

## Cell-to-cell spread of poliovirus in the spinal cord of bonnet monkeys (*Macaca radiata*)

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In order to study the spread of poliovirus in the spinal cord of bonnet monkeys, 10<sup>8</sup> TCID<sub>50</sub> Mahoney strain of poliovirus was inoculated into the ulnar nerves of monkeys that were subsequently autopsied on days 1, 2, 3, 6, 9, 12, 14, 15 and 16 post-inoculation (p.i.). Virus spread in the spinal cord, the accompanying histopathological changes and paralysis occurred in a cervico–thoraco–lumbar direction. Virus reached the cervical region of the spinal cord within the first 3 days and subsequently spread to all segments of the spinal cord. *In situ* hybridization demonstrated viral RNA initially in the cervical neurons on day 3 p.i. and in the anterior horn neurons of lumbar segments of the spinal cord by day 6 p.i. Loss of Nissl substance in some of the anterior horn neurons was apparent on day 3 p.i. in

the cervical and thoracic regions and by day 6 p.i. in the lumbar region. In the lumbar region, neurophagia was a consistent feature which was observed on days 6–9 p.i., followed by neuronal dropouts on day 12 p.i. and thereafter. In the cervical and thoracic region, reappearance of Nissl substance was apparent from day 12 p.i. Upper limb paralysis preceded lower limb paralysis (5.5–1.73 vs 8.18–2.18,  $P = 0.046$ ), further suggesting that virus spread within the spinal cord was via an intraneural route despite persistent viraemia detectable from day 2 p.i. onwards. The temporal distribution of the virus spread, distribution of viral RNA, histopathological and clinical changes indicate a cell-to-cell spread of poliovirus in the CNS, having gained access to the CNS from the peripheral nerve.

### Introduction

Much of our understanding of the pathogenesis of poliovirus infection comes from studies carried out 40–50 years ago in chimpanzees (Bodian, 1952, 1956) and cynomolgus (Faber *et al.*, 1943, 1948*a, b*, 1950*a, b*, 1951; Wenner *et al.*, 1959) and rhesus monkeys (Horstmann, 1952). However, the route of virus spread from its primary site of replication to the CNS remains unclear. The earliest animal experiments with poliovirus by Fairbrother & Hurst (1930) and by Faber *et al.* (1943, 1948*a, b*, 1950*a, b*, 1951) suggested that ingested virus enters via nerve endings of the buccopharyngeal and intestinal tract mucosa, multiplies in the neurons of regional ganglia, and then spreads centrifugally into the alimentary tract or centripetally into the CNS. However, the demonstration of viraemia in humans (Horstmann & McCoullum, 1953; Horstmann *et al.*,

1954) and experimental animals (Horstmann, 1952; Bodian, 1956) provided an alternative hypothesis. With the development of the transgenic mouse model of poliovirus infection (Ren *et al.*, 1990), the neural pathway of spread of the virus to the CNS gained favour once again, since in this model, when poliovirus was inoculated intramuscularly, the limb of inoculation was the first to be paralysed and the LD<sub>50</sub> for intramuscular and intracerebral inoculation were similar (Ren & Racaniello, 1992). Transection of the sciatic nerve blocked spread of virus to the spinal cord. Further spread to the CNS was not described. These observations suggest that after poliovirus replicates in the muscle, it travels through the nerves to reach the CNS. The transgenic mouse model also differs from human infection in that the gastrointestinal (GI) tract of the transgenic mouse does not support replication of the virus, whereas in humans, poliovirus is likely to gain entry through the microfold cells of the GI tract (Sicinski *et al.*, 1990), and in a small proportion of individuals spreads to the CNS.

In order to study the intraneural spread of poliovirus, we developed a model of paralytic poliomyelitis using the bonnet

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monkey (*Macaca radiata*), which is an unendangered species of primates, readily available in Southern India. When type 1 poliovirus (Mahoney strain) is administered either orally (Selvakumar & John, 1987), intramuscularly or intravenously (T. J. John, unpublished observation), bonnet monkeys excrete virus in the throat and faeces for periods of up to 20 days, but paralysis is rare with any of these routes of infection. However, CNS infection was achieved by direct intraneural injection of doses  $> 10^5$  TCID<sub>50</sub> of type 1 poliovirus (Mahoney strain) into the ulnar nerve. This produced flaccid paralysis of the limbs in all animals (John *et al.*, 1992), suggesting that once virus gained entry into a peripheral nerve it spread neurally.

In children with poliomyelitis, paralysis occurs predominantly in large muscles of the limbs, especially the lower limbs, with predominant involvement of the lumbar and cervical regions (Simoes, 1994). However, since anterior horn neurons of all regions of the spinal cord (cervical, thoracic, lumbar and sacral) possess poliovirus receptors (PVRs; Ren *et al.*, 1990), it is unclear why only select regions are affected. If virus was reaching the CNS through a haematogenous route, it would be expected that the neurons of all regions of the spinal cord would be equally infected. Alternatively if virus entered the spinal cord through a nerve and subsequently spread in the CNS, it could cause selective involvement of neurons in the CNS. We examined this last possibility using an intraneural route of inoculation. In this study we sought to define subsequent spread of virus in the spinal cord; to determine if this pattern of spread resembled the distribution of paralysis seen in humans; and to determine pathways of spread from segment to segment.

## Methods

**■ Monkey inoculation, observation and sample collection.** Feral juvenile bonnet monkeys weighing between 1 and 2 kg were quarantined for 4–6 weeks, serum neutralizing antibody titres were determined against the three serotypes of poliovirus, and virus cultures were obtained for enterovirus from throat swabs and stool samples. Healthy seronegative, tuberculin-negative animals were inoculated with the Mahoney strain of poliovirus type 1 in the ulnar nerve as described previously (John *et al.*, 1992). In brief, the monkeys were anaesthetized with intramuscular thiopentone sodium (25 mg/kg body weight) and the skin and underlying fascia incised between the medial epicondyle and the olecranon process and the right ulnar nerve was exposed (John *et al.*, 1992; Samuel *et al.*, 1993).  $10^8$  TCID<sub>50</sub> virus in 100  $\mu$ l minimal essential medium (MEM) was injected very slowly with a tuberculin syringe into the exposed ulnar nerve ensuring that the full inoculum was given intraneurally. The wound was then cleaned, sutured and dressed. Perioperative antibiotic prophylaxis with  $6 \times 10^5$  IU benzathine penicillin was given intramuscularly into the lateral thighs.

