Monospecific Antisera Against Capsid Polypeptides of Poliovirus Type 1 Distinguish Antigenic Structures of Poliovirus Proteins

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(Accepted 15 November 1982)

SUMMARY

Pure poliovirus polypeptides, namely VP1, VP2, VP3 and VP4, have been isolated by isoelectric focusing after dissociation of poliovirus by urea. When injected into rabbits, all four polypeptides produced monospecific antisera which were used for the characterization of poliovirus particles and poliovirus-infected cells. The specificity of these antisera was determined by immunoprecipitation of polypeptides obtained by dissociation of poliovirus with SDS, followed by characterization by polyacrylamide gel electrophoresis. The antisera revealed differences in the antigenic sites of native poliovirus particles, heated poliovirus particles and naturally occurring empty capsids. Only VP3 antiserum reacted with native poliovirus and showed some neutralization, whereas all antisera precipitated heated virus and empty capsids. These antisera reacted also with the appropriate precursors of the capsid polypeptides demonstrating their usefulness for an analysis of the cleavage pathway by monospecific antibodies and revealed a second protomer (90 kilodalton) polypeptide for the capsid proteins of poliovirus particles.

INTRODUCTION

Poliovirus polypeptides have been successfully separated by SDS-polyacrylamide gel electrophoresis (SDS–PAGE) (Summers et al., 1965). SDS–PAGE is still the most frequently applied technique for the separation of viral and other proteins (Maizel, 1971). However, the major drawbacks of this method for a preparative use are the almost irreversible denaturation of proteins, the occurrence of SDS–protein complexes and in some cases a low recovery of polypeptides from gels.

Nevertheless, SDS–PAGE has been used for the preparation of individual poliovirus polypeptides for use as immunogens (Meloen et al., 1979; Vrijsen et al., 1980). The antisera obtained were monospecific for the polypeptides VP1, VP2 and VP3 when tested by radioimmunoassay (Meloen et al., 1979) or in an immunoprecipitation test (Vrijsen et al., 1980). However, they failed to react with polypeptide VP4 or intact virus and also failed to neutralize poliovirus infectivity. Antibodies against polypeptide VP1 of foot-and-mouth disease virus (FMDV) prepared in a similar way did have neutralizing activity (Meloen et al., 1979). The observation that antisera against purified preparations of VP1 of FMDV types O and A had neutralizing activity was also reported earlier (Laporte et al., 1973; Bachrach et al., 1975; Kaaden et al., 1977).

It was also reported that antibodies against the α- and β-polypeptides of mengovirus, isolated by hydroxylapatite chromatography in the presence of SDS, reacted with intact virus; however, only the antiserum against the α-polypeptide had neutralizing activity, presumably by blocking viral attachment (Lund et al., 1977).

With coxsackie B3 virus, it was found that purified VP2 was capable of inducing neutralizing antibodies (Beatrice et al., 1980). The VP2 was isolated as a subunit by treatment of the virus with 3 M-urea at pH 9.

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Treatment of poliovirus with 9 M-urea at room temperature resulted in the dissociation of the virion followed by reconstitution after dilution. This indicates a reversible denaturation of the viral polypeptides (Drzeniek & Bilello, 1972). We have therefore used urea dissociation and isoelectric focusing in a one-step procedure for the separation of all four poliovirus polypeptides in urea-sucrose gradients (Wiegers & Drzeniek, 1980). Because of the good separation of the four viral polypeptides and the ease of recovery from urea-sucrose gradients, we have used this method for the preparative separation of poliovirus polypeptides. Using individual polypeptides as immunogens, we have obtained monospecific antisera in rabbits against all four structural poliovirus polypeptides.

The reactions of these monospecific antisera with native, heated or dissociated virus particles as well as with empty capsids and with poliovirus-infected HeLa cells are reported in this paper.

METHODS

Materials. Acrylamide, ampholytes (Servalytes), bovine pancreatic ribonuclease A (RNase I, EC 3.1.27.5), SDS, L-(1-tosylamide-2-phenyl)ethyl chloromethyl ketone (TPCK) and phenylmethylsulphonyl fluoride (PMSF) were obtained from Serva (Heidelberg, F.R.G.). Nonidet P40 (NP40) was from Fluka (Buchs, Switzerland), sucrose (ultra pure, density gradient grade) from Schwarz/Mann (Orangeburg, N.J., U.S.A.). [35S]Methionine (spec. act. > 600 Ci/mmole) was purchased from Amersham International and Buchler (Braunschweig, F.R.G.). Protein A bacterial adsorbent was obtained from Miles Laboratories (Frankfort, F.R.G.).

