The Preparation of Specific Immune Sera against Type 3 Poliovirus D-Antigen and C-Antigen and the Demonstration of Two C-Antigenic Components in Vaccine Strain Populations

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SUMMARY

Animals were immunized with purified D-antigen or C-antigen of type 3 poliovirus to produce specific antisera which were used to analyse the antigenic characteristics of the progeny virus in harvests from poliovirus type 3-infected cells.

An examination of the virus progeny present at 24 h p.i. of cells with Sabin type 3 vaccine strain virus revealed a large population of particles sedimenting at a slightly lower rate (130S) than infectious virus (155S) in addition to slowly sedimenting (80S) empty capsids. Such 130S particles were not detected in the progeny from cells infected with strains genetically unrelated to the Sabin vaccine strains. They were non-infectious, contained RNA in an RNase-resistant form unless heated, and lacked the virion protein VP4. They expressed C-antigen rather than the D-antigen of infectious virus, and, therefore, had the properties previously described for poliovirus particles eluted from cells. The amount of incorporation of radio-isotope into the proteins or nucleic acids of such particles varied from 15 to 20% to 30% of the amount incorporated into infectious virus depending on the cells and virus strains studied. Virus strains genetically related to Sabin type 3 vaccine virus which were isolated from cases of paralytic poliomyelitis produced the particles in either low or undetectable quantities.

INTRODUCTION

Poliovirus particles exist in two antigenically distinct forms, the D-antigen and the C-antigen (Mayer et al. 1957). D-antigen is associated with infectious virus whilst C-antigen is associated with empty capsids and infectious virus obtained after exposure to heat or u.v. radiation (Le Bouvier, 1955). Attempts to raise specific immune sera directed against only one of these native antigens have not been successful (Hummeler & Tumilowicz, 1960). Specific anti-D and anti-C sera would be of value in studies of the composition and antigenic properties of poliovirus preparations. The present paper describes the production of antisera to the purified D- and C-antigens of poliovirus type 3 and the evaluation of their antigenic specificities by single-radial-diffusion (SRD) techniques (Schild et al. 1980). Since radioactively labelled, purified poliovirus particles are employed in the autoradiographic zone size enhancement (ZE) techniques for identification and assay of poliovirus antigens described in the accompanying paper (Schild et al. 1980), it was important to examine these preparations to establish their homogeneity.

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The present paper also describes studies of the antigenic and biochemical characteristics of virus particles present in preparations of 'wild' and vaccine strains of poliovirus type 3. It has sometimes been found that high concentrations of vaccine virus given intraspinally to monkeys as part of the neurovirulence test of live poliovirus vaccines give fewer histological lesions than lower concentrations (P. Reeve, personal communication). This raises the possibility that the vaccine preparations contain defective interfering particles. Such particles have been reported in attenuated poliovirus vaccines (McClaren & Holland, 1974), although this finding later proved unreproducible (McClure et al. 1980). We have examined populations of virus generated by type 3 Sabin vaccine strains and 'wild' strains for defective interfering particles as part of a study of the types of particle found in harvests of infected cells and their relevance to the characterization of poliovirus vaccines.

Methods

Growth of virus and cells. HEP2c cells were grown in Eagle's minimal essential medium (MEM) supplemented with 5% foetal calf serum (Flow Laboratories, Glasgow) and sodium bicarbonate (0.44 g/l). Human diploid cells (WI38 strain) were grown in the same medium and used at a passage level less than 30. Virus pools were grown in HEP2c cells and assayed for infectivity as described (Minor, 1980). All contained in excess of $10^8$ tissue culture infectious doses 50% (TCID$_{50}$) per ml.

The poliovirus type 3 strains used had been previously characterized biochemically (Minor, 1980; P. D. Minor, unpublished results) and were: a Sabin vaccine seed strain 4a (Minor, 1980; Boulger et al. 1979); Leon, the progenitor strain for the type 3 Sabin strains (Sabin & Boulger, 1973); 553, a clone of the Sabin vaccine strain which produces fewer histological lesions in the monkey neurovirulence test than standard vaccine strains and is, therefore, thought to be more highly attenuated; and 119, a strain isolated from a fatal human case of poliomyelitis temporally associated with the administration of live type 3 poliovirus vaccine and biochemically very similar to the vaccine. Virus P3-30 was used as an example of a virulent strain genetically unrelated to the Sabin vaccine strain. Immunization of animals with purified antigen was with the non-Sabin virulent strains P3-30 or P3-694.

