Immunohistological characterisation of acute herpes simplex virus infection of lumbar dorsal root ganglia in the rat

J. M. BLONDEAU§, F. Y. AOKI† and J. I. NAGY‡

Departments of *Medical Microbiology and Infectious Disease, †Pharmacology and Therapeutics and ‡Physiology, University of Manitoba, Winnipeg, Manitoba, Canada

Summary. After inoculation of herpes simplex virus (HSV) into the dorsal skin of the hind paw of rats, HSV antigen was detected in dorsal root ganglion neurones by immunohistochemistry. Antigen was first detected 2 days after inoculation. Between 2 and 4 days after inoculation, numerous small-type unmyelinated neurones gave positive staining reactions for HSV antigens. Necrosis of neuronal cells was evident by 4 days. Antigen was not detected 6 or 9 days after inoculation. Immunohistological studies of co-cultivated ganglia removed from the infected monolayers, immediately after cytopathic effect first appeared or 24 h later, uniformly revealed virus antigen staining of small-type unmyelinated neuronal cells.

Introduction

Herpes simplex virus (HSV) causes primary, latent and recurrent infections of man and experimental animals.1-3 A unique feature of this virus is its ability to establish latency within neurones of the sensory ganglia. In 1923, Goodpasture and Teague4 postulated, and Kristensson et al.5 demonstrated experimentally, that HSV spreads within axon(s) to the central nervous system (CNS) from skin or mucosal sites of infection. Since that time, numerous investigations have studied the spread of HSV within the peripheral nervous system (PNS) and the CNS in animals experimentally infected with this virus. Cook and Stevens6 demonstrated the presence of herpes-specific fluorescence in neuronal and satellite cells of mouse dorsal root ganglia after foot pad inoculation. Furthermore, electronmicroscopy has revealed virus particles within the neurones. Itoyoma et al.7 found that 2 days after inoculation of the cornea of mice with HSV type-1, isolated neurones within the trigeminal ganglion were positive for HSV and infected satellite cells were present in ring-like arrays around infected and apparently uninfected neurones; by 8 days, HSV antigen was still detectable in neurones but not in satellite cells. We have recently characterised acute and latent HSV type-1 infections in dorsal root ganglia of a new rat model.8 We now present detailed photographic data of immunohistological studies of acute and latent infections of rat dorsal root ganglia after peripheral inoculations with HSV type-1.

Materials and methods

Rats

Sprague-Dawley outbred female rats (Charles River Canada Inc., St Constant, Quebec) weighing 70–100 g were used. They were from a strain known to be free of HSV antibody. Rats were assigned to experimental and control groups by a table of random numbers. Rats (four cage) were maintained on laboratory chow and water ad libitum. The housing temperature was 20°–21°C with a 12 h on/off light schedule. Standards of general husbandry of the Canadian Council for Animal Care were maintained throughout this study.

Virus strain

HSV-76 virus, determined to be type-1 by restriction endonuclease analysis, was used for this study.8 The virus was propagated in Vero cells in mycoplasma- and antibiotic-free Minimal Essential Medium (MEM) (Flow Laboratories) with fetal bovine serum (FBS; Gibco) 5–10% inactivated for 30 min at 56°C. When infected monolayers showed 70–80% cytopathic effect (CPE), cell cultures were freeze-thawed twice and the suspension was clarified by low speed centrifugation and passed through a 0.22-µm filter (Nalgene). Small volumes were stored frozen at −70°C. The virus was titrated by calculating the median tissue culture infectious dose (TCID50) on Vero cells in Costar 24-well plates (Costar).9 The titre
of the virus stocks was log₁₀ 8.33 TCID₅₀/ml. Virus
was used after the fourth and fifth passage following
isolation from the patient. For some experiments,
stock virus was diluted in MEM or phosphate-buffered
saline (PBS).

Inoculation of rats

Rats were anaesthetised with pentobarbitol sodium
(Chront Laboratories) 5 mg/100 g injected intra-
peritoneally and 20 μl of stock virus solution was
injected into the dorsum of the hind paw unilaterally
or bilaterally with a Hamilton microlitre syringe and a
30-gauge needle. Control animals were inoculated with
PBS or MEM. All animals were examined twice daily
for 28 days for signs of neurological abnormality and
to determine time to death.

Recovery of HSV by culture of sensory ganglia

Rats were killed with pentobarbitol sodium
15 mg/100 g injected intraperitoneally. Once anaes-
thesitised, rats were bled by cardiac puncture for plasma
and serum samples which were stored at -20°C.
Fourth, fifth and sixth lumbar dorsal root ganglia were
removed and analysed for HSV as follows. By use of a
dissecting microscope at 40 × magnification, the spinal
cord was exposed and the appropriate lumbar ganglia
were identified and removed. Ganglia were placed
individually in vials containing 1.5 ml of MEM supple-
mented with FEBS 3% and antibiotics (penicillin
100 IU/ml, gentamicin 250 μg/ml and mycostatin
25 IU/ml) and incubated for 24 h at 37°C in CO₂
5% in humidified air. Ganglia were then explanted
individually on to monolayers of human neonatal
forearm fibroblast cells and left undisturbed for 48 h
at 37°C in CO₂ 5% in humidified air. Ganglia were then
incubated for 1 h at room temperature and rinsed with
PBS containing Triton 0.3% as above. Rabbit
immunoglobulin conjugated to peroxidase anti-per-
oxidase (PAP; Miles Scientific) was then added at a
dilution of 1 in 1 in 250 in PBS containing Trion 0.3% and
BSA 1%, incubated for 1 h at room temperature and rinsed with
PBS containing Trion 0.3% as above. Rabbit
immunoglobulin was used after the fourth and fifth passage following
isolation from the patient. For some experiments,
stock virus was diluted in MEM or phosphate-buffered
saline (PBS).

