A Sensitive, Single-Radial Diffusion Autoradiographic Zone Size Enhancement Technique for the Assay of Influenza Haemagglutinin

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SUMMARY

A novel radial-diffusion technique in agarose gels is described which is applicable to the assay of small concentrations of influenza haemagglutinin (HA) antigen. The method is based on single-radial-diffusion zone size enhancement (ZE), using autoradiography to demonstrate antigen–antibody reactions. The ZE technique is capable of reliably assaying concentrations of haemagglutinin as low as 0.1 μg/ml, which represents a 20- to 40-fold increase in sensitivity over conventional single-radial-diffusion techniques. The ZE response is dependent upon the antigenic identity of the 125I-labelled ‘marker’ HA and the HA antigen being assayed. Haemagglutinins showing variation within a subtype (e.g. H3) show reduced ZE responses and thus the method has potential value for distinguishing between closely related HA antigens.

INTRODUCTION

Single-radial-diffusion (SRD) provides a reproducible and precise method for estimating antigen concentrations of serum proteins (Mancini et al. 1965, Rowe, 1969) and has been applied more recently to the assay of virus antigens including adenovirus hexon (Pereira et al. 1972) and influenza virus haemagglutinin (Schild et al. 1975; Wood et al. 1977). In this method, the antigen diffuses radially from wells punched in agarose containing specific antibody and the size of the resulting zone of immunoprecipitate, detected at equilibrium by protein staining, is directly proportional to the antigen concentration. The SRD technique has recently gained wide acceptance for standardizing the haemagglutinin (HA) concentration in inactivated influenza vaccines. It is possible to assay accurately amounts of HA antigen as low as 2 to 4 μg/ml by conventional SRD techniques (Wood et al. 1977). However, the sensitivity of conventional SRD tests is limited by the relatively high antibody concentrations required to produce stainable and visible precipitin zones.

The sensitivity of SRD tests can be enhanced by the incorporation of radioactive label into one of the reagents, either the antigen or antibody (Rowe, 1969; Pereira et al. 1972). The present paper describes a modified SRD technique of increased sensitivity for the assay of influenza HA antigen. The novel technique we describe, an autoradiographic zone size enhancement test, is based on SRD tests in which antigen–antibody reaction zones are demonstrated by autoradiographic techniques. The potency of the antigen being assayed is assessed by its ability to enhance the size of autoradiographic zones produced by small quantities of radioactively labelled HA antigen when mixtures of the labelled antigen and homologous (unlabelled) antigen for assay co-migrate radially from wells in agarose gels. Studies to determine the sensitivity, antigenic specificity, dose response characteristics and statistical reproducibility of the ZE test are described.
Preparation of purified haemagglutinin and specific antisera. High yielding recombinant influenza A H3N2 viruses were used in the studies. These were X31 (A/Hong Kong/1/68), MRC11 (A/Port Chalmers/1/73), X47 (A/Victoria/3/75) and X49 (A/England/846/75, a virus antigenically close to A/Texas/1/77). The recombinants contain surface antigens derived from their H3N2 parents and show high yield growth characteristics similar to that of the A/PR8/34 (H0N1) parent virus. A/USSR/92/77 (H1N1) and B/Hong Kong/8/73 were wild-type strains from the stocks of this laboratory.

Haemagglutinin (HA) was prepared by treatment of purified virus with bromelain and was purified by sedimentation in sucrose density gradients (Brand & Skehel, 1972).

For the preparation of potent anti-HA serum (Haemagglutination inhibition titre 1:30000 to 1:100000), sheep were immunized with multiple doses of 10 to 20 μg of purified HA in Freund’s complete adjuvant as described previously (Wood et al. 1977). A preparation of anti-A/Texas/1/77 HA serum S162, was used for all studies involving X49 virus.

Radio-iodination of HA
Chloramine-T method. Purified HA was iodinated by the method of Hunter & Greenwood (1962). Briefly, 5 μg of HA measured by the Lowry protein assay (Lowry et al. 1951) were mixed with 5 μl of 0·2 M-sodium phosphate buffer, pH 7·5, and 1 mCi Na 125I (The Radiochemical Centre, Amersham, Bucks; sp. act. 13 to 17 μCi/μg). The following reagents were added in rapid succession: 10 μl chloramine-T (CT, 5 mg/ml in 0·05 M-sodium phosphate buffer, pH 7·5), 100 μl sodium metabisulphite (1·2 mg/ml in 0·05 M-sodium phosphate buffer, pH 7·5) and 850 μl sodium iodide solution [2 mg/ml in sodium phosphate buffer containing 2% (v/v) horse serum].