Growth of labelled virus. HEP2c cells were grown at 35 °C in test tubes held in roller drums adjusted to roll the tubes horizontally. Cells were seeded at $10^7$ TCID$_{50}$ of virus in 0.3 ml, and rolled for 1 h to allow virus to adsorb to the cells before adding 1 ml of the appropriate medium. WI38 cells were infected on 250 cm$^2$ plastic flasks (Flow Laboratories). For amino acid labels ($^{35}$S-methionine, 850 Ci/mmol; $^3$H-lysine/arginine, 50 Ci/mmol; The Radiochemical Centre, Amersham) the medium added consisted of Gey's buffer supplemented with Eagle's basal medium amino acids lacking either methionine or lysine and arginine. Radioactive amino acids (25 llCi/tube, $^{35}$S-methionine; 100 llCi/tube, $^3$H-lysine/arginine) were added 6 h p.i.

For nucleic acid label ($^3$H-uridine, 25 Ci/mmol; The Radiochemical Centre, Amersham) the medium was Eagle's MEM containing 0.6 $\mu$g/ml actinomycin D but no calf serum. Isotope was added 2 h after infection. Infection was allowed to proceed for 24 h at 35 °C, after which the tubes were frozen and thawed once and cell debris removed by centrifugation at 1000 g for 5 min.

Gradient analysis of labelled virus harvests. Nonidet detergent (NP40, BDH, Poole, Dorset) was added to the supernatant harvests (1% final concentration) which was layered on to a 32 ml gradient of 15 to 45% sucrose in 10 mm-tris, 50 mm-NaCl pH 7.4 before centrifugation at 25000 rev/min for 4 h in a Beckman L5-50 uncentrifuge, SW27 head. Gradients were harvested by bottom puncture. In early experiments fractions of 0.5 ml were collected by draining into tubes. In later experiments the gradient was displaced by pumping 0.5 ml amounts of liquid paraffin into the top of the tube using an automatic syringe. Fifty $\mu$l
amounts of the fractions were counted in a Packard scintillation counter in 5 ml of scintillation fluid comprising 30% methanol, 70% toluene (both analytical reagent grade, BDH) containing 0.42 g/l PPO (Packard scintillation grade).

Preparation of specific antisera to poliovirus D- and C-particles. Poliovirus D- and C-antigens were collected as the 155S and 80S radioactive peaks from sucrose density gradients on which 35S-labelled harvests of cells infected with 'wild' poliovirus type 3 strains, P3-30 and P3-694, had been centrifuged. The harvests were examined by electron microscopy and by polyacrylamide gel electrophoresis (PAGE) and were found to have the characteristic morphology and polypeptide composition of intact infectious virus and empty capsids respectively. Rabbits and guinea-pigs were given two or three injections of 0.1 ml of undiluted gradient harvest with Freund's complete adjuvant at intervals of 2 weeks. Sera were collected 7 days after the last injection.

Single radial diffusion (SRD). Twenty μl amounts of fractions from the gradients were analysed in SRD tests employing immunoplates containing immune guinea-pig sera specific for poliovirus type 3 D-antigen and immune rabbit sera specific for C-antigen. The labelled antigens present in the antibody–antigen reaction zones were detected by autoradiography as described elsewhere (Schild et al. 1980).

PAGE and fluorography. Samples were denatured in 1% SDS, 1% 2-mercaptoethanol, 1 M-urea by boiling for 1 min and were resolved on a gel of 15% acrylamide, 0.4% methylenebisacrylamide (BDH) with a stacking gel of 5% acrylamide, 0.133% methylenebisacrylamide using the buffer system of Laemmli (1970). Electrophoresis was at 60 V for 16 h, after which the gel was treated for fluorography as described by Laskey & Mills (1975).

Nuclease treatment and determination of TCA-precipitable material. Fifty μl samples were incubated with 5 μg of pancreatic ribonuclease (Sigma, London) at 37 °C for 30 min. A 20 μl sample was then counted and another 20 μl sample spotted on to a 1 cm square of Whatman 3MM, which was dropped immediately into ice-cold 5% trichloroacetic acid (TCA; BDH). The 5% TCA was decanted and replaced three times, followed by three washes with ethanol and one with ether before the squares were dried and counted in 5 ml of scintillation fluid.

