Immunoassay of Poliovirus Antigens by Single-Radial-Diffusion: Development and Characteristics of a Sensitive Autoradiographic Zone Size Enhancement (ZE) Technique

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SUMMARY

The reactions of polioviruses in single-radial-immunodiffusion (SRD) tests were investigated with a view to developing accurate and sensitive antigen assay systems. In direct SRD tests, employing high concentrations of immune poliovirus serum in agarose gels, poliovirus D-antigens produced clear reaction zones demonstrated by protein staining. The reactions were type-specific for polioviruses of types 1, 2 and 3 but the tests were of low sensitivity, being applicable only to the assay of virus concentrates.

A novel autoradiographic zone size enhancement (ZE) test was developed which increased the sensitivity of the SRD assay 40- to 100-fold. The ZE test was dependent upon the ability of unlabelled poliovirus to co-migrate with the radioactive marker virus and so enhance the zone size detected autoradiographically. The areas of the autoradiographic zones were directly proportional to the concentration of unlabelled antigen. The ZE test was capable of detecting poliovirus D antigens in diluted cell culture fluid harvests in amounts corresponding to $10^{3.3}$ to $10^{4.8}$ TCID$_{50}$ of infectious virus.

Studies with poliovirus type 3 strains indicated that the ZE test was narrowly strain-specific for the D-antigen of poliovirus type 3 strains when homologous type 3 D-antigen was used as radioactive marker, but broadly cross-reactive for the D-antigen of type 3 viruses when heterologous poliovirus type 3 D-antigen was used as marker.

INTRODUCTION

The ability to perform precise, sensitive and specific in vitro assays of virus antigens is relevant to the understanding of the immunological characteristics of viruses, and in certain circumstances has practical applications in the standardization of virus vaccines. Single-radial-diffusion (SRD) techniques have been widely applied for antigen assays and have characteristics which enable reproducible and accurate quantification (Rumke & Thung, 1964; Mancini et al. 1965). These methods depend upon the formation of visible zones of immunoprecipitate when antigens diffuse radially from wells into agarose gels in which specific antibody is uniformly distributed. They have been applied to a limited number of virus antigens, including adenovirus hexon (Pereira et al. 1972) and influenza virus haemagglutinin (Schild et al. 1975) and foot-and-mouth disease virus (Cowan & Wagner, 1970).

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Despite the importance of polioviruses in human disease and as antigens for prophylactic use in man, little progress has been made in the development of sensitive in vitro assay systems for poliovirus antigens. The present paper describes studies on the use of conventional (direct) SRD tests for the assay of poliovirus antigens and also describes the development and characteristics of a novel, autoradiographic zone size enhancement (ZE) system for antigen assay which was of greatly increased sensitivity compared to SRD tests employing protein staining.

**METHODS**

*Polioviruses.* Poliovirus type I (Mahoney strain, P1-Mh), poliovirus type 2 (MEF strain, P2-MEF) and poliovirus type 3 [30, 190 and Saukette (P3-30, P3-190 and P3-Sauk)] were ‘wild’ strains from the collection of this laboratory. Other polioviruses employed were the Sabin vaccine strains of poliovirus types 1, 2 and 3 (P1-Sab, P2-Sab, P3-Sab) and poliovirus type 3 strains 115 and 119 (P3-115, P3-119) isolated from recipients of live Sabin vaccine, having antigenic and biochemical characteristics resembling those of the Sabin type 3 vaccine virus (Minor, 1980; A. L. Van Wezel & P. D. Minor, unpublished results).

Poliovirus stocks were prepared by infecting monolayer cultures of HEP2 (Cincinnati) cells at high virus input multiplicities. The cells were maintained at 35 °C in Eagle's basal medium (BME) with lactalbumin hydrolysate (0.5%) and harvested after 24 to 48 h when there was extensive cell destruction.

For some studies, virus was concentrated and partially purified from clarified (centrifuged 1000 g for 30 min) tissue culture fluids by treating the fluids with polyethylene glycol 6000 to precipitate the virus particles (Esposito, 1976). Sodium chloride (final concentration, 0.5 M) and polyethylene glycol (7%, w/v) were added to the clarified tissue culture fluids and the mixtures held at 4 °C for 18 h before centrifuging at 8000 g for 60 min. The resultant virus-rich precipitates were resuspended in phosphate-buffered saline (PBS) pH 6.4, to 1 to 2% of the original volume, homogenized and finally clarified by further low speed centrifugation. The infectivity titres of the final concentrates ranged from $10^{10}$ to $10^{11.5}$ TCID$_{50}$/ml.