Labelling of virus and of virus-specific proteins in infected cells. The growth of HeLa S3 cells, the purification of poliovirus, type 1, strain Mahoney, the preparation of labelled poliovirus and virus-specific proteins in infected cells has been described previously (Yamaguchi-Koll et al., 1975; Wiegers & Dernick, 1981). Briefly, suspension cultures of HeLa S3 cells were infected at high multiplicity (100 p.f.u./cell); 20 to 50 μCi/ml [35S]methionine (> 600 Ci/mmole) was added at 3·5 h after infection. For the preparation of cytoplasmic extracts, samples of infected cells were collected and immediately frozen at −80 °C. Labelled virus was prepared from cells, collected 6 h after infection and purified as described previously (Yamaguchi-Koll et al., 1975).

Cytoplasmic extracts of labelled infected cells were prepared in RSB (0·01 M-NaCl, 0·01 M-Tris-HCl pH 7·4, 0·0015 M-MgCl2) containing 0·05% NP40 and 1 mM-dithiothreitol (DTT). After 60 min on ice, the nuclei were removed by centrifugation at 10000 g for 10 min. The extracts obtained after 15 min label and 60 min label were prepared in the presence of 1 mM-PMSF and 1 mM-TPCK (Racevskis & Koch, 1977). Extracts of unlabelled infected cells for adsorption of Staphylococcus aureus were prepared in RSB containing 0·5% NP40 and 1 mM- DTT.

Preparation of viral polypeptides for immunization. A 2·5 mg amount of [35S]methionine-labelled poliovirus type 1, strain Mahoney, was disrupted in 9 M-urea in the presence of 50 μg/ml pancreatic ribonuclease A for 1 h at 25 °C and applied to four separate 7 M-urea-containing sucrose density gradients containing 2% ampholytes of pH ranges 5 to 7, 7 to 9 and 2 to 11 (2:1:0.5). Isoelectric focusing and collection of fractions was done as described previously (Wiegers & Drzeniek, 1980). Viral polypeptides were identified by SDS-PAGE. Appropriate fractions of the four gradients containing only one viral polypeptide were pooled and refocused on separate gradients. Gradients used for refocusing of VP1 and VP4 contained 2% ampholytes of pH ranges 7 to 9 and 2 to 11 (1:0:5) whereas gradients for refocusing of VP2 and VP3 contained 2% ampholytes of pH ranges 5 to 7 and 2 to 11 (1:0:5) respectively.

Refocused polypeptides were checked for purity by SDS–PAGE. Total recovery was about 200 μg for VP1, VP2 and VP3 and about 50 μg for VP4. For inoculation into rabbits, each polypeptide preparation was mixed with Freund's complete adjuvant. Booster inoculations were given 3 weeks later. The serum was collected after an additional 2 weeks.

Immunoprecipitation

Immunoprecipitation was performed according to a described procedure (Racevskis & Koch, 1978) with slight modifications.

Immunoprecipitation of polypeptides of viral particles, empty capsids and cytoplasmic extracts after dissociation in 1% SDS. [35S]Methionine-labelled viral particles or naturally occurring empty capsids were stored at −60 °C. Before dissociation, they were diluted at least tenfold in RSB containing 0·05% NP40 and 1 mM-DTT. They were dissociated by boiling in 1% SDS for 5 min. Because of disulphide bonds present in empty capsids (Heukeshoven & Dernick, 1981) the concentration of the reducing agent had to be raised to 10 mM to obtain a complete dissociation of these particles. To 20 μl virus particles, empty capsids or cytoplasmic extracts prepared as above was added 200 μl cold RSB containing 0·05% NP40 and 1 mM-DTT. Ten μl of the antiserum was then added and incubated for 1 h on ice, followed by the addition of 60 μl of a 10% suspension of heat-killed, formalin-fixed Staphylococcus aureus bearing protein A [Cowan I strain, prepared according to Kessler (1975)], which had been
Poliovirus monospecific antisera

779

pre-adsorbed with unlabelled infected cell extract (Racevskis & Koch, 1978). Incubation on ice was continued for 30 min.

