Molecular Pathogenesis of Neural Lesions Induced by Poliovirus Type 1

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SUMMARY

Using in situ hybridization techniques for viral RNA and employing a specific riboprobe, we have detected virus in neural cells of monkeys infected with poliovirus type 1 (PV-1) by the intraspinal route. In monkeys paralysed after inoculation of a neurovirulent revertant of PV-1/Sabin strain, viral RNA was detected in motor neurons and their processes, and in polymorphonuclear and small neural cells. Quantitative in situ hybridization provided evidence of viral replication in individual cells suggesting that the death of motor neurons was due to the direct effect of poliovirus replication in these cells. The histological study of neural lesions of monkeys paralysed after infection with the attenuated Sabin strain of PV-1 revealed two major differences compared to monkeys infected with a virulent strain: (i) the number of destroyed motor neurons was reduced and limited to the site of inoculation and (ii) the inflammatory reaction was localized but more intense. An account is given of the difference in histopathology induced by virulent and attenuated strains of PV-1 in the central nervous system.

INTRODUCTION

Knowledge of poliomyelitis pathogenesis is based mainly on studies by Bodian (1949, 1955), who described the major steps of viral spread in human and simian organisms. Bodian's data were substantiated by Sabin (1956), when he developed attenuated poliovirus strains for oral poliovirus vaccine. However, new molecular tools for the study of viral replication are now available, including in situ hybridization, which can provide direct evidence about the cells in which the virus replicates (Brahic & Haase, 1978; Stroop et al., 1981). Such an approach is extremely attractive for the study of poliomyelitis pathogenesis, since there is controversy about which cells of the central nervous system (CNS) are permissive for poliovirus. In the past, it was generally accepted that damage of motor neurons, inducing poliomyelitic paralysis, was generated by multiplication of poliovirus in that particular cell type (Kovacks et al., 1963; Bodian, 1964). However, Simon et al. (1970) reported that virus-specific antigen was not detected by immunofluorescence in any neurons of the CNS from monkeys infected with poliovirus, whereas antigen was observed in glial, vascular endothelial and mononuclear inflammatory cells. They inferred therefore that the nerve cell is only secondarily damaged following either the inflammatory reaction or involvement of the astrocyte foot plates and capillary endothelium. In contrast to these data, Hashimoto et al. (1984) found poliovirus antigen only in motor neurons of infected monkey CNS using an immunoperoxidase technique. In addition, by electron microscopy, they detected membrane-bound vesicles in the cytoplasm of damaged motor neurons, similar to the vesicles seen in infected cultures of cynomolgus monkey kidney cells. These authors concluded that necrosis of nerve cells was a direct consequence of
viral multiplication in these cells. Similar results were obtained by Dal Canto et al. (1986) in the CNS of paralysed mice.

The objective of our studies was the detection and quantification of viral genomes in infected cells of the CNS in order to identify poliovirus-susceptible cells and define the site(s) of virus multiplication. In situ hybridization allowed detection of the neural cells in which poliovirus replicates. We used a specific riboprobe which could detect, in histological preparations, the presence and the number of poliovirus genomes at the single-cell level. We report here results on the localization of the poliovirus genome by RNA–RNA hybridization in the CNS of monkeys inoculated with virulent and attenuated poliovirus type 1 (PV-1). We concentrated our attention mainly on motor neurons, in order to give a clear cut answer to questions raised about the site of poliovirus replication in the CNS.

METHODS

Viruses. Mahoney wild and Sabin attenuated strains of PV-1 and a neurovirulent revertant of PV-1/Sabin selected at high temperature, called S1-39-C6 (C. Christodoulou and others, unpublished results) were used. The mouse-adapted MEF1 strain of poliovirus type 2 (PV-2) was also used in this study. All viruses were plaque-purified three times on HEp-2c cells.