Monkeys were observed daily for paralysis. Shedding of virus from the GI tract was monitored daily by virus isolation from throat swabs and stool samples until autopsy. Viraemia was assayed daily in heparinized blood for the first week (or until autopsy if sacrificed earlier). Poliovirus-specific antibody response was measured in serum collected on the day of inoculation and at autopsy, or on day 7 post-inoculation (p.i.) for animals sacrificed after 6 days. A sample of cerebrospinal fluid (CSF) was

obtained just prior to autopsy by cisternal puncture for virus isolation and antibody determination.

Virus-inoculated monkeys were autopsied on days 1, 2, 3, 6, 9, 12, 14, 15 and 16 p.i. A control monkey was inoculated with MEM and autopsied on day 7 p.i. At autopsy a laminectomy was performed and the distance from medial epicondyle of the olecranon process to the cervical spinal cord was measured. Spinal cord segments were collected from the upper and lower regions of the cervical, thoracic and lumbar cord in MEM for virus isolation, and in Carnoy's fixative for histology. Specimens from monkeys autopsied on days 3, 6 and 9 p.i. were also collected in paraformaldehyde-lysine-periodate fixative for *in situ* hybridization.

**■ Virus culture and serological assays.** Primary monkey kidney cell cultures were used for virus isolation from throat swabs, stool, plasma, buffy coat and spinal cord (10% w/v) suspensions, and Hep-2 cells were used for subsequent titration using standard methods (WHO, 1992). Virus isolates were confirmed to be poliovirus type 1 using type 1 antiserum in a microneutralization assay. Neutralizing antibody titres against type 1 poliovirus in sera and CSF samples were determined with the quantal microtitre assay using  $10^2$  TCID<sub>50</sub> virus (WHO, 1992).

**■ Histology.** Specimens collected in Carnoy's fixative were dehydrated in graded ethanol, cleared in chloroform and embedded in paraplast. Five  $\mu$ m and 10  $\mu$ m sections were stained with Harris's haematoxylin and eosin (H&E) and cresyl violet (CV) respectively. Histological changes were graded with a modification of the WHO system for scoring the spinal cord lesions for neurovirulence testing of vaccine strains of poliovirus: 0, no infiltration and no neuronal damage; 1, cellular infiltration with no neuronal damage; 2, cellular infiltration with minimal neuronal damage; 3, cellular infiltration with extensive neuronal damage; 4, massive neuronal damage with or without cellular infiltration (WHO, 1982). The hemisectional scores were determined blindly and averaged for each region of the spinal cord.

**■ In situ hybridization.** The anti-sense riboprobe was generated from a cloned cDNA fragment (nt 1–1809) of the 5' end of poliovirus type 1 (Mahoney strain) in a pDS14 plasmid using SP6 polymerase. Transcription was performed in the presence of 0.04 M Tris-HCl pH 8.0, 6 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 2 mM spermidine, 10 mM NaCl, 0.1 unit RNase inhibitor, 1 mM each of ATP, GTP and CTP, 0.65 mM UTP and 0.35 mM digoxigenin-11-UTP (DIG-11-UTP). Following transcription, the mixture was treated with DNase, phenol-extracted, ethanol-precipitated, and the RNA suspended in RNase-free water and subjected to alkaline hydrolysis in carbonate buffer (pH 10.2) for 20 min at 60 °C (Karr *et al.*, 1995). After neutralization with acetic acid, the fragments of 100–150 nucleotides were precipitated in ethanol and resuspended in RNase-free water. Specimens for *in situ* hybridization were dehydrated and embedded as for histology and 10  $\mu$ m sections were mounted on Superfrost Plus microscope slides (Fisher Scientific) and *in situ* hybridization performed as described earlier (Rotbart *et al.*, 1988). Briefly, sections were treated with 0.2 M HCl for 20 min followed by 0.38 mg/100 ml proteinase K at 37 °C for 20 min and hybridization was performed overnight at 42 °C with the digoxigenin-labelled probe in prehybridization solution. The next day, slides were washed in decreasing concentrations of saline sodium citrate ( $2 \times$ ,  $1 \times$ ,  $0.5 \times$  SSC) and treated with 20  $\mu$ g/ml RNase for 20 min at 37 °C. Specific binding of RNA was detected as described previously (Rotbart *et al.*, 1988). Anti-digoxigenin antibody tagged to alkaline phosphatase (Boehringer Mannheim) was used to detect DIG-11-UTP-labelled hybridized probe and coloured with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (Boehringer Mannheim). *In situ* hybridization studies were performed only on animals sacrificed on days 3, 6 and 9 p.i. Sections from each anatomical site of the control monkey were included as

negative controls in each hybridization experiment. Each experiment included a positive control containing sections from the upper lumbar cord of a monkey inoculated with Mahoney strain of poliovirus and autopsied on day 7 p.i. The specificity of the probe was checked by testing the positive control with a non-specific RNA probe (derived from a segment of varicella zoster virus DNA cloned into a pDS14 plasmid).

■ **Statistical methods.** A Student *t*-test was used to test the significance of the differences in the mean time from intraneural inoculation to onset of paralysis in the upper or lower limbs and for measurement of virus titres in different regions of the spinal cord. An analysis of variance was used to test the significance of differences in mean titres of virus isolated from cervical and lumbar regions of the cords.

**Results**

**Clinical observations**

Two monkeys were autopsied on each of the first 3 days p.i., none of which developed paralysis (Fig. 1). Nine of 10 monkeys observed thereafter developed paralysis from 4–11 days after inoculation, mostly of the lower limbs (7) and/or of the inoculated upper limb (4). The mean ( $\pm 1$  SD) incubation period for the onset of paralysis of the inoculated upper limb was 5.5 ( $\pm 1.73$ ) days and that of lower limbs was 8.18

( $\pm 2.18$ ) days ( $P = 0.046$ ). The mean ( $\pm 1$  SD) distance between the site of inoculation and the cervical spinal cord was 152 ( $\pm 24$ ) mm.