Bacterial-adsorbed immune complexes were collected by centrifugation at 10 000 g for 3 min and washed with TEN (0.15 M-NaCl, 0.01 M-Tris–HCl pH 7.4, 0.001 M-EDTA) containing 0.5% NP40 and once with TEN high salt (0.6 M-NaCl) containing 0.5% NP40. Proteins were eluted by incubation at room temperature for 15 min with 100 µl sample buffer (0.059 M-Tris–HCl pH 6.7, 1% SDS). The bacterial ghosts were removed by centrifugation. The supernatant was boiled in the presence of 1% 2-mercaptoethanol and 10% glycerol and analysed by SDS-PAGE.

**Immunoprecipitation of native virus particles, empty capsids and heated virus.** Virus particles and empty capsids were taken from the same stock in CsCl as above. Heated virus was prepared by incubation of virus particles, diluted tenfold in RSB containing 0.05% NP40 and 1 mM-DTT for 1 h at 56 °C. Immunoprecipitation was carried out as above, but omitting the dissociation step in 1% SDS.

**SDS-PAGE.** Samples were analysed on 10% polyacrylamide slab gels in SDS using the discontinuous Tris-glycine buffer system originally described by Laemmli (1970) and Maizel (1971). After electrophoresis the gels were dried and fluorographs prepared as described by Chamberlain (1979).

**RESULTS**

**Monospecific antibodies against structural polypeptides**

To prepare monospecific antisera rabbits were immunized with poliovirus polypeptides purified by preparative isoelectric focusing in urea-containing sucrose gradients. To ensure homogeneity of the polypeptides, pooled fractions containing only one polypeptide were refocused a second time (see Methods).

The antisera obtained were tested by immunoprecipitation of a mixture of radioactively labelled polypeptides obtained by dissociation of poliovirus by SDS. The SDS-dissociation procedure was chosen because it dissociates poliovirus particles completely within a few minutes, with no tendency to reaggregate.

Preliminary experiments have demonstrated that only very low amounts of antigen–antibody precipitates were formed in the presence of 0.5% SDS. The concentration of SDS had to be lowered to 0.1% to obtain an acceptable amount of antigen–antibody complex bound to the staphylococcal protein A antibody adsorbent as described in Methods.

Fig. 1 demonstrates the monospecificity of the antisera tested. The complete absence of any non-specific precipitation under these conditions is demonstrated by the use of preimmune

![Fig. 1. Fluorogram SDS-polyacrylamide gel of [35S]methionine-labelled poliovirus polypeptides after immunoprecipitation with monospecific antisera against the VP1, VP2, and VP3 and VP4 polypeptides. Virus particles (lanes 2 to 6) and empty capsids (lanes 7 to 14) were dissociated in 1% SDS at 100 °C in the presence of 1 mM-DTT (lanes 2 to 12) or 10 mM-DTT (lanes 13 to 17). Immunoprecipitation was carried out in 0.1% SDS, 0.05% NP40 and 1 mM-DTT. Lane 1, purified poliovirus particles; lane 7, purified empty capsids; lanes 2, 8 and 13, control reaction, preimmune serum added; lanes 3, 9 and 14, VP1 antiserum; lanes 4, 10 and 15, VP2 antiserum. Lanes 5, 11 and 16 contained the immunoprecipitates with VP3 antiserum, and lanes 6, 12 and 17 the precipitates with the VP4 antiserum. Note that this figure is a composite of two separate experiments, with lanes 1 to 6 from experiment 1 and lanes 7 to 17 from experiment 2.](image-url)
serum (lanes 2 and 13). The VP1 antiserum precipitated only the polypeptide VP1 from a mixture of polypeptides obtained from viral particles (lane 3) or from empty capsids dissociated in the presence of 10 mM-DTT (lane 14). When empty capsids were dissociated in the presence of 1 mM-DTT, some non-specific precipitation of VP0 was seen (lane 9). Under these conditions a faint band of VP0 was also detected, when preimmune serum was used (lane 8). The VP2 antiserum precipitated VP2 from dissociated virus particles (lane 4) and VP0 and traces of VP2 from empty capsids (lanes 10 and 15). The VP3 antiserum precipitated only VP3 from dissociated viral particles and from empty capsids dissociated in the presence of 10 mM-DTT (lanes 5 and 16 respectively). Again, after dissociation in the presence of 1 mM reducing agent, some coprecipitation of VP0 occurred (lane 11).