In vivo studies. In the course of routine tests of poliovaccines for neurovirulence, cynomolgus monkeys (Macaca fascicularis) were injected intraspinally between the first and second lumbar vertebrae by the 'standard technique' described by Beswick & Coid (1961) with 3.0 x 10^6 TCID50 of PV-1/S1-39-C6 and 1.0 x 10^6 or 1.3 x 10^6 TCID50 of PV-1/Sabin. Monkeys inoculated with PV-1/S1-39-C6 were sacrificed at the time of paralysis (2 or 3 days after injection). One of the monkeys inoculated with 1.3 x 10^6 TCID50 of PV-1/Sabin developed paralysis 7 days post-inoculation (p.i.) and was sacrificed at that time. None of the monkeys inoculated with 1.0 x 10^6 TCID50 of PV-1/Sabin developed paralysis and animals were sacrificed 21 days p.i. The CNS of monkeys was fixed in situ by acetic–formol saline perfusion, then embedded in paraffin by routine histological techniques (Boulger, 1973). Tissues of two monkeys injected with the virulent strain and of three monkeys infected with the attenuated strain were processed for histological examination and/or in situ hybridization.

Preparation and synthesis of the riboprobe. cDNA (Kean et al., 1986) from the 5' non-coding region (nucleotides 220 to 670) of PV-1/Mahoney was inserted into the polylinker of a Gemini 1 Riboprobe vector (Promega Biotec) at the BamHI site between the SP6 and T7 promoters. The resulting recombinant plasmid was purified on a CsCl gradient, treated with RNase A and then with proteinase K. Plasmid was linearized with EcoRI and transcripts of negative polarity were synthesized, essentially as described by Butler & Chamberlin (1982) with 20 units (U) SP6 RNA polymerase (Promega Biotec) in the presence of 12 μM[13P]UTP, [35S]UTP or [3H]UTP (400 Ci/mmol, 1000 Ci/mmol and 40 Ci/mmol, respectively) (Amersham) for about 1 μg of plasmid DNA. Afterwards, 1 U of RQI DNase (Promega Biotec) was added for 15 min and transcripts were purified on a G75 Sephadex column. After extraction with phenol–chloroform and chloroform, RNA was precipitated with 0.3 M LiCl and ethanol. The length of the riboprobe was checked by electrophoresis on a polyacrylamide gel and found to correspond to the expected length of 450 nucleotides.

Preparation of cells and tissues. HEp-2c cells were infected with PV-1/Mahoney or PV-2/MEF1 for different times (indicated in the text) then washed three times with sodium phosphate buffer (P/NaCl) and fixed for 20 min in 0.05 M-sodium periodate in lysine (pH 7.4) containing 4% paraformaldehyde and 1% glutaraldehyde (PLFG) (Moench et al., 1985). Cells were then rinsed twice with distilled water. The microscope glass slides for tissue sections were treated by sequential immersions in 1 M HC1 for 30 min, distilled water briefly, 95% ethanol for 30 min and wiped with gauze to reduce non-specific binding of the probe, as described by Haase et al. (1984). Slides were then dipped in 1 x Denhardt's medium and 3 x SSC at 65 °C for 3 h, dipped briefly in distilled water and transferred to ethanol–acetic acid (3:1 v/v) for 20 min, then air-dried. Tissue sections of 10 μm embedded in paraffin were deposited on treated slides and deparaffinized, then treated with colloion (1 vol.) in ether (25 vol.) and methanol (25 vol.) for 1 min in order to avoid loss of sections (J.-G. Fournier, personal communication). Pretreatment of fixed samples was performed as described by Brahic et al. (1984) with minor modifications. In order to denature viral RNA before quantitative hybridization, both cells and tissues were incubated in 0.2 M-glyoxal, 0.1 M-phosphate buffer pH 6.9 and 50% dimethyl sulphoxide at 50 °C for 1 h, then dipped twice in water at 4 °C for 1 min each. To reduce electrostatic binding of the probe to sections, slides were incubated in 0.1 M-triethanolamine pH 8.0 and 0.25% (v/v) acetic anhydride for 10 min, then washed in distilled water. Cell permeability was increased by sequential immersions in 1 M-HCl for 20 min and in 10 mM-Tris–HCl pH 7.4, 2 mM-CaCl2 containing 1 μg/ml proteinase K for 15 min at 37 °C. Slides were dipped in distilled water after each step. Sections and cells were then dehydrated by sequential immersions of 5 min each in 70% ethanol twice and 95% ethanol, and air-dried.