*Virus infectivity and infectivity neutralization (VN) tests.* These were performed on HEP2 cell cultures using microtiter techniques as described by Dömök & Magrath (1979).

*Preparation of purified 35S-labelled poliovirus.* 35S-labelled poliovirus antigens were prepared as described by Minor et al. (1980). Radioactive D- and C-antigens (Mayer et al. 1957; Roizman et al. 1957, 1958), harvested as the 155S and 80S radioactive peaks from sucrose density gradients and shown to be antigenically pure by autoradiographic SRD tests with anti-D and anti-C sera (Minor et al. 1980), were used as markers in the ZE tests.

*Preparation of immune sera*

Antiserum to unfractionated poliovirus antigen (anti-V sera). Rabbits or guinea-pigs were immunized with 0.2 ml vol. of concentrated poliovirus antigens. Animals were given two or three intramuscular injections of antigen at intervals of 2 weeks. Sera were collected 7 days after the last injection. Antisera selected for use in the studies possessed virus neutralization (VN) titres of 1:5000 against the homologous virus and gave a single well-defined precipitin line when tested against concentrated virus in double-immunodiffusion (DID) tests (Van Wezel & Hazendonk, 1979).

Antiserum to purified poliovirus D- and C-antigens. The preparation and properties of these specific anti-D and anti-C sera for poliovirus type 3 antigens are described elsewhere (Minor et al. 1980). Antisera to D-antigens were prepared in guinea-pigs and antiserum to C-antigens were prepared in rabbits.

*Direct single-radial-diffusion (SRD) tests.* SRD tests were carried out as described for influenza virus haemagglutinin by Wood et al. (1977) except that Sarcosyl detergent was...
Sensitive assay for poliovirus antigen

added to the agarose gels in order to facilitate uniform diffusion of poliovirus antigens. Briefly, immunoplates were prepared using agarose gels (1% Seakem, medium EEO, Marine Colloids, Rockland, U.S.A.: prepared in PBS pH 7.1, containing 0.01% sodium azide and 0.025% sodium sarcosyl, NL 97) to which was added immune poliovirus serum. The poliovirus antigens for assay were added to 4 mm diam. wells in the immunoplates. After a diffusion period of 2 to 7 days, the immunoplates were washed, dried and stained with Coomassie brilliant blue G-250 (Serva Feinbiochemica, Heidelberg, Germany), and diffusion zones were measured using a Transidyne Calibrating Viewer (Transidyne General Corp., Ann Arbor, U.S.A.) coupled to Autodata digital caliper equipment (Autodata, Hitchin, U.K.).

Autoradiography of immunoplates. Immunoplates for autoradiography were washed in PBS for 24 h to remove free 35S-labelled antigen, pressed, dried (Wood et al. 1977, 1980) and contact exposures prepared on X-ray film (Fuji Photo Film Co., Japan) using exposure times of 2 to 10 days. The diameters of the autoradiographic zones indicating limits of diffusion of the 35S-labelled antigens were measured as described above for stained SRD zones.

Zone size enhancement (ZE) assays. Immunoplates for ZE tests were prepared as for direct SRD tests (above) but with lower concentrations of immune poliovirus sera (anti-V, anti-D or anti-C; final serum dilution in gel 10^-4 to 10^-6). Twenty µl vol. of appropriate dilutions of unlabelled poliovirus antigen in PBS were added to each well, followed 30 to 60 min later by 10 µl of a standard dilution of 35S-labelled poliovirus D- or C-antigen as marker. Standard 'challenge' dilutions of the labelled antigens were selected which, in the absence of added unlabelled antigen, produced small (5 mm diam.) autoradiographic zones. Such dilutions had radioactive counts of 200 to 400 ct/min per 10 µl vol. The plates were left at room temperature for 2 to 3 days to allow for diffusion of the antigens and the antibody-antigen reaction zones detected autoradiographically. Zones surrounding wells containing unlabelled assay antigen and marker, which were 0.5 mm greater in diam. than the zones surrounding control wells containing radioactive marker and PBS only, were considered to be enhanced.