The VP4 antiserum precipitated small amounts of VP4 of dissociated viral particles, only visible on the original X-ray film (lane 6). But it reacted exclusively with VP0, when empty capsids were dissociated in the presence of 1 mM-DTT (lane 12). Raising the concentration of the reducing agent during the dissociation step, however, abolished this reactivity (lane 17).

Because of the low reaction of the VP4 antiserum with dissociated poliovirus, we determined the amount of empty capsids precipitated by the VP4 antiserum. We found that up to 20% of radioactively labelled empty capsids were precipitated. Since this antiserum did not precipitate VP1, VP2, or VP3, but small amounts of VP4 from virus particles or VP0 from empty capsids, this is clear evidence for VP4 antibodies in our antiserum obtained by immunization with a pure VP4 polypeptide preparation.

Further reactions of the VP4 antiserum are demonstrated with heated virus as antigen (see below). The problems associated with this particular antiserum are dealt with in the discussion of this paper.

**Reaction of poliovirus, heated virus and empty capsids with monospecific antiserum**

The monospecific antiserum were tested against highly purified viral particles, viral particles heated for 1 h at 56 °C and naturally occurring empty capsids. The reaction was assayed by immunoprecipitation without prior dissociation of the particles by 1% SDS. From the antiserum tested, only the VP3 antiserum precipitated virus particles, which is demonstrated by the presence of VP1 to VP4 in the gel (Fig. 2, lane 4). This indicates that our VP3 antiserum is
Poliovirus monospecific antisera

Directed against an antigenic site of VP3, which is present on the surface of native virus particles. From this set of antisera, only the VP3 antiserum exhibited a low neutralizing activity. Undiluted VP3 antiserum neutralized 1000 p.f.u./ml of poliovirus type 1, whereas a 1:10 dilution gave a 50% reduction of the plaque number. No reduction was found with undiluted VP1 and VP2 antisera. Therefore, antigenic sites reacting with our VP1 and VP2 antisera must be inaccessible to these antisera on native virus particles.

However, heated virus particles and naturally occurring empty capsids were precipitated by all four monospecific antisera used (Fig. 2, lanes 7 to 14). This demonstrates the occurrence of antigenic sites on these particles not present on native virus particles. It is known that incubation of poliovirus particles for 1 h at 56 °C leads to a gross conformational change of the virus surface (Rueckert, 1976). This conformational change between native and heated virus particles is demonstrated by the differential reactions with our antisera. Heated virus was precipitated with VP1 and VP2 antisera (lanes 7 and 8 respectively), in addition to the VP3 antiserum (lane 9) indicating that this altered conformation makes antigenic sites available also for the VP1 and VP2 antisera. Furthermore, heated virus is precipitated, but to a lesser extent, with the VP4 antiserum (lane 10). This figure also demonstrates that VP4 was still present in the heated virus preparation, as can be seen from its occurrence in lanes 7 to 10.

Naturally occurring empty capsids resemble heated virus particles in their reactions with these antisera. The antisera against VP1, VP2, VP3 and, to a lesser extent, also the VP4 antiserum precipitated empty capsids, detected by the occurrence of VP0, VP1 and VP3 in the gel (lanes 11 to 14) (see above).

Immunoprecipitation of poliovirus polypeptides of infected cells with monospecific antisera

Poliovirus capsid polypeptides in infected cells are derived from a common precursor by proteolytic cleavage (Rueckert et al., 1979). To determine whether the antisera tested would recognize antigenic sites on larger precursor polypeptides they were assayed with [35S]methionine-labelled extracts of infected cells. Infected HeLa cells were labelled for 15, 60 and 120 min starting 3.5 h after infection.

Cytoplasmic extracts were prepared with 0.05% NP40 in hypotonic buffer (RSB). The extract obtained after labelling cells for 60 min was assayed by immunoprecipitation with VP1 antiserum. The result is shown in Fig. 3. The VP1 antiserum precipitated the structural polypeptides VP0, VP1 and VP3 and their precursor, the non-structural polypeptide la, if the cytoplasmic extract was immunoprecipitated without prior dissociation with SDS (lane 2). The addition of 1% SDS at room temperature prior to immunoprecipitation leads to the same result (lane 3). However, when the cytoplasmic extract was boiled with 1% SDS and then subjected to immunoprecipitation carried out as in Fig. 1, only VP1 and la are precipitated (lane 4). The coprecipitation of VP0 and VP3 with VP1 must be due to a stable complex, consisting of VP0, VP1 and VP3. Whether this complex is a protomer, a 14S subunit (pentamer) or 74S particle (empty capsid) has not been determined.