In situ hybridization. Slides were hybridized in situ with 35S-riboprobe (sp. act. 2-4 x 10^8 d.p.m./μg) or
Poliovirus-induced neural lesions

Specificity of the riboprobe analysed by dot blot hybridization. RNA samples of PV-1/Mahoney (Mah), PV-2/MEF1 (MEF1) and brome mosaic virus (BMV) were spotted onto nitrocellulose filters and hybridized to a 32P-riboprobe corresponding to 450 nucleotides of the 5' non-coding region of PV-1/Mahoney. Positive hybridization was revealed by autoradiography.

RESULTS

Probe specificity and optimization of in situ hybridization

To verify the specificity of the riboprobe for poliovirus RNA, we performed a dot blot test as described by Thomas (1980) (Fig. 1). As expected, a positive signal was obtained with RNA from both PV-1/Mahoney and PV-2/MEF1 because the probe corresponded to nucleotides 220 to 670 of the 5' non-coding region (Cova et al., 1988) which is a highly conserved region of the genome of all three poliovirus serotypes. No signal was obtained with brome mosaic virus RNA, used as negative control.

We determined the optimal conditions of fixation, pretreatment and hybridization of samples by conducting tests on poliovirus-infected HEp-2c cells. We found that acetic-formol saline or PLPG fixation (described in Methods) gave similar in situ hybridization signals per cell but PLPG preserved cellular morphology better. Treatment before hybridization with 0.2 M-HCl and proteinase K significantly increased (about 1.5-fold) the number of grains, whereas heating at 70 °C in 2 x SSC had no effect on the signal compared to untreated, PLPG-fixed cells. The hybridization efficiency with the 35S-RNA probe (described above) was twofold higher than...
Fig. 2. Detection of poliovirus genomes in infected cultured HEp-2c cells by *in situ* hybridization with
\(^{35}\)S-riboprobe. HEp-2c cells were infected at an m.o.i. of 100 p.f.u. with PV-1/Mahoney at 37 °C then *in situ* hybridization was carried out using 0.5 ng \(^{35}\)S-riboprobe/µl. The probe is described in the legend to Fig. 1. Autoradiographic exposure was for 96 h in all cases. Cells were counterstained with Giemsa. (a) Uninfected HEp-2c cells, (b) HEp-2c cells at 3 h p.i. and (c) HEp-2c cells at 5 h p.i. Note the alteration of cellular morphology which consisted of cytoplasm and nuclear retraction. Bar markers represent 5 µm.

with nick-translated \(^{35}\)S-cDNA probe specific for poliovirus (with a mean length of 500 nucleotides covering the 3′ end of PV-1 genome and a sp. act. of 1.2 × 10^8 d.p.m./µg). Moreover, the non-specific signal obtained after hybridization with the riboprobe was about fivefold lower compared to that with the cDNA probe (not shown). The reduced background probably resulted from RNase A digestion performed during washing after riboprobe hybridization. For all these reasons we chose to use the riboprobe in further studies.

At the cellular level, the specificity of the probe was shown by comparative *in situ* hybridization of infected and uninfected HEp-2c cells. Cell cultures infected with 100 p.f.u. per cell of PV-1/Mahoney for 3 or 5 h were hybridized with the \(^{35}\)S-riboprobe. Silver grains, demonstrating positive hybridization, were detected only in the cytoplasm of infected HEp-2c cells, whereas uninfected cells did not contain grains. The number of grains was proportional to the time course of infection (Fig. 2). Similar data were obtained with PV-2/MEF-1-infected cells (not shown).

To avoid the high non-specific signal produced by the \(^{35}\)S-labelled riboprobe on histological preparations of CNS, we used a \(^{3}\)H-labelled riboprobe that gave a greatly reduced background signal.