**Virus isolation from the spinal cord**

Poliovirus was isolated only from the cervical or upper thoracic cord prior to day 3 p.i. (Fig. 1), but from all segments of the spinal cord by days 6–9 p.i. The highest titres of virus were obtained from the upper lumbar region of three of those monkeys ( $P = 0.014$ , upper cervical vs upper lumbar;  $P = 0.06$ , lower cervical vs upper lumbar). On day 12 p.i., virus was present in very low titres; by day 14 p.i. there was only patchy isolation at very low titre. By days 15 and 16 p.i. no poliovirus was isolated from these three animals and very low titres of virus were present in the upper lumbar region of the fourth animal.

**Virus isolation from other sites**

Poliovirus was isolated from the CSF of one monkey on day 2 p.i., but was not isolated prior to or thereafter. Viraemia occurred regularly in all 16 monkeys in the first week after

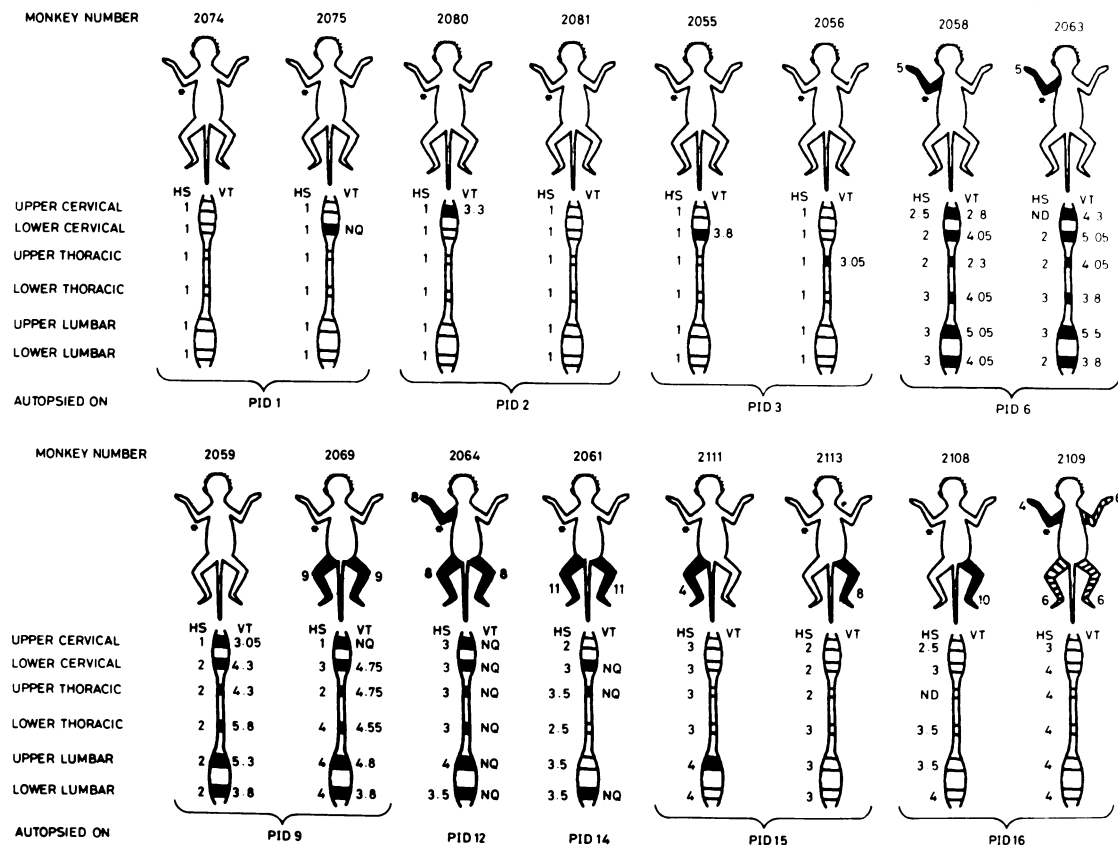


Fig. 1. Clinical picture and virological and histological findings in the spinal cord after intraneural inoculation of Mahoney strain of poliovirus type 1. The arm of inoculation is indicated with an asterisk, the limb of initial paralysis is completely shaded, the limb affected subsequently is hatched. The day p.i. when paralysis occurred is indicated by a number beside the limb. Positive virus isolation is indicated by shading on the spinal cord diagram; others areas were virus isolation negative. HS, Histological scores; VT, virus titres expressed as log<sub>10</sub> TCID<sub>50</sub>/g tissue (virus detected in the upper lumbar segment of monkey 2111 was not titrated); NQ, virus isolated but too low to be quantified; ND, not done.