For the immunoprecipitation reaction with all four antisera, extracts were prepared from infected cells labelled for 15 min and 2 h with [35S]methionine. Prior to immunoprecipitation they were heated to 100 °C in the presence of 1% SDS. The result is shown in Fig. 4. The VP1 antiserum (lanes 3 and 8) precipitated VP1 and its precursor la, as already shown in Fig. 3. The VP2 antiserum precipitated la, VP0 and VP2 when cells were labelled for 2 h (lane 9). After a short pulse, only traces of VP2 were detected (lane 4), due to the small amount present after 15 min labelling. The immediate precursor of VP0 and VP3, the polypeptide 3a, was also precipitated with antiserum against VP2. The VP3 antiserum reacted with VP3 and its precursors la and 3a (lanes 5 and 10). VP4 antiserum did not visibly precipitate VP0 or VP4 bands when incubated with dissociated cytoplasmic extract (lanes 6 and 11).

The VP1 to VP3 antiserum precipitated in addition to polypeptide la (95K) a second polypeptide of about 90K antigenically related to the capsid precursor la. A similar second capsid precursor protein (88K) was first reported for poliovirus by Vrijsen et al. (1980). A faint spot of a 90K unnamed poliovirus protein just below NCVPla was detected also on two-dimensional gels (Wiegers & Dernick, 1981). The occurrence of two precursor proteins differing
Fig. 3. Fluorogram of SDS-polyacrylamide gel of [35S]methionine-labelled poliovirus proteins of infected HeLa cells immunoprecipitated with VP1 antiserum. Labelling time was 60 min. Cytoplasmic extracts were prepared with 0.05% NP40 and 1 mM DTT in RSB. Lane 1, control reaction, preimmune serum; lanes 2 to 4, immunoprecipitates formed with VP1 antiserum, without prior dissociation (lane 2) or dissociated with 1% SDS and 1 mM DTT at room temperature (lane 3) or at 100 °C for 5 min (lane 4).

Fig. 4. Fluorogram of SDS–polyacrylamide gel of [35S]methionine-labelled viral proteins of poliovirus-infected HeLa cells after immunoprecipitation with monospecific antisera against the VP1, VP2, VP3 and VP4 polypeptides. Cytoplasmic extracts were prepared from infected cells labelled for 15 min (lanes 2 to 6) or 2 h (lanes 7 to 11). The extracts were boiled for 5 min in 1% SDS with 1 mM DTT. Immunoprecipitation (lanes 2 to 11) was done in 0.1% SDS as in Fig. 1. Lane 1, extract of poliovirus-infected HeLa cells; lanes 2 and 7, control reaction with preimmune serum added; lanes 3 and 8, immunoprecipitates formed with VP1 antiserum; lanes 4 and 9, with VP2 antiserum; lanes 5 and 10, with VP3 antiserum; lanes 6 and 11, with VP4 antiserum. Left side, poliovirus polypeptides detected in cytoplasmic extract (lane 1); right side, poliovirus polypeptides detected by immunoprecipitation (lanes 2 to 11).
Poliovirus monospecific antisera

only slightly in molecular weight (by 10K or less) were described earlier for encephalomyocarditis virus (Butterworth et al., 1971), mengovirus (Paucha et al., 1974) and rhinovirus (Rueckert et al., 1979).

DISCUSSION

Poliovirus polypeptides purified by isoelectric focusing in high concentrations of urea produce in rabbits monospecific antisera against the four structural polypeptides. The specificity of the antisera was determined by immunoprecipitation of radiolabelled polypeptides after SDS disruption of poliovirus particles. This has some implications for the nature of the antigenic sites detected, suggesting that the antibodies reacting in our system are directed against antigenic sites which are stable or only reversibly denatured by urea and SDS. The VP1, VP2 and VP3 antisera precipitated almost equal amounts of polypeptides from SDS-disrupted virions, taking into account the methionine content of the individual polypeptide.