**Viral replication in the CNS of monkeys infected with poliovirus**

Monkeys were infected with PV-1/S₁-39-C₉, a neurovirulent revertant of the attenuated PV-1/Sabin strain selected at high temperature. In the study of the neural lesions induced by PV-1, we preferred to use this virus instead of PV-1/Mahoney to facilitate comparison of viral genomic structures involved in neurovirulence and in attenuation. Compared to attenuated PV-1/Sabin, PV-1/S₁-39-C₉ became neurovirulent after three amino acid mutations in the capsid protein coding region (two in VP1 and one in VP3) and one mutation in the polymerase, as determined by sequencing 80% of the viral RNA (C. Christodoulou and others, unpublished results).

The intensity of CNS lesions in monkeys after intraspinal inoculation of PV-1 was revealed by lesion scores estimated according to World Health Organization (1981) criteria (Table 1). Lesions in the lumbar cord were more intense than those in the cervical cord and in the brain. To identify cells of cervical and lumbar cords that supported viral replication, we performed *in situ*
Table 1. *Comparison of lesion extension, histological changes and infected cells of CNS of monkeys injected with attenuated or neurovirulent strains of poliovirus type 1*

<table>
<thead>
<tr>
<th>Poliovirus strain</th>
<th>Clinical symptoms</th>
<th>CNS lesion scores*</th>
<th>Overall mean lesion score†</th>
<th>Histology‡</th>
<th>Infected cells§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virulent PV-1/S-39-C₆</td>
<td>Both limbs paralysed (2 or 3 days p.i.)</td>
<td>Lumbar (4:00−3:68)</td>
<td>3:29−2:08 (4 monkeys)</td>
<td>Meningitis</td>
<td>Motor neurons</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cervical (3:50−2:15)</td>
<td></td>
<td>Inflammation of the whole grey matter</td>
<td>Polymorphonuclear cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Brain (2:37−1:44)</td>
<td></td>
<td>Motor neuronal necrosis</td>
<td>Small neural cells</td>
</tr>
<tr>
<td>Attenuated PV-1/Sabin</td>
<td>Right limb paralysed (7 days p.i.)</td>
<td>Lumbar (2:36)</td>
<td>1:85 (1 monkey)</td>
<td>Meningitis</td>
<td>Motor neurons</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cervical (1:70)</td>
<td></td>
<td>Inflammation of the anterior horn</td>
<td>Polymorphonuclear cells</td>
</tr>
<tr>
<td></td>
<td>Non-paralysed (sacrificed 21 days p.i.)</td>
<td>Lumbar (2:07, 2:54)</td>
<td>1:08−1:34 (2 monkeys)</td>
<td>Meningitis</td>
<td>Motor neurons</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cervical (0:05, 0:60)</td>
<td></td>
<td>Inflammation of the anterior horn</td>
<td>Polymorphonuclear cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Brain (0:62, 1:12)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Values in parentheses correspond to lesion scores of infected monkeys.
† The figures represent lesion scores for the whole of the CNS. Virulent strain gave a mean lesion score of > 2.5 and attenuated strain gave a mean value of less than 2.0.
‡ Observations for lumbar spinal cord only.
§ Infected cells correspond to cells positive for viral replication detected by in situ hybridization of lumbar cord.
Fig. 3. Detection of poliovirus genomes in lumbar and cervical cords of monkeys by in situ hybridization with \(^{3}\)H-riboprobe. Transverse sections of spinal cord of monkeys paralysed after intraspinal inoculation of neurovirulent PV-1/S
\(_{-1}\)-39-C\(_{6}\) or attenuated PV-1/Sabin were processed for in situ hybridization. Tissues were hybridized with 0-5 ng/ml of \(^{3}\)H-riboprobe (see Fig. 1 legend), exposed for 4 weeks and counterstained with haematoxylin-eosin. (a) Normal motor neurons in lumbar cord of an uninfected monkey. (b) Infected motor neuron in the interior horn of a lumbar cord of a monkey infected with PV-1/S
\(_{-1}\)-39-C\(_{6}\). Note the presence of silver grains in large processes (small arrows). (c) Degenerating, infected motor neuron in the anterior horn of a lumbar cord of a monkey infected with PV-1/S
\(_{-1}\)-39-C\(_{6}\). Note retraction of the cytoplasm and the nucleus. (d) Ghost of a motor neuron and a cluster of polymorphonuclear cells containing silver grains in the anterior horn of a cervical cord of a monkey infected with PV-1/S
\(_{-1}\)-39-C\(_{6}\). Note the presence of silver grains in empty cytoplasm and in processes of the ghost motor neuron (small arrows). (e) Silver grains inside a polymorphonuclear cell cluster around a lysed motor neuron in the anterior horn of a lumbar cord of a monkey infected with PV-1/Sabin. (f) Infected small neural cell with processes in the posterior grey horn of a cervical cord of a monkey inoculated with PV-1/S
\(_{-1}\)-39-C\(_{6}\). Silver grains in processes are indicated by arrows. Bar markers represent 10 \(\mu\)m.
hybridization on slides containing CNS preparations of monkeys injected intraspinally with PV-1/S₁₃₉-C₆ or PV-1/Sabin. The results for histological lesions and target cells for both viruses in the CNS of monkeys are summarized in Table 1.