Infectivity assays. Infectivity was determined by plaque formation on confluent monolayers of HEP2c cells in RB6 six-well plates (Linbro). The overlay consisted of 1% Nobel's agar (Difco Laboratories, Detroit, Mich., U.S.A.) in Eagle's MEM supplemented with 4% foetal calf serum. Plates were incubated for 3 to 4 days before staining with naphthalene black.

RESULTS

Specificity of immune sera for poliovirus type 3 D- and C-antigens

Immune sera were characterized by autoradiographic SRD tests (Schild et al. 1980) and by their ability to neutralize virus infectivity in microtitre assays (Dömök & Magrath, 1979). D-antigen-specific antisera had high homologous virus-neutralizing titres (> 1: 10,000) and gave clear autoradiographic SRD reactions with homotypic 35S-labelled poliovirus D-antigen purified by sucrose density-gradient centrifugation (155S component) but produced no SRD reaction with the corresponding C-antigen (80S component). C-antigen-specific antisera lacked virus-neutralizing activity (titre < 1:10) and produced SRD reactions with purified C-antigen, but not with D-antigen. They did, however, react with D-antigen preparations which had been previously heated at 56 °C for 45 min to convert D-antigen to C-antigen (Le Bouvier 1955; Le Bouvier et al. 1957). The heated D-antigens failed to react in immunoplates containing anti-D serum. Additional characterization of the anti-D and anti-C antisera will be described elsewhere (G. C. Schild et al., unpublished results).
Fig. 1. Autoradiographic SRD reactions of $^{35}$S-labelled poliovirus antigens in immunoplates containing antisera to purified poliovirus type 3 antigens. Anti-D: immune guinea-pig serum to the D-antigen of P3-30, 2 μl/ml gel; anti-C: immune rabbit serum to the purified C antigen of P3-694, 5 μl/ml gel; anti-V: immune rabbit serum to unfractionated P3-Mahoney, 2 μl/ml gel. $^{35}$S-labelled purified D- and C-antigens (155S and 80S radioactive peaks) from sucrose density gradients were added to the wells. Poliovirus type 3 antigens: P3-30, D and C; P3-10, D and C; P3-Sabin, D and C. Poliovirus type 1: Mahoney D antigen. The plates were left for 2 days to allow diffusion of the antigen, washed and autoradiographs prepared.

A proportion of the guinea-pigs immunized with strain P3-30 D-antigen yielded sera with specific anti-D activity only. The other guinea-pigs produced antisera with both anti-C and anti-D activity. Rabbits immunized with the same antigen preparation yielded sera with both anti-C and anti-D activity. Rabbits immunized with strain P3-694 C-antigen exhibited specific anti-C activity only. Guinea-pigs immunized with the same preparation gave sera with both anti-D and anti-C activity. The differences in the responses of guinea-pigs and rabbits to immunization with purified type 3 C- and D-antigens are under investigation. The autoradiographic SRD reactions of representative specific anti-D guinea-pig and anti-C rabbit sera are shown in Fig. 1 and compared with the reactions of sera from rabbits immunized with unfractionated poliovirus type 3 (anti-V sera; Schild et al. 1980).

**Particles found in infected cell harvests**

HEP2 cells were infected at high multiplicity (5 to 10 TCID$_{50}$/cell) with strain P3-30 and labelled with $^{35}$S-methionine. A gradient analysis of the harvest 24 h p.i. prepared as described in Methods is shown in Fig. 2. Only two distinct peaks of radioactivity were visible. Other strains unrelated to the Sabin vaccine strain also gave only two peaks (data not shown). In contrast, a gradient analysis of a harvest from cells infected with a Sabin type 3 vaccine seed strain (4a; Minor, 1980) consistently gave three distinct peaks of radioactivity (Fig. 3). The slowest and the fastest moving components corresponded to empty capsids and mature infectious virions with sedimentation coefficients of 80S and 155S (Fernandez-Tomas & Baltimore, 1973) respectively. The third component in the vaccine harvest had a sedimentation coefficient of approx. 130S.
Properties of the 130S component

The character of the poliovirus antigens in the various radioactive peaks was identified using autoradiographic SRD tests with immunoplates containing antisera specific for poliovirus type 3 C-antigen and D-antigen (Schild et al. 1980). The results are shown in the lower panels of Fig. 2 and 3. The fastest sedimenting component contained only D-antigen.
while the slowest component contained only C-antigen. This is consistent with their containing mature infectious virus and empty capsids, respectively (Le Bouvier et al. 1957). The 130S component was only detectable in the vaccine strain harvest and contained only C-antigen.