RESULTS

Direct SRD assays

The sensitivity and specificity of direct SRD tests for the detection of poliovirus antigens were examined using Coomassie blue staining to demonstrate the zones of antibody-antigen reaction. Potent rabbit antisera prepared against concentrated, unfraccionated poliovirus antigens (anti-V sera) for polioviruses of types 1, 2 and 3 or guinea-pig antisera to purified D-antigen of poliovirus type 3 (anti-D sera) were incorporated into agarose gels at a range of dilutions from 10^-2 to 10^-4 and poliovirus concentrates added to wells in the immunoplates.

The plates were examined at intervals over a 7 day period unstained, by dark ground illumination and after staining. Table 1 shows the minimum concentration in agarose gel of each of the sera required for the production of well-defined, stained zones in SRD tests with homologous antigen and the homologous VN titres of the sera. The minimal concentrations of immune rabbit sera required for SRD tests were between 20- and 50-fold higher than the homologous VN end-point titres. A guinea-pig anti-D serum (Table 1) with potent VN activity was relatively poor in SRD tests. The most potent serum with a VN titre of 1:100000 gave clear SRD reactions when used at a concentration of 0.5 to 0.7 µl serum/ml gel. In tests with limiting concentrations of antisera the SRD zones were not visible before staining. However, well-defined opalescent zones were seen with homologous antigens on unstained immunoplates when concentrations of sera two- to fourfold higher than these minimal
Fig. 1. Specificity of direct SRD reactions with poliovirus antigens. Immune rabbit sera (anti-V) to poliovirus type 1 (Mahoney and Sabin), type 2 (MEF) and type 3 (Sabin) were incorporated into agarose gels at concentrations indicated in Table 1. Concentrates of poliovirus antigens of type 1 (Mahoney and Sabin), type 2 (MEF and Sabin) and type 3 (30 and Sabin) were added to wells. The reaction zones were stained with Coomassie blue. Zones were produced only by homotypic antigens and their intensity was greater for homologous strains than for heterologous strains.

Table 1. Relationship between virus neutralization titre and minimal concentration of antiserum required for SRD tests

<table>
<thead>
<tr>
<th>Immune sera</th>
<th>Virus neutralization titre*</th>
<th>Minimal dilution of serum required for SRD tests†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-V sera (rabbit)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1-Sabin</td>
<td>3000</td>
<td>1:150</td>
</tr>
<tr>
<td>P1-Mahoney</td>
<td>18,000</td>
<td>1:800</td>
</tr>
<tr>
<td>P2-Sabin</td>
<td>32,000</td>
<td>1:800</td>
</tr>
<tr>
<td>P2-MEF</td>
<td>2000</td>
<td>1:70</td>
</tr>
<tr>
<td>P3-Sabin</td>
<td>2000</td>
<td>1:90</td>
</tr>
<tr>
<td>P3-115</td>
<td>100,000</td>
<td>1:2000</td>
</tr>
<tr>
<td>P3-30</td>
<td>28,000</td>
<td>1:750</td>
</tr>
<tr>
<td>P3-Saukette</td>
<td>6,000</td>
<td>1:300</td>
</tr>
<tr>
<td>P3-119</td>
<td>17,000</td>
<td>1:500</td>
</tr>
<tr>
<td>P3-190</td>
<td>8,500</td>
<td>1:500</td>
</tr>
<tr>
<td>P3-30</td>
<td>5,000</td>
<td>1:200</td>
</tr>
<tr>
<td>Anti-D serum (guinea-pig)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P3-30</td>
<td>&gt;30,000</td>
<td>1:200</td>
</tr>
</tbody>
</table>

* Neutralization titre in microtitration assays with homologous poliovirus strain.
† Minimal final dilution of antiserum in gel required to give clearly visible stained zones in SRD tests with homologous or closely related poliovirus antigen.
Fig. 2. Direct SRD assay of poliovirus type 3, strain 115, concentrates. Twenty μl vol. of dilutions of two preparations (1 and 2) of virus were added to 4 mm diam. wells in immunoplates containing 0.7 μl/ml of rabbit antiserum (anti-V) to strain 115. After allowing 5 days for diffusion, the reaction zones were clearly visible after staining with Coomassie blue.