Infected neural cells detected by in situ hybridization are shown in Fig. 3. The very weak background signal seen in uninfected CNS tissues after in situ hybridization confirmed the specificity of the hybridization reaction (Fig. 3a).

Examination of the CNS from monkeys injected with PV-1/S₁₃₉-C₆ revealed poliovirus genomes in the cytoplasm of motor neurons of anterior grey horns of cervical and lumbar cords and in motor nuclei of medulla oblongata. Both the motor neuron body and the proximal extremity of their axonal or dendritic processes contained viral RNA (Fig. 3b). The linear collections of silver grains not connected to a cell-body might represent processes separated from their soma by the plane of section (not shown). Infected motor neurons exhibited different morphologies which seemed to correspond to different stages of viral infection. One could observe infected motor neurons with a normal morphology (Fig. 3b), a necrotic aspect with retracted cytoplasm and pyknotic nucleus (Fig. 3c), or empty spaces created by motor neuron lysis resembling motor neuron ghosts (Fig. 3d) which were often invaded by inflammatory cells (Fig. 3d and e). No autoradiographic grains were detected in sensory neurons or in the white matter of spinal cord and medulla oblongata. These observations are in agreement with histological aspects which revealed neuronal necrosis, neuronophagia and severe inflammation of the whole grey matter, mainly in motor areas of cervical and lumbar cords (Table 1).

Poliovirus RNA was frequently found inside clusters of inflammatory cells in the anterior horn, and sometimes in the posterior horn. These cells seemed to delimit the area of the destroyed cells (Fig. 3d and e). We identified them as neutrophil polymorphonuclear cells associated with some histiocytes. Taken as a whole, the results suggest that invading polymorphonuclear cells might remove infected, necrotic neural cells and become passive carriers of the virus. In contrast, we did not observe any significant positive signals for viral genome in perivascular cuffs composed of lymphocytes and polymorphonuclear cells, in vascular endothelial cells or in white matter.

Interestingly, silver grains were found in small neural cells and their processes in the anterior and posterior grey horns of the spinal cord (Fig. 3f). The presence of infected neural cells in the posterior grey horn might explain the above mentioned inflammatory reaction at this site.

Examination of CNS from monkeys, with or without clinical signs after receiving the attenuated PV-1/Sabin vaccine strain, showed that only some motor neurons and 'ghosts' of motor neurons invaded by clusters of inflammatory cells located at and around the site of inoculation, contained poliovirus genomes (Fig. 3e). Moreover, none of the preparations we examined contained other neural cells infected by poliovirus. This corroborates histological observations indicating that inflammation of the spinal cord was mainly confined to the anterior horn and that most of the neurons exhibited a normal aspect. No noticeable difference was observed by the examination of the CNS of paralysed and clinically asymptomatic monkeys infected with PV-1/Sabin.