Harvests from cells infected with the vaccine virus 4a and labelled with 3H-lysine/arginine were analysed on sucrose gradients and samples from the three peaks of radioactivity analysed on a 15% SDS–polyacrylamide gel. A representative fluorogram is shown in Fig. 4. The fastest component contained four proteins (VP1, VP2, VP3 and VP4) and the slower component contained an additional protein (VP0) and lacked VP4. The 130S component contained VP1, VP2 and VP3, but lacked VP4.

VP4 was not labelled with 35S-methionine for any of the type 3 polioviruses examined (P. D. Minor, unpublished results).

Virus 4a was grown in WI38 cells, labelled with 3H-uridine, and the harvests analysed as before (Fig. 5a). 35S-methionine-labelled harvests yielded the three components, as found in HEP2c harvests. Identical results were obtained with another human diploid cell line, MRC5 (data not shown). The relative amount of the 130S component was consistently greater for human diploid than for HEP2c cell harvests. Only two components were labelled with 3H-uridine as the lightest (80S) component consists of empty capsids. The labelled material in both components was precipitable with cold 5% TCA and insensitive to RNase (Fig. 5b).
Fig. 5. Sucrose density-gradient analysis of $^3$H-uridine-labelled harvests from WI38 cells infected with Sabin vaccine seed strain 4a. Sedimentation is from right to left. (a) Radioactivity in 50 μl amounts of fractions. (b) TCA-precipitable material in 20 μl amounts after treatment with RNase without heating (●—●) or after boiling for 10 min (○—○). (c) Infectivity of fractions (p.f.u./ml).

Fig. 6. Sucrose density-gradient analysis of $^{35}$S-methionine-labelled harvests from cells infected with strain (a) P3-553, (b) P3-Leon and (c) P3-119. Radioactivity was assayed in 50 μl amounts. Sedimentation is from right to left.

If the fractions were boiled (100 °C for 10 min), the material in both radioactive peaks became RNase sensitive (Fig. 5b), suggesting that both contain RNA which is normally in a protected form. Infectivity was associated only with the faster moving component (Fig. 5c).
Table I. Comparison of radioactivity in poliovirus 130S particle peak and the infectious virus peak

<table>
<thead>
<tr>
<th>Virus seed</th>
<th>Host cell</th>
<th>Ct/min in 130S peak</th>
<th>Ct/min in infectious virus peak × 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>4a*</td>
<td>WI38</td>
<td>120%</td>
<td></td>
</tr>
<tr>
<td>4at</td>
<td>WI38</td>
<td>104%</td>
<td></td>
</tr>
<tr>
<td>4at</td>
<td>HEP2c</td>
<td>22%</td>
<td></td>
</tr>
<tr>
<td>553t</td>
<td>HEP2c</td>
<td>312%</td>
<td></td>
</tr>
<tr>
<td>Leon†</td>
<td>HEP2c</td>
<td>12.6%</td>
<td></td>
</tr>
<tr>
<td>119†</td>
<td>HEP2c</td>
<td>10%</td>
<td></td>
</tr>
</tbody>
</table>

* 3H-uridine-labelled material.
† 35S-methionine-labelled material (gradient not shown).
‡ Mean of at least two determinations.

No poliovirus-like particles were found on electron microscopic examination of sucrose density-gradient fractions containing the 130S component. In addition, the 130S component did not form a band when recentrifuged on a 0 to 60% (w/w) caesium chloride density gradient, while the 155S infectious virus peak formed a single sharp band. These findings suggested that the 130S component is more fragile than infectious virus. The 130S component did not reduce the yield of infectious virus when added to cell cultures co-infected with purified infectious particles. Therefore, 130S particles did not appear to interfere with infection. Finally, a triply plaque-purified virus from a live vaccine preparation produced 130S particles, which, therefore, cannot form a replicating subpopulation within the population of vaccine viruses.

Factors influencing the yield of 130S particles

Comparisons of the yields of 130S particles for several poliovirus type 3 strains are illustrated in Fig. 6(a to c). In these studies the antigenic activity (C- or D-antigen) associated with the radioactive peaks was tested by SRD. Virus 553 is a clone derived from the Sabin type 3 vaccine and has a lower neurovirulence for monkeys than standard vaccines (P. Reeve, personal communication), Leon is the paralytic strain from which the currently used type 3 vaccine strain was derived by Sabin (Sabin & Boulger, 1973) and 119 is a strain isolated from a fatal paralytic case associated with the use of oral polio vaccine. It is indistinguishable from the vaccine by its oligonucleotide map or the polypeptide pattern produced in infected cells, while being highly neurovirulent in monkeys (Minor, 1980).