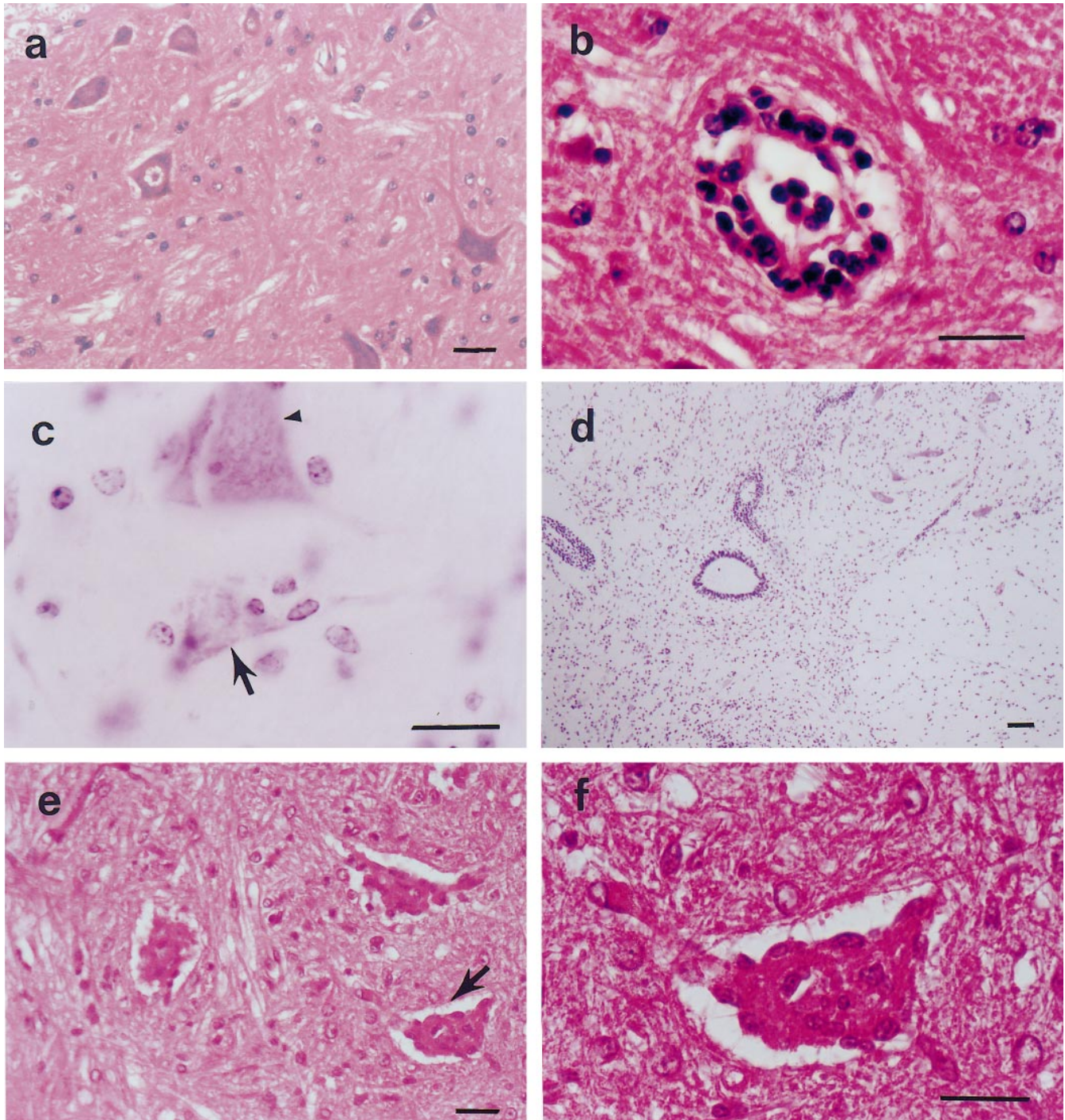


Fig. 2. Photomicrographs of histological changes. (a) Areas of cervical cord where foci of infiltration are seen in monkey no. 2081 on day 2 p.i. (H&E stain). (b) Focal infiltration under higher magnification (H&E stain). (c) Right cervical cord of monkey no. 2058 on day 6 p.i. showing neuron with loss of Nissl substance (arrow) and another with no loss of Nissl substance (arrowhead) after CV staining. (d) Infiltration seen in the lumbar cord on day 6 p.i. Note areas of heavy infiltration around vessel wall (CV stain). (e) Lumbar cord showing foci (arrow) of inflammatory cells which are phagocytosing neurons (H&E stain). (f) Higher magnification of a neuron undergoing neuronophagia in lumbar cord on day 6 p.i. (H&E stain). Bars, 20  $\mu\text{m}$  (a, b, c, e, f); 50  $\mu\text{m}$  (d).

inoculation with titres of  $10^{2.05}$ – $10^{4.55}$  TCID<sub>50</sub>/ml plasma. Peak titres occurred on day 2 and declined thereafter. Virus was detected in the throats of monkeys from day 1 p.i. (2 of 10

monkeys tested) to day 10 p.i. (3 of 4 tested), and in the faeces from day 2 p.i. (1 out of 14 monkeys) up to day 13 p.i. (2 out of 6 monkeys).