As compared to the CNS of monkeys paralysed after inoculation of virulent PV-1/S₁₃₉-C₆, the number of infected neurons in the lumbar cord of monkeys inoculated with attenuated PV-1/Sabin was reduced about 10- to 20-fold and limited to the site of inoculation, and the inflammatory reaction in anterior horns was significantly more intense.

Quantification of poliovirus genomes in motor neurons

Since the histological appearance of infected neurons described above seems to indicate that the difference in the number of genomes per neuron corresponded to different intervals p.i., we attempted to confirm this by studying poliovirus genome synthesis in these cells. For this purpose, we applied a previously described quantitative in situ hybridization technique (Szabo et al., 1977; Brahic & Haase, 1978) to poliovirus-infected cells.

We established a calibration curve, i.e. we determined the relationship between the number of silver grains and the number of genomes per cell in fully permissive PV-1/Mahoney-infected HEp-2c cells, according to the method described by Brahic et al. (1978, 1984). The results
Fig. 4. Quantification of poliovirus genomes in HEp-2c cells by \textit{in situ} hybridization. HEp-2c cells were infected at an m.o.i. of 5 p.f.u. with PV-1/Mahoney and incubated for 2, 4 and 6 h. Cytoplasmic RNA was extracted from aliquots of each sample and the mean number of RNA copies per cell was measured by dot blotting with the \textit{S}32P-riboprobe and compared to known amounts of cytoplasmic RNA and purified poliovirus RNA. Another aliquot of cells was deposited on a slide and hybridized \textit{in situ} for the presence of viral RNA using an excess of \textit{S}3H-riboprobe. The number of silver grains was counted over 100 randomly selected cells per slide on four different slides after different times of autoradiographic exposure ranging from 7 to 56 days. The background, determined by hybridizing uninfected cells, was subtracted in each case and the mean values of grains per cell per min of autoradiographic exposure was calculated. The straight line was computed by linear regression and gives the relationship between viral genome content per cell and the number of grains found per cell. Correlation coefficient, 0.99.

obtained are presented in Fig. 4 and show a linear relationship between the number of grains per cell per min of autoradiographic exposure and the number of copies of viral RNA per cell. These results are in agreement with those of Brahic \textit{et al.} (1984) on BHK cells infected with Theiler's virus, another member of the picornavirus group.

Using this calibration curve, we compared the number of viral genomes per cell and the histopathology of the \textit{in vitro} poliovirus-infected HEp-2c cells at different times p.i. (from 2 to 7 h) with that of motor neurons from cervical and lumbar spinal cords of monkeys infected with PV-1/S1-39-C6. \textit{In vitro}, a relationship could be established between the number of genomes per cell and cellular morphology, on the one hand, and the time of infection, on the other (Table 2). Motor neurons of intraspinally infected monkeys exhibited a wide range of viral RNA content from $2.0 \times 10^3$ to $1.0 \times 10^5$ genomes per cell. These values were close to those observed in HEp-2c cell cultures during the course of infection. The maximum number of viral genomes in motor neurons ($1.0 \times 10^5$) was comparable to that in HEp-2c cells 6 h p.i. with 5 p.f.u. per cell ($6.5 \times 10^4$). The latter number is in agreement with studies on infected HeLa cells (Baltimore \textit{et al.}, 1966).