Bolton and Hunter method. Iodination was also performed as described by Bolton & Hunter (1973). Bolton and Hunter (BH) reagent [N-succinimidyl 3-(4-hydroxy, 5-125I-iodophenyl) propionate, sp. act. 1600 Ci/mmol] was obtained from The Radiochemical Centre, Amersham. After dialysis against 0·1 M-borate buffer, pH 8·5, 150 μg of HA was added to the dry BH reagent (1 mCi) and after 15 min gentle mixing the reaction was stopped by addition of 500 μl of 0·2 M-glycine solution on 0·1 M-borate buffer, pH 8·5, followed by 250 μl of 0·05 M-sodium phosphate buffer, pH 7·5, containing 0·25% (w/v) gelatin as a carrier protein.

Purification of labelled HA. 125I-HA was separated from small mol. wt. radio-iodinated products by gel filtration on G25 Sephadex. A representative analysis of fractions eluted from gel filtration of BH-labelled X49 HA is shown in Fig. 1. Fractions were analysed for HA antigen content by the SRD technique (Wood et al. 1977) followed by autoradiography. A peak of radioactivity corresponding to HA antigen appeared in the void volume (fractions 2 to 8) and two further peaks of radioactivity (fractions 21 and 33) corresponded to unreacted BH reagent. Similar purification procedures have been carried out with CT-labelled X49 HA. Fractions containing HA antigen were pooled for further examination. The specific radioactivity of labelled X49 HA for a number of preparations has been in the range 2·4 to 24·6 μCi/μg, the value being dependent upon the quantity of HA protein iodinated.

Zone enhancement radial diffusion (ZE) tests. ZE assays were based on the single-radial-diffusion (SRD) technique described by Wood et al. (1977). For routine ZE assays, the radioactively labelled HA and the unlabelled test virus were antigenically homologous. Agarose gels (1%, Seakem medium EEO Marine Colloids Inc. Rockland, U.S.A.; prepared in phosphate-buffered saline, pH 7·1, containing 0·1% sodium azide) containing antiserum
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Fig. 1. Gel filtration of $^{125}$I-X49 HA. A 10 x 1 cm column was equilibrated with 0.05 M-sodium phosphate buffer, pH 7.5, containing 0.25% (w/v) gelatin, followed by 100 mg/ml of bovine serum albumin. Reaction mixture (0.25 ml) was loaded on to the column and 0.2 ml fractions were collected into polystyrene tubes containing 0.2 ml 0.05 M-sodium phosphate buffer, pH 7.5, with 2% horse serum. ○ --- ○, Radioactivity; Ω --- Ω, SRD HA activity. Inset shows analysis of fractions 1 to 12 for HA activity by SRD autoradiography. HA antigen activity corresponding to a peak of radioactivity was detected at the void volume (fractions 2 to 8); two further peaks of radioactivity occurred at fractions 21 and 33.

to HA were cast on glass plates and 20 μl of serially diluted virus, treated for 30 min at 20°C with detergent [20% (v/v) Mulgofen, Gaf (Great Britain) Ltd. Manchester, England] to release the HA subunits, were added to 4 mm diam. wells in the agarose. $^{125}$I-HA was added either to virus in the wells or to the virus dilutions before addition to the wells. After allowing 18 h for diffusion, the gels were dried and autoradiographs were prepared using Kodirex X-ray film (Kodak Ltd). After exposure of the film for 2 to 10 days, the autoradiographs were developed and zones were measured using a Transidyne calibrating viewer (Transidyne General Corporation, Ann Arbor, U.S.A.) coupled to Autodata digital caliper equipment (Autodata, Hitchin, England).

RESULTS

Establishment of ZE test conditions

The amount of antiserum used in ZE tests was much lower (10- to 30-fold) than that used in conventional SRD tests; consequently, the resulting diffusion zones were no longer visible by conventional staining techniques. However, small amounts of $^{125}$I-HA, added to gels containing low concentrations of antiserum, produced zones which were clearly visible by autoradiography. If unlabelled HA dilutions were allowed to co-migrate with constant
Concentration of unlabelled virus (μg/ml)

10  5  2.5  1:2  0.6  0.3  0

Fig. 2. Autoradiograph illustrating the ZE technique using X49 125I-HA. Concentrations of unlabelled X49 virus (0.3 to 10 μg HA/ml) were added to 1:100, 1:500 and 1:1000 dilutions of X49 125I-HA. The size of the zone produced by X49 125I-HA alone was enhanced by the addition of unlabelled X49 virus. Antiserum concentration (St62) was 0.38 μl per 1 ml gel.

amounts of labelled HA, the autoradiographic zone sizes were enhanced. The degree of enhancement was proportional to the amount of unlabelled HA in the mixture.