The VP4 antiserum precipitated only a faint band of VP4 when SDS-dissociated poliovirus particles were used as a source of viral polypeptides. This is partly due to the low amount (by weight) of VP4 present in virus particles, partly to its low radioactivity when [35S]methionine is used as label, and partly to the low titre of the antiserum (see below). The reactivity of the VP4 antiserum was demonstrated easily when naturally occurring empty capsids were dissociated with SDS and the mixture obtained was precipitated with VP4 antiserum. In this case the VP4 antiserum precipitated the polypeptide VP0 which is the structural precursor of VP4. The VP4 antiserum also precipitated heated poliovirus particles or naturally occurring empty capsids which were not treated with SDS. The amount of material precipitated by VP4 antiserum was much lower than that precipitated by antisera against VP1 to VP3, indicating a low titre of the VP4 antiserum. This is understandable, because of the smaller amount of VP4 used for immunization (see Methods). Earlier attempts to obtain monospecific antisera against VP4 were unsuccessful (Meloen et al., 1979; Vrijisen et al., 1980).

The observation that the monospecific antiserum against VP3 recognizes an antigenic site on the surface of native virions is striking. Chemical labelling methods have demonstrated that VP1 seems to cover a great proportion of the surface of native virus (Carthaw & Martin, 1974; Rowlands et al., 1975; Lonberg-Holm & Butterworth, 1976; Beneke et al., 1977; Lund et al., 1977; Wetz & Habermehl, 1979). The antiserum against VP3 has only a very low titre (1-0 log10) in the neutralization assay. Antiserum against VP1 and VP2, besides reacting with their homologous SDS-polypeptides, reacted with naturally occurring empty capsids and heated virus particles. However, they did not precipitate native virus particles and did not neutralize viral infectivity. This demonstrates that the antigenic sites recognized by our VP1 and VP2 antibodies must be buried in native virions. The differential reaction of native virus and heated virus or empty capsids with the VP1, VP2 and VP3 antisera can be used as a sensitive marker for the detection of conformational changes of the virus particle. Thus, naturally occurring empty capsids or virions heated for 1 h at 56 °C change the orientation of antigenic sites of VP1 and VP2 with respect to the particle’s surface, making them accessible to our monospecific antisera.

Virus preparations which were stored in isotonic phosphate buffer (PBS) and were frozen and thawed twice, showed a slight reaction with the VP1 and VP2 antiserum (data not shown) although virus stored at −20 °C in CsCl gradient solution did not react with these antisera (as described above).

Monospecific antiserum, obtained by immunization with individual polypeptides purified by SDS-PAGE, precipitated precursor polypeptides from infected cell extracts (Vrijisen et al., 1980). Our antiserum also recognized antigenic sites on the appropriate precursor polypeptides in infected cell extracts. The VP1, VP2 and VP3 antisera reacted with their common precursor polypeptide 1a (96K), and with a smaller antigenically related polypeptide of about 90K. The reaction of the 90K protein with the VP1, VP2 and VP3 antisera has also been noted earlier for poliovirus (Vrijisen et al., 1980), and for other picornaviruses (Rueckert et al., 1979; Butterworth et al., 1971; Paucha et al., 1974). The demonstration of a second precursor for all four structural polypeptides could have consequences for the established cleavage pathway. Proteolytic cleavage of 1a yields polypeptide 3a and VP1 (Rueckert et al., 1979); thus, 3a contains the
sequences of VP0 and VP3. This relationship is demonstrated by precipitation of 3a by the VP2 and VP3 antisera.

In addition to the results reported above, with cytoplasmic extracts dissociated by SDS into the monomeric polypeptides, we have analysed also the immunoprecipitation of cytoplasmic extracts not treated with SDS by our monospecific antisera against VP1. When the dissociation precipitated, in addition to polypeptide 1a, VP0, VP1 and VP3. This finding suggests the existence of complexes of structural polypeptides and of their precursor(s) which are resistant to 1% SDS at room temperature. It indicates that the polypeptide 1a exhibits the same antigenicity as empty capsids which is, however, different from the antigenicity of native virus because VP1 antiserum does not react with native virus.

The differences observed in the reactions of our antisera may be a useful probe to look for antigenic differences of poliovirus polypeptides in infected cells during their assembly into infectious virus particles.

We are grateful to Marianne Hilbrig for technical assistance; we thank Peter Nobis for a gift of Staph. A material and thank him and John Bilello for introducing us to special immunoprecipitation techniques. The Heinrich-Pette-Institut is financially supported by Freie und Hansestadt Hamburg and Bundesministerium für Jugend, Familie und Gesundheit, Bonn.

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Poliovirus monospecific antisera


(Received 28 July 1982)