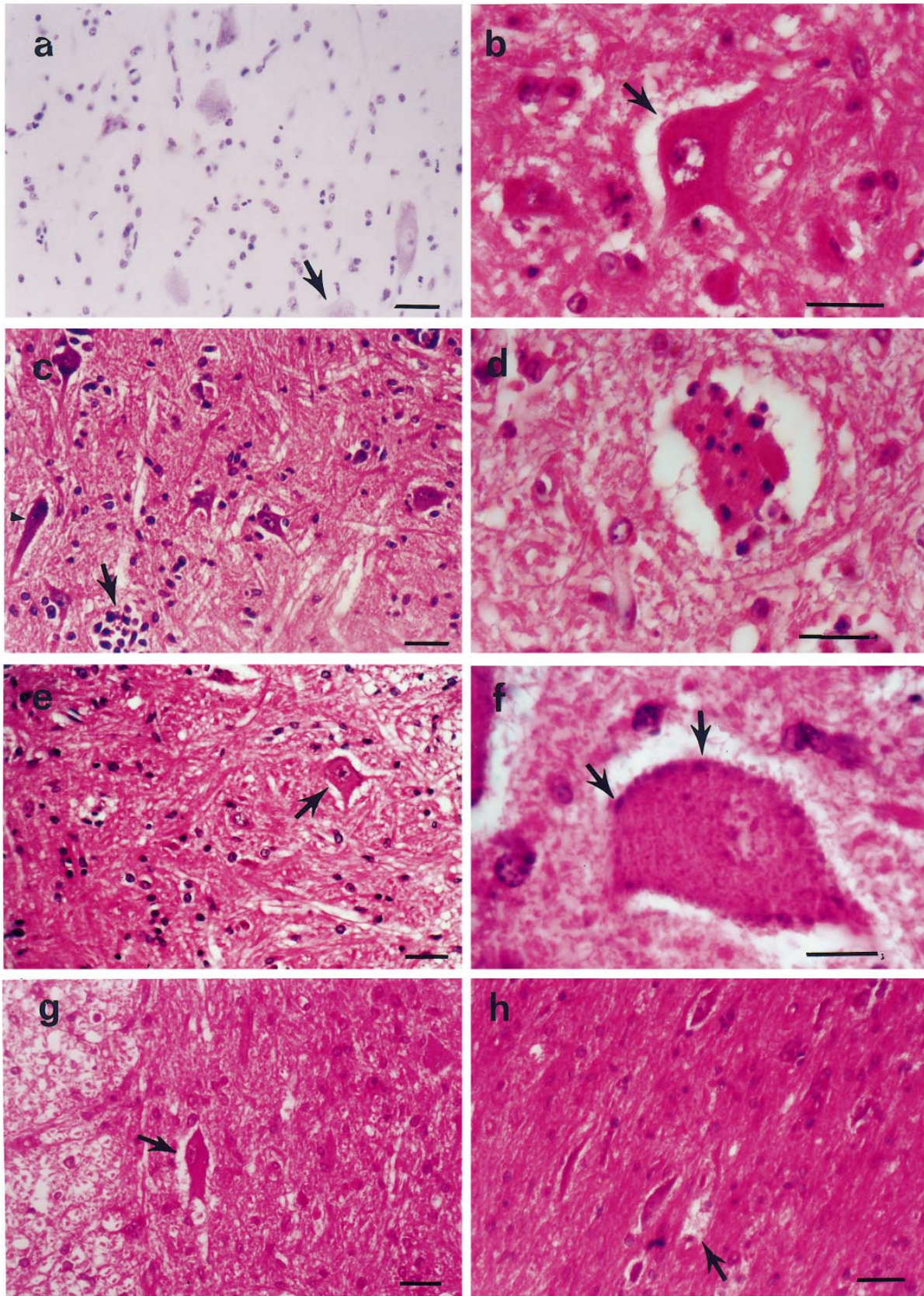


Fig. 3. For legend see p. 2398.

### Neutralization antibody response

All monkeys not sacrificed prior to day 6 p.i. developed antibodies by day 7 p.i., with titres ranging from 2 to 1024. The geometric mean titre of neutralizing antibody on day 7 p.i. was 70.

### Histopathological findings in the spinal cord

On day 1 p.i. there was generalized infiltration throughout the cord, primarily with mononuclear cells surrounding normal-appearing neurons. In the cervical and lumbar posterior horn region, streaming of lymphocytes was observed. The meninges were normal. On day 2 p.i. there was an increase in generalized infiltration with scattered foci of infiltrating cells around vessel walls only on the right side of the cervical cord of both monkeys examined. The infiltrating cells were neutrophils and macrophages (Fig. 2*a, b*). By day 3 p.i. there was mild loss of Nissl substance in some of the neurons in the anterior horns of cervical and thoracic regions and the neutrophilic infiltration was more intense. On day 6 p.i., nuclei of the cervical cord were pyknotic with loss of Nissl substance (Fig. 2*c*). There was remarkable surrounding infiltration (Fig. 2*d*). In the thoracic cord, neurons demonstrated loss of Nissl substance with glial cell proliferation, while neuronophagia (Fig. 2*e, f*) with surrounding glial cell proliferation was visualized in the lumbar cord. On day 9 p.i., neuronophagia was not apparent in the cervical and thoracic cord (Fig. 3*a, b*), although it was a prominent feature in the lumbar region (Fig. 3*c, d*). On day 12 p.i., the cervical (Fig. 3*e, f*) and thoracic (Fig. 3*g*) cords showed peripheral staining of Nissl substance in neurons, while there were neuronal dropouts in the lumbar region (Fig. 3*h*). On day 14 p.i., the lumbar cord demonstrated extensive neuronal dropout on the right side with some neuronal damage on the left side, but there was a decrease in inflammatory cells. By days 15 and 16 p.i. there were fewer persisting inflammatory cells in the spinal cord of both monkeys with anterior horn neuronal dropouts in the lumbar cord. The neurons in the cervical and thoracic regions were recovering, as demonstrated by the reappearance of Nissl substance in their periphery.

### *In situ* hybridization for the demonstration of poliovirus replication

On day 3 p.i., viral RNA was detected only in a few neurons of the anterior horn of the cervical region in both

monkeys. (Fig. 4*a, b, c*). By day 6 p.i., aside from occasional neurons in the cervical region, signals were seen in a few axons in the intermediate column and in occasional neurons of the thoracic cord and in an appreciable number of neurons of the lumbar cord (Fig. 4*d, e, f*). On day 9 p.i. only a few neurons showed viral RNA in the cervical and thoracic regions. In the lumbar region, neurons, dendritic processes and axons demonstrated strong hybridization signals (Fig. 5*a*) and inflammatory cells in the gray matter contained viral RNA. Some of the neurons in the lumbar region, in various stages of degeneration, had strong signals (Fig. 5*b, c*). Others undergoing neuronophagia did not contain viral RNA, but signal was detected within the surrounding phagocytic cells (Fig. 5*d*).

## Discussion

### Virus entry into the spinal cord

We used assays of virus replication and virus-induced cytopathology in the CNS to study pathogenesis of poliomyelitis in bonnet monkeys after ulnar nerve infection. Early after infection virus was isolated only from the cervical or the upper thoracic spinal cord segments. Since the ulnar nerve originates from these segments, we hypothesize that virus reached the CNS via the ulnar nerve, although viraemia was also an early event. However, had spread been haematogenous, the lower half of the cord should contain virus. Furthermore, viraemia was present at early time-points before virus was demonstrated in the spinal cord. Although virus was transported to the spinal cord in all inoculated animals, virus was isolated from the CSF in only one animal, thereby eliminating the CSF as a predominant mode for spread.

Virus was reproducibly isolated, and replicative intermediates indicative of active replication of the virus were demonstrable within the cervical spinal cord, by 72 h. The average length of the upper limb axon in these monkeys from the site of inoculation to the cervical spinal cord was found to be 152.5 mm. Thus, the rate of transport of the virus is 152.5 mm per 72 h, or 2.1 mm/h. This matches well with the rate of transport of virus calculated by earlier workers (2.4 mm/h; Bodian & Howe, 1941).

Two major theories have been invoked to explain how poliovirus reaches the CNS: that it enters the CNS from blood across the blood-brain barrier (BBB) (Nathanson & Bodian, 1961*a, b*) or is transmitted to the CNS via a peripheral motor nerve (Bodian & Howe, 1941; Ren & Racaniello, 1992;

**Fig. 3.** Photomicrographs of histological changes. (*a*) Cervical cord of monkey no. 2069 on day 9 p.i. showing neurons with loss of Nissl substance (arrow) (CV stain). Note there is no neuronophagia. (*b*) Damaged thoracic cord neuron (arrow) with no neuronophagia on day 9 p.i. (H&E stain). (*c*) Lumbar cord on day 9 p.i. showing extensive neuronophagia (arrow). An occasional neuron is spared (arrowhead) and is not surrounded by the inflammatory cells as in the case of other neurons undergoing neuronophagia (H&E stain). (*d*) Neuronophagia in the lumbar region on day 9 p.i. (H&E stain). (*e*) Cervical cord of monkey no. 2064 on day 12 p.i. showing neurons in the recovery phase (arrow) (H&E stain). (*f*) Neuron with Nissl substance in the periphery (arrows) on day 12 p.i. shown under higher magnification (H&E stain). (*g*) Lower thoracic cord of monkey no. 2064 on day 12 p.i. Note the damaged neuron (arrow) with nucleus pushed to the side, and loss of Nissl substance and the absence of neuronophagia (H&E stain). (*h*) Lumbar cord of monkey no. 2064 on day 12 p.i. showing loss of neurons (arrow) (H&E stain). Bars, 20  $\mu$ m.