To examine the dose response characteristics of the test, 125I-X49 HA was incorporated into ZE assays according to the following procedure. Twenty μl of detergent-treated X49 virus dilutions (0.3 to 10 μg HA/ml) were introduced into wells punched in agarose gels which incorporated 0.38 μl of St62 antiserum per 1 ml gel. This antiserum concentration was 10-fold lower than that used in conventional SRD tests. After allowing time for the wells to empty, 5 μl of a 1:100, 1:500 or a 1:1000 dilution of 125I X49 HA were added to the wells. The resulting diffusion zones were not visible after staining but were clearly seen by means of autoradiography (Fig. 2). It was clear that the size of the zone formed by labelled HA had been enhanced by addition of antigenically homologous virus and that there was a clear dose-response effect; the area of the autoradiographic zone was directly proportional to the concentration of unlabelled antigen.

Antigenic specificity of ZE

Studies were performed to investigate the antigenic specificity of the ZE test using immunoplates containing antiserum to X49 HA and employing 125I-HA from X49 virus. The unlabelled challenge antigens comprised several H3N2 virus strains including A/Hong Kong/1/68 (X31), A/Port Chalmers/1/73 (MRC 11) and A/Victoria/3/75 (X47) together with A/USSR/92/77 (H1N1) and B/Hong Kong/8/73 virus. The test viruses were used over a range of several dilutions corresponding to 1 to 50 μg HA/ml and their activities in
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Fig. 3. Dose–response studies showing antigenic specificity of ZE reactions. Twenty µl of detergent-treated X49 (A/Texas/1/77-like strain, H3N2) (●), A/Victoria/3/75 (H3N2) (▲), A/Port Chalmers/1/73 (H3N2) (■), A/Hong Kong/1/68 (H3N2) (○), A/USSR/92/77 (H1N1) and B/Hong Kong/8/73 (▲) viruses at concentrations of 50 µg HA/ml were added to wells in immunoplates containing antiserum to X49 HA (SI62) at 1/2 µl per 1 ml gel. This was followed by addition of 10 µl of a 1:10 dilution of X49 125I-HA. The degree of autoradiographic zone size enhancement was determined by comparison of test virus zones with zones produced by X49 125I-HA alone (— — —). Most enhancement was produced by the antigenically homologous X49 virus and varying degrees of enhancement, depending on the antigenic similarity to X49 virus, were given by the remaining H3N2 viruses. No enhancement was produced by A/USSR/92/77 virus or B/Hong Kong/8/73 virus.

ZE tests were compared with those of similar concentrations of X49 HA. The amounts of HA antigen in the purified, unlabelled viruses were estimated by biochemical analysis using polyacrylamide gel electrophoresis (Wood et al. 1979). The results of the ZE tests are shown in Fig. 3. It is seen that the degree of zone enhancement was largest for the homologous X49 HA antigen, smaller for the closely related H3N2 virus A/Victoria/3/75 while only minor degrees of enhancement were detected for the more distantly related H3N2 strains A/Port Chalmers/1/73 and A/Hong Kong/1/68. No enhancement was detected for the H1N1 and influenza B viruses. These findings indicated that the ZE reaction is narrowly specific for closely related variants within an HA antigen subtype.

Quantitative aspects

It was important to establish whether ZE techniques could be used for quantitative measurements of influenza HA and to what extent the sensitivity of the technique compared with conventional SRD techniques. Consequently, various low concentrations of X49 virus (0.1 to 0.5 µg HA/ml) and infected allantoic fluids were assayed in ZE tests. The antiserum concentrations used (0.09 µl/ml gel) were approx. 30-fold less than those used in conventional SRD tests. All of the preparations gave visible autoradiograph zones and when these were measured, a graph of zone diameter² against virus dilution was linear for each preparation (Fig. 4). All of the preparations gave responses which were statistically valid by the normal criteria employed for SRD slope ratio assays (Finney, 1952; Wood et al. 1977). Thus it was established that ZE could be used for quantitative measurements of virus concentrations as
Fig. 4. ZE dose response of X49 virus preparations containing 0.5 (●—●), 0.3 (■—■) and 0.1 (□—□) μg HA/ml. Each preparation gave a linear dose response and the three dose response curves met at a statistically significant common intercept. Ten μl of 1:280 X49 125I-HA were added to dilutions of unlabelled X49 virus and 20 μl of the mixture was added to wells in SRD gels containing 0.09 μl S162 antiserum per 1 ml gel.

low as 0.1 μg HA/ml. An infected allantoic fluid with a haemagglutination titre of 1:640 was found to contain 0.4 μg HA/ml.