Fig. 3. SRD dose–response curves for poliovirus type 3, strain 115, virus concentrates tested as indicated for Fig. 2.

concentrations were used. The zones produced by the most potent antigens with infectivity titres 10^{11.5} TCID_{50}/ml reached a maximum diam. of approx. 10 mm after allowing 4 to 5 days for diffusion.

Studies on the specificity of the SRD reactions with anti-V sera (Fig. 1) and anti-D sera (not shown) indicated they were poliovirus type-specific; no intertypic reactions were observed. Within a poliovirus type the intensities of the stained zones were greater for homologous than for heterologous antigens, and for heterologous antigens multiple reaction rings were sometimes apparent (Fig. 1 and 5).

Studies of the dose–response characteristics of direct SRD assays were performed with several preparations of antisera and homotypic antigens. A representative assay is illustrated in Fig. 2. With decreasing concentrations of antigen, reduction in zone size but not intensity was observed. Linear dose–response curves (Fig. 3) were obtained when antigen dilution was plotted against the square of zone diameter (d^2). Curves for different preparations of an antigen tested on the same immunoplate converged to a common intercept on the d^2 axis as has been observed for similar assays of influenza haemagglutinin (Wood et al. 1977).
Studies were performed to determine the nature of the poliovirus antigen reacting in SRD tests. Fractions from sucrose density gradients on which harvests of $^{35}$S-labelled poliovirus P3-3O had been centrifuged (Minor et al. 1980) were tested on SRD immunoplates containing homologous anti-V serum. The immunoplates were examined after staining for reaction zones. Radioactive counts and virus infectivity assays were also performed on the gradient fractions. A peak of SRD activity was detected which coincided with the 155S radioactive peak and with the peak of infective virus (Fig. 4). No SRD activity corresponding with the 80S radioactive peak was detected. The 155S and 80S components were further characterized by analysis of their polypeptide compositions by polyacrylamide gel electrophoresis (PAGE) (Fig. 4). The patterns of polypeptides detected were characteristic of D- and C-antigen respectively (Minor et al. 1980). It was concluded that the SRD zones detected on immunoplates containing anti-V or anti-D sera were produced by antigens associated with intact infectious virus, i.e. D-antigen (Mayer et al. 1957; Roizman et al. 1957). This
was supported by the observation (data not shown) that antigens of all three types of poliovirus lost all activity in SRD tests with anti-V or anti-D sera after heating at 56 °C for 45 min, a procedure known to convert D-antigen to C-antigen (Le Bouvier, 1955; Mayer et al. 1957).

**Autoradiographic SRD tests: effect of addition of unlabelled poliovirus antigen on autoradiographic zones produced by 35S-labelled antigen**

As reported elsewhere (Minor et al. 1980) SRD reaction zones could be demonstrated by autoradiography when 35S-labelled poliovirus was added to immunoplates containing specific antisera. Studies were performed to determine the effect on autoradiographic SRD zones when the labelled antigen was added to wells which contained unlabelled homotypic poliovirus antigens. The mixtures were tested in immunoplates containing concentrations of serum suitable for use in direct SRD tests so that stained and autoradiographic zones could be detected for the same immunoplate.