In the few motor neurons in which genomes of infecting PV-1/Sabin were detected, the number of viral RNA molecules was similar to that found after infection with virulent strain PV-1/S1-39-C6. No difference in the number of genomes per cell was found between paralysed or clinically asymptomatic monkeys inoculated with attenuated PV-1/Sabin.
Table 2. Quantification of poliovirus genomes in motor neurons of paralyzed monkeys and in infected HEp-2c cells by in situ hybridization

<table>
<thead>
<tr>
<th>Cell morphology*</th>
<th>HEp-2c cells†</th>
<th>Motor neurons‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time p.i.</td>
<td>Genome number/cell</td>
</tr>
<tr>
<td></td>
<td>(days)</td>
<td>number/cell</td>
</tr>
<tr>
<td>Normal</td>
<td>2</td>
<td>2.0 x 10³</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.0 x 10⁴</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5.5 x 10⁴</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>6.0 x 10⁴</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6.5 x 10⁴</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>5.0 x 10⁴</td>
</tr>
</tbody>
</table>

* Necrotic cell morphology corresponded to retracted cytoplasm and pyknotic nuclei.
† HEp-2c cells were infected at an m.o.i. of 5 p.f.u., hybridized in situ and the number of grains per cell was counted over 200 cells after autoradiographic exposure times of 7, 14, 21, 28 and 56 days. Figures are converted into number of genomes per cell per min of exposure using a calibration curve. Cellular morphology was noted for each time of infection.
‡ Viral genome number per motor neuron was estimated, as described above for HEp-2c cells, in spinal cord sections from paralyzed monkey infected with virulent strain PV-1/S1-39-C₆ for more than 500 motor neurons and cellular morphology was noted.

**DISCUSSION**

In the present study, in situ hybridization was used for the first time to localize and quantify poliovirus genomes in the CNS of infected monkeys. Indirect evidence of viral replication in motor neurons of primates and mice has already been reported by other authors, who detected poliovirus by electron microscopy (Bodian, 1964; Blinzinger et al., 1969), by immunofluorescence (Kovacks et al., 1963; Kanamitsu et al., 1967; Jubelt et al., 1980a) or by immunocytochemical methods (Hashimoto et al., 1984; Dal Canto et al., 1986). Using quantification of silver grains in motor neurons of monkeys paralyzed after infection with virulent PV-1/S1-39-C₆, we have shown that the maximum number of viral genomes found in degenerating motor neurons was quite similar to the maximum number of viral genomes in poliovirus-infected cells in vitro just before the cytopathic effect. These results, together with the existence of degenerated, infected neurons in the absence of any inflammatory cells, indicate that death of motor neurons is due to a direct effect of virus multiplication inside the cytoplasm of infected neurons, rather than to an indirect effect of inflammation, as previously stated by Simon et al. (1970).

Cells in which poliovirus replicated were found far from the inoculation site, demonstrating viral dissemination within the CNS. The mechanism of poliovirus spread within the CNS is not yet clear. It seems unlikely that the virus is taken up, amplified and transported by migrating inflammatory or microglial cells because we found no indication of virus multiplication in inflammatory cells. The observation of a few poliovirus genomes in neutrophil polymorphonuclear cells confined to cellular ghosts suggests a passive uptake of the virus rather than virus replication in polymorphonuclear cells. This is strengthened by the fact that in none of the experiments performed over the last 10 years (Jubelt et al., 1980a; Hashimoto et al., 1984) has viral antigen been detected in inflammatory cells of the CNS from poliovirus-infected monkeys or mice. The role of these cells would therefore be restricted to removal of dead cells.

An alternative hypothesis assumes axonal transport of poliovirus as the dissemination mechanism within the CNS. This is a normal pathway of diffusion for other neurotropic viruses such as herpes simplex type 1 (Kristensson & Lycke, 1971), measles (Fournier et al., 1985) and rabies viruses (Murphy et al., 1973; Bijlenga & Heaney, 1978; Gillet et al., 1986) and seems probable for poliovirus. Our results show the presence of viral genomes in neuronal processes of poliovirus-infected monkeys. Previous work by Dal Canto et al. (1986) showed the presence of poliovirus antigen in motor neuron processes and in synaptic elements in poliovirus-infected...
mice by ultrastructural immunochemistry. Indirect evidence of axonal transport of poliovirus within the CNS of mice comes from the studies of Jubelt et al. (1980b, 1986) reporting that (i) cordectomy prevented virus dissemination despite intact cerebrospinal fluid circulation, (ii) dissemination of poliovirus from the sciatic nerve to the CNS was inhibited by soaking the nerve in colchicine (a fast axonal transport inhibitor) 24 h before virus inoculation into the gastrocnemius muscle, and (iii) the relative resistance of newborn mice to intracerebral inoculation of poliovirus could be explained by maturational changes in the fast axonal transport system. All these results support the involvement of axonal transport as a probable mechanism for virus diffusion within and towards the CNS.