In comparisons of X49 HA labelled with either CT or BH reagent, no differences could be found in the specificity or quantitative aspects of ZE reactions. Similar ZE data have been obtained using A/USSR/92/77 virus HA iodinated using the BH technique.

With prolonged storage of 125I-HA at 40°C, the loss in radioactivity meant that increasingly large amounts had to be added to ZE immunoplates in order to maintain zone intensity. This procedure was possible over a period of about 100 days storage, after which time the amount of 125I-HA added to the plates became excessive for quantitative tests. However in qualitative tests, 125I-HA stored for 9 months has been used with no detectable changes in antigenic specificity.

The reproducibility of the technique was assessed in replicate tests carried out on each of 3 days using several different X49 and A/USSR/92/77 virus preparations (laboratory purified virus; whole virus, B-propiolactone inactivated; whole virus, formalin inactivated; detergent disrupted, formalin inactivated). The inactivated virus preparations were commercially available inactivated influenza vaccines.

All virus preparations were first assayed by SRD (Wood et al. 1977) where stained zone sizes of virus dilutions were compared with those of a reference virus antigen calibrated in μg of HA. The HA concentration of virus preparations could then be calculated by constructing a graph of zone diameter^2 against virus dilution and using the following equation:

\[
\text{HA concentration of virus (μg/ml)} = \frac{\text{virus dose response slope}}{\text{reference dose response slope}} \times \text{HA concentration of reference (μg/ml)}
\]
Table 1. Reproducibility of ZE technique

<table>
<thead>
<tr>
<th>Virus preparation*</th>
<th>Strain</th>
<th>Dilution</th>
<th>SRD‡</th>
<th>Test 1</th>
<th>Test 2</th>
<th>Test 3</th>
<th>Mean (95% CL)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPL WVV</td>
<td>X49</td>
<td>1:20</td>
<td>1.7</td>
<td>1.89</td>
<td>1.88</td>
<td>1.91</td>
<td>1.89 (1.82-1.97)</td>
</tr>
<tr>
<td>WVC</td>
<td>X49</td>
<td>1:1500</td>
<td>2.2</td>
<td>2.08</td>
<td>1.87</td>
<td>2.01</td>
<td>2.00 (1.92-2.07)</td>
</tr>
<tr>
<td>FORM WVV</td>
<td>X49</td>
<td>1:80</td>
<td>1.7</td>
<td>1.71</td>
<td>1.74</td>
<td>1.76</td>
<td>1.73 (1.66-1.80)</td>
</tr>
<tr>
<td>BPL WVV (i)</td>
<td>A/USSR/92/77</td>
<td>1:20</td>
<td>2.4</td>
<td>2.13</td>
<td>2.16</td>
<td>2.25</td>
<td>2.17 (2.1-2.3)</td>
</tr>
<tr>
<td>BPL WVV (ii)</td>
<td>A/USSR/92/77</td>
<td>1:20</td>
<td>1.8</td>
<td>1.43</td>
<td>1.24</td>
<td>1.45</td>
<td>1.40 (1.3-1.5)</td>
</tr>
<tr>
<td>FORM DVV</td>
<td>A/USSR/92/77</td>
<td>1:20</td>
<td>1.5</td>
<td>1.30</td>
<td>1.34</td>
<td>1.37</td>
<td>1.33 (1.3-1.4)</td>
</tr>
</tbody>
</table>

* Abbreviations: BPL WVV, β-propiolactone-inactivated, whole virus vaccine; FORM WVV, formalin-inactivated, whole virus vaccine concentrate; WVC, laboratory-purified, whole virus concentrate; FORM DVV, formalin-inactivated, disrupted virus vaccine; BPL WVV (i) & (ii), vaccines from different manufacturers.

‡ HA concentrations calculated using either X49 reference antigen Lot 2 (134 μg HA/ml), supplied by the Bureau of Biologics, Bethesda, Md., U.S.A., or NIBSC A/USSR/92/77 reference antigen 78/508 (40 μ HA/ml).