The results of a study with several poliovirus type 3 antigens are illustrated in Fig. 5. The immunoplates contained antiserum to poliovirus P3-115 (Sabin vaccine-like). Unlabelled concentrates of 'wild' polioviruses P3-30 and P3-190, and vaccine-like strains P3-Sabin and P3-115, were added to wells in the immunoplates followed by 35S-labelled P3-Sabin D-antigen (homologous marker system) or 35S-labelled P3-30 D-antigen (heterologous marker system). Comparisons were made of the size of the stained zones with the autoradiographic zones detected on autoradiographs of the same plates. Identity of the stained and autoradiographic zones indicated co-migration of the unlabelled and labelled antigen, i.e. that the unlabelled antigen had enhanced the size of the radioactive zone produced by the labelled antigen (a positive ZE response). Where the size of an autoradiographic zone surrounding a well which contained both reagents was the same as that produced by labelled virus alone, this indicated that co-migration of the two antigens did not occur, i.e. the unlabelled antigen had produced no ZE response. For the homologous marker system, only the unlabelled viruses antigenically homologous with the serum and marker virus (P3-Sabin and P3-115) produced ZE activity whilst P3-30 and P3-190 produced no ZE activity. In contrast, in the heterologous system employing 35S-labelled P3-30 D-antigen, each of the unlabelled viruses produced a clear ZE response. In studies with the same unlabelled antigens and markers, but employing immunoplates containing anti-V serum for P3-30, the converse pattern of reactions was observed. For the homologous (P3-30 D-antigen) marker system P3-30 and P3-190 antigens gave ZE responses, indicating their antigenic similarity, but P3-Sabin and P3-115 did not produce ZE activity. It was concluded from these investigations with other antisera and a larger collection of poliovirus 3 strains (G. C. Schild, unpublished results) that ZE reactions with polioviruses were narrowly strain-specific when a homologous 35S-labelled D-antigen marker was used and broadly reactive within a subtype when heterologous D-antigen marker was used.

**Dose–response characteristics and sensitivity of ZE assays**

It was established from studies with poliovirus type 3 antigens that maximal sensitivity for ZE assays, consistent with suitable dose–response characteristics, was achieved using antisera concentrations approx. 100-fold lower than the minimal concentrations necessary for direct SRD assays (Table 1) and using concentrations of 35S-labelled, purified D-antigen marker which contained 200 to 400 ct/min per 10 μl. Fig. 6 illustrates ZE assays with serial dilutions of several poliovirus type 3 antigens (cell culture fluid harvests and high dilutions, from 10^{-2}, of virus concentrates) performed on immunoplates containing anti-V serum to
P3-115, and using a heterologous marker system, 35S-labelled P3-3o D-antigen. All poliovirus type 3 antigens tested, including P3-Sauk (data not shown), produced ZE responses whilst poliovirus type 1 and type 2 antigens gave no ZE response. The most potent cell culture fluids could be diluted 1:64 before extinction of ZE activity was reached whilst the most potent poliovirus type 3 concentrate produced ZE activity at a dilution of 1:3200.

Fig. 5. Reactions of poliovirus type 3 antigens employing homologous and heterologous 35S-labelled D-antigen marker systems showing intratypic differentiation of virus strains. Unlabelled concentrates of poliovirus type 3; Sabin vaccine-like strains (Sabin and 115) and ‘wild’ strains (30 and 190) were added to wells in immunoplates containing rabbit antiserum (anti-V) to strain 115 (0.7 µl serum/ml gel). 35S-labelled D-antigen (Sabin or 30) was added to each well and the plates left for 2 days to allow antigen diffusion before Coomassie blue staining and autoradiography. The reactions demonstrated by staining and autoradiography of the same plates are shown. All virus concentrates produced clear SRD zones with zone intensity greatest for homologous virus strains. Only virus strains which were homologous (Sabin, 115) with the antiserum and marker showed ZE in the homologous system (Sabin marker) whereas all four strains showed ZE in the heterologous system (30 marker). The marker viruses above were in too low concentrations to produce stained zones. The tests indicate the existence of antigenic differences between the D-antigens of the vaccine and ‘wild’ strains.
Sensitive assay for poliovirus antigen

Antigen dilution

Fig. 6. Dose–response studies of ZE assays of poliovirus type 3 antigens. Twenty µl vol. of serially diluted preparations of poliovirus P3-30 cell culture fluid (P3-30tc, ○—○); strain 30 concentrate, diluted $10^{-2}$ (P3-30 concn. $10^{-2}$, ●—●); $10^{-2}$ dilutions of two strain 115 concentrates [P3-115 (1) concn. $10^{-8}$, □—□; P3-115 (2) concn. $10^{-8}$, ■—■] and poliovirus strain Mahoney concentrate diluted $10^{-8}$ (P1-Mh concn. $10^{-8}$, ---) were added to wells in immunoplates containing rabbit antiserum (anti-V) to poliovirus P3-115 (0.01 µl/ml gel). This was followed by addition of 10 µl of 1:50 dilution of $^{35}$S-labelled strain 30 D-antigen marker. The autoradiographic ZE responses (a) and the resulting dose–response graph (b) are illustrated. Each type 3 preparation gave a linear dose–response with all dose–responses meeting at a common intercept on the d² axis. Type 1 virus showed no ZE response.