The presence of poliovirus RNA in small neural cells of the grey matter of monkey CNS indicates that motor neurons are not the only cells susceptible to poliovirus, as described by Simon et al. (1970). We have not tested specific markers to identify these nerve cells, but they might be astrocytes because of their non-specific localization in the grey matter. Moreover, as processes of astrocytes are tightly associated with the endothelial cells of capillary walls (Goldstein & Betz, 1986; Janzer & Raff, 1987) and with the surface of the axon (Raff, 1989), they would be a pathway for viral spread within the CNS.

Results similar to those for the CNS of paralysed monkeys infected with PV-1/S1-39-C6 were obtained for the CNS of mice paralysed after intracerebral inoculation with PV-2/MEF1 or PV-2/Lansing (Couderc et al., 1989a).

In monkeys intraspinally inoculated with attenuated PV-1/Sabin, viral RNA was found in motor neurons only at and around the site of inoculation at the level of the lumbar cord, whether clinical symptoms appeared or not. As compared to monkeys paralysed after intraspinal inoculation of the virulent strain, a significantly smaller number of infected motor neurons was found in monkeys inoculated with PV-1/Sabin, which corresponds to the absence or the weakness of clinical symptoms. It is not clear why an animal inoculated with PV-1/Sabin developed paralysis. However, clinical signs in monkeys receiving attenuated viruses do not always parallel the severity of lesions found upon microscopic examination. The infected neurons of monkeys inoculated with PV-1/Sabin (sacrificed 7 or 21 days p.i.) harboured a number of viral genomes similar to that observed after inoculation of the neurovirulent strain (sacrificed 2 or 3 days p.i.). This suggests that attenuated virus can carry out efficient RNA replication in the nerve cells which it infects. La Monica & Racaniello (1989) reported that, in human neuroblastoma cells in vitro, attenuated poliovirus displayed kinetics of viral RNA synthesis similar to those of the virulent strain, which had only a single amino acid difference in the 5' non-coding region. The origin of the significantly more intense inflammatory reaction found in monkeys infected with the attenuated strain is obscure. It might be a non-specific inflammatory reaction to the trauma of the intraspinal injection of the PV-1/Sabin. Monkeys infected with this strain were sacrificed 7 or 21 days p.i., but the animals injected with the virulent strain were sacrificed only 2 or 3 days p.i. In the latter case, the time lapse would be too short for non-specific immunological responses to the trauma. An alternative to the above mentioned hypothesis might be that slower multiplication of attenuated virus would leave enough time for the immune system to develop an effective defence, contrary to the rapidly multiplying virulent revertant.

At the present time, we have no answer to the question of how poliovirus gets from the blood to the CNS. Some of our results showed that poliovirus replication in human endothelial cells might be involved in poliovirus transport across the blood–brain barrier (Couderc et al., 1989b). The study of poliomyelitis pathogenesis will be benefited by using knowledge acquired from new data emerging from molecular characterization of the cellular poliovirus receptor (Shepley et al., 1988; Mendelsohn et al., 1989). Molecular probes which detect poliovirus receptor transcripts will help define human tissues susceptible to virus infection. In situ hybridization, as developed and applied here, will provide a complementary tool necessary for the demonstration of poliovirus replication in cells of the living organism. By its specificity and its simplicity for the detection of poliovirus genomes in animal tissues, this technique can be further exploited for testing the neurovirulence of oral poliovaccine and for defining the aetiology of lethal human paralysis of unknown viral origin.
Poliovirus-induced neural lesions

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