Gromeier & Wimmer, 1998). Evidence favouring entry across the BBB includes the following observations: (a) viraemia appears necessary for spread of virus to the CNS (Nathanson & Bodian, 1961a, b); (b) poliovirus virulence correlates with the duration of viraemia (Bodian, 1954, 1955); and (c) passively administered poliovirus antibody protects against paralysis (Nathanson & Bodian, 1962; Sabin, 1978; Salk *et al.*, 1984). The mechanisms for virus entering the CNS via the BBB have not been identified. It has been suggested that poliovirus infection of endothelial cells with subsequent CNS invasion may be an explanation. In favour of this hypothesis, virus antigen has been detected on vascular endothelial cells of poliovirus-infected monkeys (Kanamitsu *et al.*, 1967) and PVRs are present in endothelial cells (Couderc *et al.*, 1990). A second explanation invokes human monocytes that are susceptible to poliovirus infection and may transmit poliovirus across the BBB (Freistadt & Eberle, 1996). Recent experiments using radioactively labelled poliovirus to study distribution in tissues of PVRTg mice have questioned both of these mechanisms (Yang *et al.*, 1997). Poliovirus distribution in the CNS was not dependent on PVR expression since poliovirus was detected in equal quantities in PVRTg mice and non-PVRTg mice and monoclonal antibodies against PVR had no effect on CNS distribution in the former. In this experiment poliovirus did not associate with the monocytes and they were not implicated as carriers of the virus into the CNS. While entry of the virus at the BBB is an attractive hypothesis, poliovirus is very rarely isolated from the CSF of children with poliomyelitis (Simoes, 1994), and in children with a breach in the BBB (shunt infections), poliovirus causes an aseptic meningitis but not paralytic poliomyelitis (Gutierrez & Abzug, 1990).

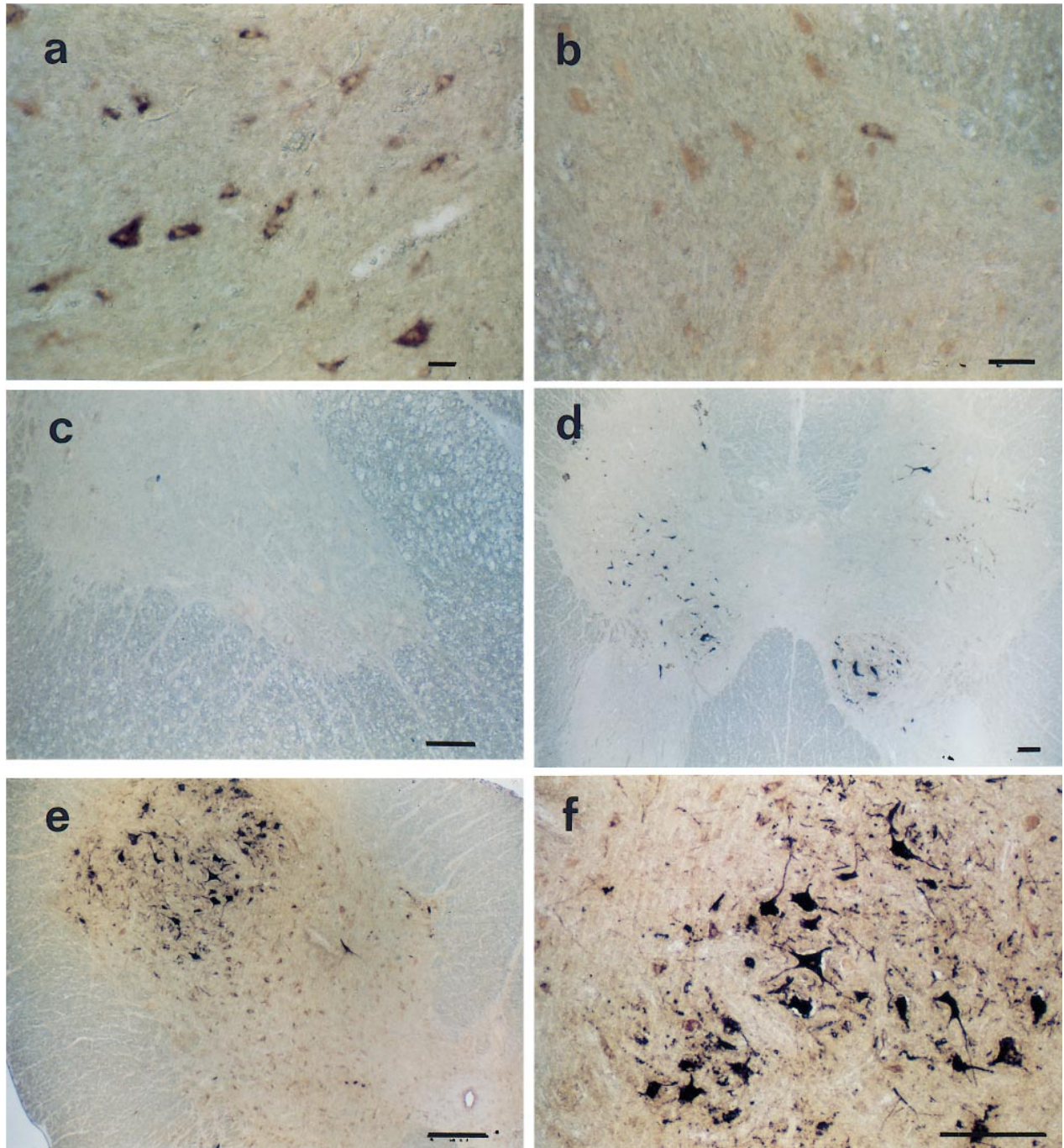
Evidence of virus entry through peripheral nerves was supported by the observation of 65 cases of paralytic polio occurring 4 weeks following the inoculation of 400 000 persons with an inadequately inactivated vaccine (the Cutter incident) (Nathanson & Langmuir, 1963). Poliomyelitis occurred in the inoculated limb in 2/3 of the cases. In the PVRTg mice, transection of the sciatic nerve abrogates spread of poliovirus from infected muscle to CNS and prevents CNS infection (Ren & Racaniello, 1992). It has been observed for many decades that stimulation of a muscle either by overuse (exercise induced) or injection (provocation) during the incubation period of poliomyelitis provoked paralysis in that limb (Auld *et al.*, 1960; Lucchesi, 1934). These observations have now been extended to oral poliovirus vaccine, since it was demonstrated that intramuscular injections provoked paralysis in recipients with vaccine strains of poliomyelitis (Strebel *et al.*, 1995). An explanation for this phenomenon is that skeletal muscle injury stimulates the retrograde axonal transport of poliovirus to the CNS at an enhanced rate, resulting in provocation paralysis (Gromeier & Wimmer, 1998). We have been able to prevent retrograde axonal transport of poliovirus in the ulnar nerves of monkeys by pretreating the nerve with colchicine (that selectively blocks retrograde transport) (E. M. Ponnuraj, un-