The diameters of the autoradiographic zones were measured and graphs plotted of $d^2$ against antigen dilution (Fig. 6b). Each poliovirus type 3 antigen showed a linear dose–response and the response lines for different antigen preparations converged to a common intercept on the $d^2$ ordinate. Thus ZE potency assays could be analysed in a similar manner to direct SRD assays for which the slopes of the dose–response curves are proportional to the antigen concentration (Schild et al. 1975; Wood et al. 1977). For similar ZE assays but using homologous D-antigen as marker (P3-115), only Sabin vaccine-like strains gave ZE responses.

The relative sensitivities of direct SRD and ZE assays for several poliovirus type 3 preparations are shown in Table 2. The viruses were titrated for infectivity and were assayed for antigen by direct SRD and ZE tests in comparison with a commercial poliovirus type 3
Table 2. Relative sensitivities of direct SRD and ZE assays for poliovirus type 3 antigens

<table>
<thead>
<tr>
<th>Antigen preparation</th>
<th>Infectivity titre (log TCID&lt;sub&gt;50&lt;/sub&gt;/ml)</th>
<th>Assay system employed</th>
<th>Antiserum concentration used in assay*</th>
<th>Limiting dilution at which significant antigenic activity detected†</th>
<th>Antigen concn. in D units at limiting dilution‡</th>
<th>Infectivity titre at limiting dilution (log&lt;sub&gt;10&lt;/sub&gt; TCID&lt;sub&gt;50&lt;/sub&gt;/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-antigen reference</td>
<td>Inactivated virus</td>
<td>Direct SRD</td>
<td>5 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>1:4</td>
<td>100</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ZE</td>
<td>3 × 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>1:160</td>
<td>2.5</td>
<td>–</td>
</tr>
<tr>
<td>Virus concentrate</td>
<td>11.0</td>
<td>Direct SRD</td>
<td>5 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>1:64</td>
<td>140</td>
<td>9.2</td>
</tr>
<tr>
<td>115 strain</td>
<td></td>
<td>ZE</td>
<td>3 × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>1:6400</td>
<td>3.5</td>
<td>7.2</td>
</tr>
<tr>
<td>Cell culture fluid</td>
<td>9.5</td>
<td>Direct SRD</td>
<td>5 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>&lt; 1:1§</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>30 strain</td>
<td></td>
<td>ZE</td>
<td>3 × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>1:256</td>
<td>3.5</td>
<td>7.1</td>
</tr>
</tbody>
</table>

* Rabbit serum (anti-V) to poliovirus P3-115 (Sabin vaccine-like strain), homologous VN titre of 1:80000.
† Highest antigen dilution showing significant activity in direct SRD (stained zones) or ZE (autoradiographic) tests.
‡ D-antigen units determined by comparison with poliovirus type 3 (Saukette) reference antigen, with an assigned potency of 400 D-antigen units/ml (Glaxo Laboratories, Greenford, U.K.).
§ < 1:1 no detectable activity with undiluted antigen.
Sensitive assay for poliovirus antigen

Fig. 7. An unlabelled harvest of HEP2 cells infected with poliovirus type 3, strain 30 was centrifuged in a 15 to 45% sucrose density gradient (35000 rev/min for 4 h) and 2 ml fractions collected by bottom puncture. The fractions were tested for ZE activity in (a) employing 35S-labelled, strain 30, D-antigen as marker in immunoplates containing homologous immune rabbit serum (anti-V) and (b) employing 35S-labelled, strain 30, C-antigen as marker in immunoplates containing specific anti-C rabbit serum. The figure shows the distribution of ZE activity in the gradient in each of the immunoplates.

reference antigen (arbitrary potency of 400 D-antigen units/ml; Beale & Mason, 1962). For the purposes of these studies a direct SRD or ZE response was judged to be significant when $d^2$ was greater than 2 mm$^2$ above the common intercept on the $d^2$ axis of the dose-response graph.

