td>(4 × 10^4 H.A.U./ml)*</td>
<td>(H0)</td>
<td>(H2)</td>
<td>(H3)</td>
<td>(N2)</td>
</tr>
<tr>
<td>A/BEL/42 (H0 N1)</td>
<td>5.9† i</td>
<td>&lt; 2.0</td>
<td>&lt; 2.0</td>
<td>&lt; 2.0</td>
</tr>
<tr>
<td>A/SINGAPORE/1/57 (H2 N2)</td>
<td>&lt; 2.0</td>
<td>4.8 i</td>
<td>2.2 d</td>
<td>6.5 d</td>
</tr>
<tr>
<td>A/BEL/42(H0)–A/SING/57(N2)</td>
<td>5.9 i</td>
<td>&lt; 2.0</td>
<td>&lt; 2.0</td>
<td>6.4 d</td>
</tr>
<tr>
<td>X-31 (H3 N2)</td>
<td>&lt; 2.0</td>
<td>2.2 d</td>
<td>4.9 i</td>
<td>3.8 d</td>
</tr>
</tbody>
</table>

* The origin and character of these recombinants have been described (Schild et al. 1970; Kilbourne et al. 1971).
† Zone diameter (mm). i, Intense zone with sharp margins; d, Relatively diffuse zone. Sera giving no reactions are recorded as < 2.0 (diameter of well).

Statistical analyses of the results of dilutions anti-H3 and anti-N2 sera multiply tested by single-radial-diffusion on immunoplates containing X-31 virus have indicated that differences in relative antibody concentrations as small as 15 to 20% could be detected and were of statistical significance. With conventional methods of detection of antibody to influenza virus (HI, NI and CF tests) only differences in titre of 400% or greater (4-fold) are regarded as significant.

In studies with paired human sera from individuals with clinical A/HONG KONG/1/68 (H3N2) infections, the single-radial-diffusion tests appeared as sensitive as conventional
serological diagnostic tests for influenza infection (complement fixation or haemagglutination-inhibition tests) and the results were unambiguous. In samples of sera from 70 individuals no zones were detected in tests with X-31 virus on the ‘acute’ serum samples collected during the first one or two days of illness. Zones ranging from 2-3 mm. diameter up to 10 mm. diameter were detected in convalescent sera taken 10 to 14 days later.

The specificity of single-radial-diffusion reactions was examined with a number of influenza A virus strains (Table 1). Antiserum against the purified haemagglutinin, H0 (Schild, 1970), of A/BEL/42 (H0N1) virus reacted equally well with BEL and with a recombinant virus A/BEL/42(H0)-A/SING/57(N2) known to contain H0 haemagglutinin and N2 neuraminidase. This antiserum gave no reaction with A/SING/57(H2N2) parent virus or with X-31(H3N2). Antiserum against SINGAPORE neuraminidase (N2) reacted equally well with A/SINGAPORE and with the BEL(H0)-SING(N2) recombinant; it also gave a reaction with X-31(H3N2) virus which contains neuraminidase of the same subtype but gave no reaction with A/BEL/42(H0N1). As in the other studies, the zones produced by antiserum to neuraminidase were relatively less intense and had less distinct margins than those produced by anti-haemagglutinin sera. The single-radial-diffusion tests in general indicated a lack of cross-reaction between different haemagglutinins (H0 with H2 or H3) or neuraminidase (N1 and N2) subtypes. The significance of the minor cross-reactions between H2 and H3 is not clear. The ability of radial-diffusion tests to distinguish between variants within a given H or N subtype is under investigation.

In an attempt to elucidate the mechanism of the single-radial-diffusion test, electron micrographs of thin sections of agarose gels containing 4 × 10^4 H.A.U./ml. of X-31 virus were examined. These indicated that the virus was present in the gel as single particles or as small clumps of up to eight particles. Intact particles or clumps were separated by at least 50 times the particle diameter (5000 nm.). Sections through zones produced by anti-haemagglutinin or anti-neuraminidase antibody showed virus particles with haloes of antibody but did not indicate any change in the distribution of virus particles. It seems therefore that the test depends upon an increase in the light scattered by virus after primary attachment of antibody and is independent of secondary agglutination or precipitation. This conclusion is supported by the finding (Schild & Pereira, 1969) that intact influenza virus particles do not diffuse in gels of the present type.

The single-radial-diffusion test appears to have considerable advantages over conventional tests for antibodies to influenza envelope proteins. The test is simple to perform and is not susceptible to the effects of non-specific inhibitors. Very small volumes of antisera are required for the test and assays of antibody have been made with small samples of whole blood obtained from finger pricks. This has obvious practical advantages for routine diagnostic and epidemiological studies since venepuncture may be avoided. In addition, by the choice of appropriate antigenic hybrid influenza viruses, antibody reacting with a particular envelope antigen may be assayed separately. In studies employing a recombinant influenza virus containing A/HONG KONG/1/68 neuraminidase (N2) and haemagglutinin (HAV1) derived from fowl plague virus (Schild & Newman, 1969b) antibody to A2/HONG KONG neuraminidase in human and animal sera were assayed independently of anti-haemagglutinin antibody. Using the reverse type of recombinant containing A/HONG KONG/68 haemagglutinin (H3), antibody to haemagglutinin may be assayed. Techniques previously available have not enabled the independent assay of haemagglutinin and neuraminidase antibodies in a single system.

Since agarose gels containing X-31 virus have been stored at 4°C for up to 6 months before use and have given reproducible results, and since the opalescent zones once formed
are stable for several weeks, the single-radial-diffusion test seems to be well suited to field studies on influenza. In a pilot study, immunoplates containing X-3I virus were distributed by mail to laboratories in Hong Kong, Johannesburg and The Gambia for the addition of paired human sera from individuals with Hong Kong influenza. On return of the plates to this laboratory 6 weeks later the reactions of the test sera were read easily and showed no deleterious effects due to transportation. A collaborative survey is in progress in which the single-radial-diffusion test will be used for serological epidemiological studies of influenza in a number of areas of the world.

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