published observations). In the current study, despite an abundant viraemia, virus spread in the CNS was not random, but followed a cranio-caudal progression, with the site of initiation being the neurons of origin of the ulnar nerve. In our study, it is very unlikely that the intramuscular penicillin injections that the monkeys received in the thighs influenced the entry of poliovirus from the ulnar nerve into the spinal cord, its interneural transmission within the cord, or the recovery of cervical neurons after infection. It is, however, possible that neuronal death and neuronophagia in the lumbar region could have been enhanced.

### Spread of the virus within the spinal cord

We observed a cranio-caudal spread of the virus in the spinal cord as evidenced by the clinical picture, the pattern of paralysis, the sequence of histological changes and the temporal appearance of virus. The arm of inoculation was the first limb to be paralysed with a mean incubation period of  $5.5 \pm 1.73$  days, whereas the mean incubation period for the onset of paralysis of the lower limbs was  $8.18 \pm 2.18$  days ( $P = 0.046$ ). The inoculated upper limb was involved significantly earlier than the lower limbs in these animals with multiple limb involvement, which suggested that virus reached the cervical cord first and subsequently spread caudally to the lumbar cord. Histology demonstrated that there was a loss of Nissl substance in the neurons of the cervical cord on day 3 p.i., while the anterior horn cells of the lumbar region were still normal. By day 6 p.i. the lumbar anterior horn cells were not only deficient in Nissl substance, but were also undergoing neuronophagia, which progressed further on day 9 p.i., leading to neuronal dropouts by day 12 p.i., while neurons in the upper cord were recovering. Virus was first isolated from the cervical or upper thoracic cord during the first 3 days of infection, followed by isolation of virus from the lumbar cord by day 6. *In situ* hybridization studies also supported a cranio-caudal progression of spread. Viral RNA was detected in the cervical cord neurons on day 3 p.i., with a caudal spread to the lumbar region by day 6 p.i. Viral RNA was present in the neurons of the cervical and lumbar regions, but only in axons in the thoracic region. This correlated well with higher virus titres in the lumbar cord than in the thoracic region on day 6 p.i.

*In situ* hybridization revealed that the supportive cells of the CNS did not contain poliovirus genome, and that only neurons, their processes and some of the inflammatory cells contained viral RNA. Taken together with the observation that only axons and not neuronal bodies appear to have viral RNA in the thoracic cord, we concluded that intraneural spread was mainly via the neural bodies and their processes. This spread was most likely via intrasegmental tracts within the anterior, posterior and lateral funiculi which form a network of intersegmental connecting tracts. The alpha and gamma motor neurons receive connections from these intersegmental tracts (Gray, 1985).