ZE assays were shown to be 40- to 100-fold more sensitive than direct SRD tests. The most potent cell culture fluid harvests, which had no detectable activity in direct SRD tests, had limiting ZE activity at a 1:64 to 1:128 dilution. The limits of sensitivity in terms of infectious virus, for detection of D-antigen corresponded to approx. $10^9$ TCID$_{50}$/ml for direct SRD assays, approx. $10^7$ TCID$_{50}$/ml ($10^4.3$ TCID$_{50}$/20 µl) for ZE assays.

D- and C-antigen specificity of ZE assays

To determine the nature of the poliovirus antigen reactive in ZE tests, unlabelled harvests of HEP2 cell cultures infected with poliovirus type 3 were centrifuged in sucrose gradients and gradient fractions were examined for ZE activity on immunoplates containing homologous anti-V serum with 35S-labelled, purified D-antigen as marker and in immunoplates containing anti-C serum for poliovirus 3 with 35S-labelled, purified C-antigen as marker. The distribution of ZE activity in the gradient for the ‘D-antigen’ and the ‘C-antigen’ immunoplates is shown in Fig. 7. Only a single peak of ZE activity was detected on each immunoplate. The D-antigen immunoplate detected a band of ZE activity with a sedimentation coefficient of approx. 155S whilst the C-antigen immunoplate detected a band of activity at approx. 80S. These findings provided evidence that the ZE tests with anti-V serum (or anti-D serum, data not shown) using D-antigen as the radioactive marker were D-antigen specific. This finding was supported by the fact that ZE activity in this system was not detected when the assay antigen was heated at 56 °C for 45 min to denature D-antigen. The ‘C-antigen system’ detected C- but not D-antigen as evidenced by the sedimentation characteristics of the peak of ZE activity detected in this system and the finding that the ZE activity was stable on heating at 56 °C for 45 min.
Fig. 8. Relationship between infectivity titre (TCID<sub>50</sub>/ml) and D-antigen activity detected in ZE assays for poliovirus type 1 preparations. Poliovirus type 1 strain 30, concentrate diluted 10<sup>-3</sup> (□), strain Mahoney cell culture fluid (▲), strain Mahoney concentrate (a) diluted 2× 10<sup>-1</sup> (●), concentrate (b) diluted 2× 10<sup>-1</sup> (■), and Sabin strain, cell culture fluid (○) were assayed for ZE activity using rabbit antiserum to poliovirus 1 (Mahoney) and <sup>35</sup>S-labelled type 1, strain 1/67 D-antigen as marker. ZE activity was expressed in D-antigen units by comparison with the ZE activity of type 1 D-antigen reference preparation (880 D-antigen units/ml) supplied by Glaxo Laboratories. Virus infectivity assays were performed in HEP2 cell cultures.

**ZE assays with poliovirus type 1 antigens**

Studies with poliovirus type 1 antigens employing ZE techniques produced similar results in terms of antigenic specificity and dose-response characteristics, to those described for type 3 viruses. Several preparations of different type 1 virus strains of known infectivity were examined for ZE antigen activity which was measured in relation to a poliovirus type 1 D-antigen reference preparation (arbitrary potency of 880 'D' units/ml). The results indicate a good correlation between virus infectivity (log TCID<sub>50</sub>) and ZE activity (log D units/ml) (Fig. 8). Virus preparations with infectivity titres of 10<sup>-9</sup> TCID<sub>50</sub>/ml (approx. 10<sup>3.3</sup> TCID<sub>50</sub>/20 μl) and above showed good ZE responses, whereas preparations with titres (TCID<sub>50</sub> < 10<sup>-6</sup>) gave minimal ZE activity. The extinction end-point for poliovirus type 1 D-antigen was approx. 10<sup>6</sup> TCID<sub>50</sub>/ml (10<sup>3.8</sup> TCID<sub>50</sub>/20 μl) and corresponded to about 5 D-antigen units.