† HA concentrations calculated by conventional SRD tests on undiluted virus material.

§ 95% CL, 95% confidence limit.

The virus preparations were diluted for ZE tests so that they contained amounts of HA ranging from 1.3 to 2.2 μg/ml. The results (Table 1) were judged to be homogeneous by χ² analysis demonstrating ZE to be a reproducible technique. The nominal potencies of the diluted virus preparations calculated from SRD data, which are given for comparison in Table 1, show that ZE and SRD tests may be compared quantitatively.

**DISCUSSION**

The conventional SRD test (Schild et al. 1975; Wood et al. 1977), which is based on stained diffusion zones, is routinely used for standardizing the amount of HA in inactivated influenza vaccines. The SRD test, which depends upon the comparative titration of a vaccine and a reference antigen calibrated in μg of HA, can assay HA antigen concentrations as low as 2 to 4 μg/ml. This provides adequate sensitivity for the standardization of HA content in vaccines which conventionally contain 7 to 20 μg HA per dose. However, amounts of HA antigen as low as 3 μg HA per dose may stimulate satisfactory antibody responses in primed individuals (Nicholson et al. 1979) and it is therefore desirable to develop more sensitive assay systems which are capable of accurately quantifying small amounts of HA.

The ZE technique described in this communication provides for the accurate and reliable assay of HA at concentrations down to 0.1 μg/ml and will detect concentrations as low as 0.02 μg/ml. This represents a 20- to 40-fold increase in sensitivity over conventional SRD tests where the zones are revealed by staining. Further increases in the sensitivity of the test are possible. For example, the use of intensifying screens for autoradiography could enable the test to be performed with lower concentrations of ¹²⁵I-HA thus increasing sensitivity and reducing the time needed for autoradiographic exposure. These modifications are under study in our laboratory. The ZE assays we describe here produce data which are statistically valid by accepted criteria (Finnecy, 1952).

The ZE reactions are antigenically strain specific, reacting narrowly within an HA antigen subtype. In contrast, the conventional SRD test is broadly reactive within an HA antigen subtype. The mechanism of the ZE test appears to be based on competition for antibody between the labelled and unlabelled HA antigens and in particular on the availability of
anti-HA antibody in the gel capable of reacting with and arresting the diffusion of the labelled HA antigen. When both the labelled and unlabelled antigens are homologous with the antiseraum they co-migrate in the gel and the size of the antibody-antigen reaction zone, demonstrated by autoradiography, corresponds to the total amount of HA present. In contrast, unlabelled HA antigenically related but not homologous with the labelled HA and the antiseraum, would be expected to react with only a proportion of the anti-HA antibody molecules surrounding the well from which it diffuses. Its addition would thus cause partial reduction in the local concentration of anti-HA molecules capable of reacting with the labelled HA. This would enable the labelled HA to diffuse further from the well before reaching equilibrium with antibody than from the corresponding control wells, to which no unlabelled HA is added. This supposition is consistent with the finding that ZE activity of the closely related A/Victoria/3/75 HA was less than that of the homologous A/Texas/1/77 (X49) HA for a similar quantity of HA antigen and that only a minor degree of ZE activity was detected for the HA antigens of more distantly related viruses A/Port Chalmers/1/73 and A/Hong Kong/1/68 while unrelated HA antigens did not affect the diffusion of the labelled HA. This explanation of the mechanism of the ZE test is supported by our findings (J. M. Wood & G. C. Schild, unpublished data) that if comparisons of the sizes of stained zones and autoradiographic zones are made on the same SRD immunoplate, the stained zones corresponding to unlabelled HA, and autoradiographic zones corresponding to labelled HA, are exactly the same size when the labelled and unlabelled HA are antigenically homologous. However, with heterologous unlabelled HA the sizes of the autoradiographic zones are always smaller than those of the stained zones.

ZE tests appear to be very sensitive methods for poliovirus antigen assay and for the intratypic differentiation of poliovirus strains which cannot readily be distinguished by other techniques such as virus neutralization or immuno-double-diffusion (G. C. Schild, unpublished data). An exact correspondence of the size of autoradiographic and stained SRD zones occurred only when the labelled and unlabelled poliovirus antigens were antigenically identical. For influenza virus the ZE method may also provide a sensitive method for the detection of antigenic variants and its value for this is being investigated in our laboratory.

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