The amount of radioactivity in the 130S peak, expressed as a proportion of the amount in the infectious virus peak, is given in Table 1 for these three strains. Comparisons were also made (Table 1) for virus 4a grown in HEP2c cells labelled with 35S-methionine, (Fig. 2), grown in WI38 cells labelled with 3H-uridine (Fig. 5a) and grown in WI38 cells labelled with 35S-methionine (gradient not shown). The virus–cell system used clearly influenced the amount of 130S component produced. Vaccine-related but virulent strains of poliovirus type 3 produced relatively less 130S component than Sabin vaccine strains, while non-vaccine strains produced no detectable 130S material (Fig. 3a).

DISCUSSION

Antisera specifically directed against poliovirus type 3 D-antigen or poliovirus type 3 C-antigen have been prepared by immunizing animals with purified infectious particles or purified empty capsids respectively. The preparation of such specific immune sera using native antigens has not previously been described. Specific anti-C and anti-D sera are of great potential value in the quantification and identification of poliovirus antigens. It was shown that the sera were C- or D-antigen-specific in SRD tests with purified D-antigen
Immune sera to poliovirus D- and C-antigens

(pooled 155S component from a sucrose density gradient) and purified C-antigen (pooled 80S component) and by examination of the antigen present in sucrose density-gradient fractions containing 35S-methionine-labelled harvests of poliovirus type 3. Our findings cannot, however, be interpreted as evidence that the 155S and 80S poliovirus particles have no common antigenic determinants but rather that their major antigenic determinants are distinctive.

For many strains of type 3 poliovirus, such analyses of sucrose density gradients give a single peak of D-antigen activity at 155S and a single peak of C-antigen activity at 80S. This is in agreement with the two peaks of radioactivity found in such gradients and provides confirmation of the specificity of the immune sera for a single component (either 80S C-antigen- or 155S D-antigen-specific) in sucrose density gradients of harvests of cells infected with type 3 poliovirus. However, three peaks of radioactivity have been found in harvests from cells infected with type 3 Sabin vaccine strain poliovirus. They corresponded to mature infectious virus, empty capsids and a component sedimenting at approx. 130S. This third component reacted as C-antigen. It contained RNA which was in an RNase-resistant form unless heated, had the virion proteins VP1, VP2 and VP3 but lacked VP4, the smallest of the four poliovirion proteins. It was not infectious, did not interfere with infection by mature virus and was produced by triply plaque-purified virus. It was, therefore, not a defective interfering particle. No such particles have been detected in these experiments. Collectively, the properties of the 130S particles are those of partially uncoated (Longberg-Holm et al. 1975; De Sena & Mandel, 1976), sloughed (Joklik & Darnell, 1960 or eluted poliovirus (Fenwick & Cooper, 1962). Their presence in virus harvests to the extent found here has not been previously reported, and is of practical significance in both quantitative and qualitative studies of the immunological properties of poliovirus in that for certain type 3 polioviruses D-antigen preparations obtained by sucrose gradient centrifugation may be easily contaminated with C-antigen.

It is not clear how the 130S particles are generated. All cases described here involved high m.o.i. (5 to 10 infectious units/cell) and were, therefore, single-cycle infections. Radioactive virus formed in a first cycle of infection could, therefore, not adsorb to and elute from uninfected cells to give 130S particles. Moreover, the quantity of particles formed depends on the host cell and virus strain studied. For strains genetically related to the Sabin vaccine strain it appeared that viruses which are less neurovirulent in monkeys produced greater quantities of 130S particles. Strains neurovirulent for monkeys and unrelated to the Sabin type 3 vaccine strain, failed to produce any 130S particles. It is possible that the formation of 130S particles under the conditions described here reflect some failure in the virus–host cell interaction which also results in the virus being less virulent in monkeys. Infection of human diploid cells with attenuated virus generates roughly equal quantities of infectious virus and 130S particles. This is of interest in view of the use of such cells in the production of polio vaccines, suggesting that much of the vaccine virus produced may be non-infectious. Since immunity is believed to be induced by the D-antigen, inactivated vaccines based on Sabin type 3 strains grown in human diploid cells could include a high proportion of material of the wrong antigenic specificity.

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REFERENCES


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