**DISCUSSION**

A disadvantage of SRD assays for virus antigens, which are often present in low concentration in biological materials, is their relative insensitivity when reaction zones are detected by protein staining. For influenza haemagglutinin, the limit of sensitivity is 1 μg antigen/ml (Wood et al. 1977). The studies described here indicated that intact poliovirus particles diffused well in agarose gels and gave well-defined SRD reactions which were type-specific and had satisfactory dose-response characteristics. The tests were, however, of limited use being insufficiently sensitive to detect antigen in non-concentrated virus preparations. In the studies using immunoplates containing immune (anti-V) sera to unfractionated poliovirus antigen or with specific anti-D sera, it was shown that the SRD reactions corresponded to D-antigen. Direct SRD studies using potent specific anti-C sera (G. C. Schild, unpublished observations) have shown that only when very high concentrations of anti-C antibody are used in immunoplates are stainable zones corresponding to C-antigen detectable. The lack of such reactions with anti-V sera may thus be related to their
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relatively low content of antibody to C antigen (G. C. Schild, unpublished results). Sucrose density gradient analysis reported here and in the accompanying paper (Minor et al. 1980) failed to demonstrate antigenic activity (SRD or ZE) in the slow sedimenting region of the gradient. This is in contrast with previous reports of an antigenic 14S component in poliovirus preparations (Ghendon & Yakobson, 1971).

The ZE assay system for poliovirus antigens described here appears to offer considerable potential for the sensitive quantification of poliovirus D antigens, applicable to concentrations of antigen present in conventional cell culture fluids and inactivated poliovirus vaccines. It is also of potential value as a means of fine antigenic characterization of polioviruses. The narrow strain specificity of the homologous ZE system for poliovirus D antigens demonstrated in the present studies is in contrast to the broad type specificity exhibited by direct SRD tests with poliovirus D-antigens and the broad subtype specificity exhibited by such tests for influenza haemagglutinin antigens.

The ZE assay depends essentially on competition for antibody in the agarose gel between the unlabelled and radioactively labelled poliovirus antigen and upon the availability in the gel of antibody molecules capable of binding with and halting the diffusion of the labelled antigen. When it is antigenically homologous with the antiserum and the labelled antigen (homologous marker system) the unlabelled antigen is capable of reacting with antibody molecules in the gel which are specific for the labelled antigen thus effectively reducing their concentration. Under these conditions it is shown that the labelled and unlabelled antigen co-migrate in the gel since the autoradiographic and stained SRD zones are identical in size (Fig. 5). The degree of enhancement of autoradiographic zone size, compared to zones surrounding wells containing labelled antigen only, is proportional to the amount of unlabelled antigen.

When the labelled virus and antiserum are antigenically homologous and the unlabelled (homotypic) virus is antigenically heterologous, the unlabelled virus would be expected to react with only a proportion of antibody molecules in the gel, leaving other strain-specific molecules to react with the labelled antigen so arresting its diffusion. This is consistent with the finding that in the homologous marker system only homologous unlabelled antigens give ZE responses.

For ZE systems in which the labelled virus and antiserum are antigenically heterologous all homotypic poliovirus D-antigens produce ZE responses. An explanation for this finding is that in the heterologous system only antibody molecules of cross-reactive specificity within a poliovirus type are involved. Such cross-reactive antibody is depleted by binding with all poliovirus D-antigens within a type. This suggested mechanism for the ZE test is supported by studies described in the present paper and by studies of influenza haemagglutinin using similar ZE techniques (Wood et al. 1980).

An interesting feature of the ZE assay for poliovirus antigens is that, under appropriate conditions, it may be adapted for the detection of either the D- or the C-antigens of polioviruses. However, detailed information on the sensitivity or strain specificity of the ZE assay for poliovirus C antigen is not yet available.

An important potential application of ZE assays is the standardization of potency of inactivated polio vaccines for use in man. Immunological potency of polio vaccines is related to D-antigen content which has been evaluated for vaccine concentrates by quantitative immunodiffusion techniques (Le Bouvier, 1959, Beale & Mason, 1962). However, the lack of sensitivity of these methods has precluded their use for final vaccines which are routinely evaluated by assessment of antibody responses in experimental animals (Gard et al. 1956). Studies are in progress in our laboratory to evaluate the use of ZE assays for poliovaccine standardization and satisfactory assays have been performed on D-antigens present in conventional formalin-inactivated vaccines.
A special feature of the poliovirus ZE system is its high degree of specificity which is largely independent of the properties of the antibody employed but directed by the marker antigen. This is not the case for other antigen assay systems such as SRD, radioimmunoassay and enzyme-linked immunosorbence where the assay is dependent upon the specificity of the antibody employed and which generally shows broad antigenic reactivity.

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REFERENCES


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