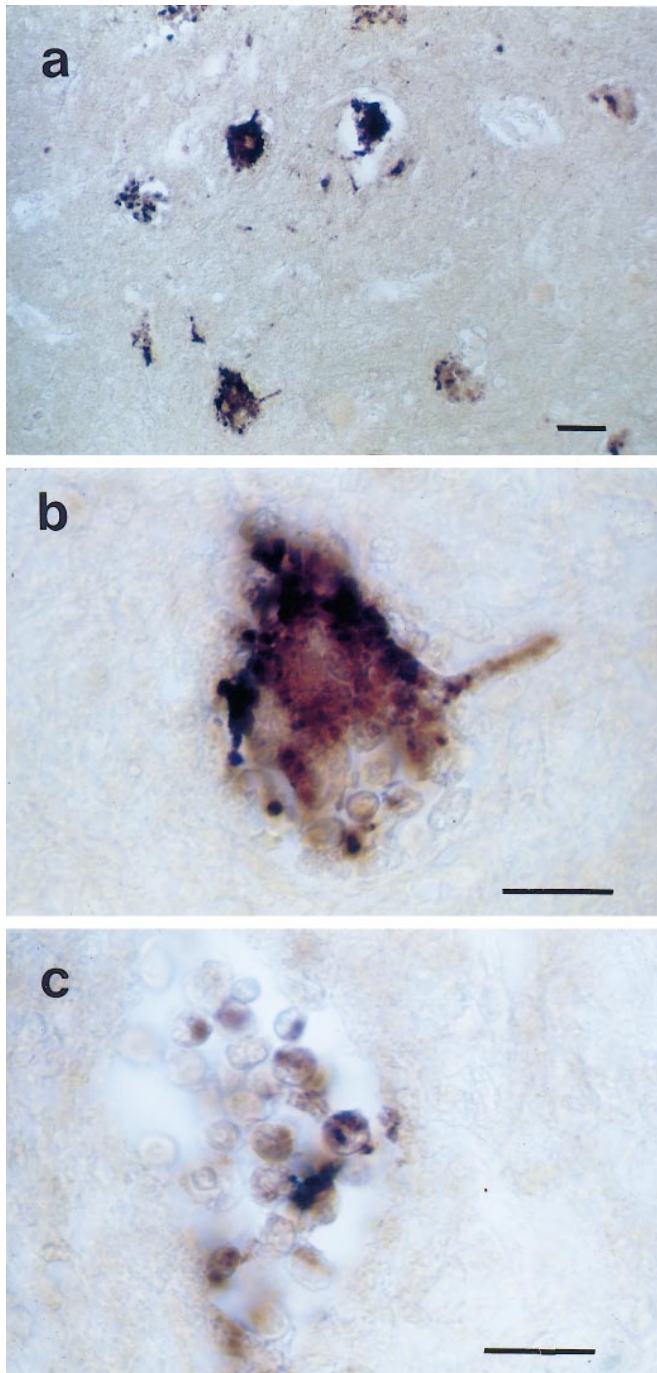


**Fig. 4.** Photomicrograph of *in situ* hybridization of spinal cord tissues. (a) Upper cervical region showing neurons with signals in the anterior horn region of monkey no. 2055 on day 3 p.i. (b) Lower cervical region of monkey no. 2056 with signals on day 3 p.i. (c) Lumbar cord without signals on day 3 p.i. (d) Upper lumbar segment showing signals in neurons and a few axons on day 6 p.i. (e) Lower lumbar segment with signals in the neurons and its processes on day 6 p.i. (f) Higher magnification of lower lumbar segment with signals in the neurons and its processes. Bars, 20  $\mu$ m.

The inflammatory cells do not appear to play a role either in the spread of infection from the ulnar nerve to the spinal cord or within the spinal cord. Thus, there was no evidence of infection of the inflammatory cells on day 3, when polioviral mRNA was only demonstrated within neurons. Polioviral

mRNA was demonstrated in inflammatory cells in the lumbar region only following neuronophagia on the day 9 p.i., suggesting that the inflammatory cells were responsible for the clearance of the virus and infected neurons and not directly involved in spread of the virus.





**Fig. 5.** Photomicrograph of *in situ* hybridization of spinal cord tissues collected on day 9 p.i. (a) Lumbar cord neurons showing varying stages of neuronophagia. (b) Lumbar neuron with some areas with signals and some areas without signals and phagocytosing cells with signals. (c) Lumbar region showing neuronal dropout and signals present in phagocytes. Bars, 20  $\mu$ m.

#### Site-specific neuronal damage

The decline in virus titre in the lumbar cord correlated with virus clearance by phagocytes and the presence of viral RNA in phagocytes on day 9 p.i. On day 12 p.i. virus was

undetectable and phagocytes were no longer seen in the areas of neuronophagia, where neuronal dropouts were demonstrated. Neurons in the cervical and thoracic cord did not undergo massive neuronophagia, and the content of virus in this region decreased gradually. Reappearance of Nissl substance in the cervical cord at a later time-point, indicative of neuronal recovery (Bodian, 1949), suggested that virus–cell interactions were different in the neurons at different anatomical sites. PVRs are distributed throughout the spinal cord (Brown *et al.*, 1987). Since we found that virus replicated efficiently in both cervical and lumbar regions of the spinal cord, the difference in pathology may not be at the level of the receptor. Hence, we hypothesize that steps in virus replication leading to cell death or virus clearance are different in neurons located in different anatomical sites. In the lumbar region, phagocytic cells removed the neurons and viral RNA was present in the cytoplasm of these phagocytic cells. In contrast, this was not observed in the cervical region and cervical neurons did not appear to undergo cytolysis. The absence of cytolysis in the neurons of the cervical cord was associated with the reappearance of Nissl substance in the periphery. Thus, the mechanism of clearance of virus in the neurons of the cervical cord may be different from that seen in the lumbar region. From these studies it appears that poliovirus has a non-cytolytic cycle of infection in the cervical neurons. Replication of an attenuated strain of poliovirus in tissue culture also has shown that the outcome of infection may be a conventionally observed cytolytic infection in Vero and Hep-2 cell cultures, or may remain as a noncytolytic infection in neuroblastoma cell cultures (Agol *et al.*, 1989).

#### Extraneural spread of virus after intraneural inoculation

Viraemia, a consistent feature in every monkey, was also prolonged, and lasted longer than that seen in perorally fed chimpanzees (Bodian, 1956) and bonnet monkeys (T. J. John, unpublished data). All intraneurally inoculated monkeys demonstrated a viraemia with peak titres of plasma virus on day 2 p.i., considerably earlier than peak CNS titres, at days 6–9 p.i. This could reflect different rates of travel from the site of inoculation to the CNS or to extraneural sites of replication responsible for the viraemia. It is likely that intraneural spread is limited because of physical constraints, and the viraemia reflects the seeding from multiple sites of replication. Viraemia may have originated from spill-over into non-neural tissues at the time of intraneural inoculation. However, virus is more likely to have entered the blood through the blood vessels lining the ulnar nerve and the spinal cord. We have demonstrated viral RNA in blood vessels of the ulnar nerve infected with the Mahoney strain of poliovirus of another group of experimental monkeys (E. M. Ponnuraj & E. A. F. Simoes, unpublished data). This may explain why viraemia occurs from day 1 p.i. and how such high titres of virus are seen

in the blood in comparison to monkeys infected either orally or intramuscularly.

GI excretion was present in some of these intraneurally inoculated monkeys. Virus was found in the throat as early as day 1 p.i. and in the stool by day 3 p.i. The titres in faeces were not as high as measured in bonnet monkeys given 100 ID<sub>50</sub> poliovirus type 1 by mouth (Selvakumar & John, 1989). Thus, it appears that excretion from the GI tract in this model is secondary to the viraemia. Even when the virus is introduced into a peripheral nerve as we did, thereby bypassing the primary site of replication, virus enters the bloodstream from the nervous tissue and seeds the GI tract, from where it is shed. The predilection of poliovirus for the GI tract may be related to the survival of this virus in nature.

The antibody titres in monkeys inoculated intraneurally were surprisingly much higher than those seen in monkeys fed poliovirus type 1 orally (Selvakumar & John, 1987). This is probably due to the higher inoculum in the intraneurally inoculated monkeys, and the prolonged viraemia, which would have been responsible for greater stimulation of the immune system.

Not only has this study demonstrated that poliovirus spreads through a neural route after entry into the CNS, it also has demonstrated that replication of poliovirus and the outcome of infection is dependent on the host cell. It is likely that infection with poliovirus leads either to a cytolytic infection as seen conventionally or to an unconventional non-cytolytic replicative cycle which is apparently host-cell dependent.

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