Immunohistochemistry

Rats were anaesthetised with 0.5–1 ml of chloral
hydrate 25% injected intraperitoneally. Immediately
following anaesthesia and before death, rats were
placed on ice, the heart was exposed and the right
atrium was severed. A 15-gauge needle was inserted
into the left ventricle and the rat was perfused with
50 ml of ice-cold prefix consisting of distilled H₂O
80%, saline (0.15 M NaCl) 10%, 0.5 M phosphate
buffer 10%, NaNO₃ 0.1% w/v and heparin 100 units.
Following this prefix, the rats were perfused with ice-
cold fixative consisting of distilled H₂O 80% and 0.5 M
phosphate 20% with paraformaldehyde 4% w/v. The
spinal cord was then exposed by dissection and
removed along with the left and right fourth, fifth and
sixth lumbar ganglia. In some rats, the brain was also
removed. Tissues were placed in fixative overnight and
transferred the following day to 0.1 M phosphate
(pH 7.4) containing sucrose 15% w/v for 1 day.

Tissues were mounted in O.C.T. mounting medium
on a metal chuck placed on dry ice. The chuck with
was placed at -26°C for 30 min to allow
temperature equilibration, and cryostat sections were
cut at a thickness of 15 μm and mounted on to gelatin-
coated microscope slides. Slides were removed from
-26°C one at a time and a border of plastic cement
was placed around the tissue to contain the antibody
solution. The tissue was covered with a polyclonal
rabbit antibody to HSV (Dimension Laboratory),
diluted 1 in 1000 in PBS containing Triton 0.3% and
bovine serum albumin (BSA) 1%, incubated for 48 h
at 4°C in a humidified chamber and then rinsed for
two 30-min periods in PBS containing Trion 0.3%.
The sections were then covered with goat anti-rabbit
immunoglobulin (Miles Scientific) at a dilution of 1 in
20 in PBS containing Trion 0.3% and BSA 1%,
incubated for 1 h at room temperature and rinsed with
PBS containing Trion 0.3% as above. Rabbit
immunoglobulin was used after the fourth and fifth passage following
isolation from the patient. For some experiments,
stock virus was diluted in MEM or phosphate-buffered
saline (PBS).

The characteristics of infection after unilateral
injection of 6.4 × 10⁶ TCID₅₀ of HSV-1 in the dorsal
skin of the hind paw were reported previously.³
Fig. 1. Immunoperoxidase staining for HSV in sections of dorsal root ganglia at various times after subcutaneous injection of $6.4 \times 10^6$ TCID$_{50}$ of HSV-1 into the ipsilateral dorsal hind paw: a, 2 days after inoculation—the arrow points to an area staining positive for HSV antigens; b, 58 h after inoculation—the small arrow (→) points to a small-type unmyelinated neurone stained positive for HSV antigen, the large arrow (●) to an unstained large-type myelinated neuron; c, 3 days after inoculation—the small arrow (→) points to a small-type unmyelinated neurone stained positive for HSV antigen, the large arrow (●) to an unstained large-type myelinated neuron; d, 4 days after inoculation—the small arrow (→) points to a small-type unmyelinated neurone stained positive for HSV antigen, the large arrow (●) to an unstained large-type myelinated neuron; e, 5 days after inoculation—the arrow points to a small-type unmyelinated neurone stained positive for HSV antigen (× 400).
Results

Immunohistological studies

HSV antigen was not detected in lumbar ganglia 1 day after inoculation. After 2 days, nerve fibres gave positive results for virus antigen in at least one ganglion of three rats tested (fig. 1a). Between 2 and 4 days, viral antigen was detected in numerous small-type neurons (fig. 1b, c and d). At 2-4 days, one or two cells/section were stained positive and at 4 days, 1-20 cells/section were stained positive for antigen. Necrosis of neuronal cells was evident by day 4 (fig. 1d). By day 5 (fig. 1e), only one ganglion had positive

cells and at days 6 and 9, no antigen was detected (data not shown).

Immunohistological studies of co-cultivated ganglia removed from the infected monolayers, either immediately after CPE first appeared or 24 h later, showed uniform virus antigen staining—primarily of small-type unmyelinated neuronal cells (figs. 2a and b). In addition, there was consistent staining around the peripheral edge of the ganglia corresponding to the tissue closest to the infected monolayer. Ganglia removed from monolayers not showing CPE after 5, 6 and 7 days of co-cultivation gave negative results for HSV antigens (data not shown).

Discussion

Subcutaneous injection of HSV into the dorsum of the hind paw of rats yielded predictable acute and latent HSV dorsal root ganglion infection that conformed to our current knowledge of this infection in otherwise healthy human subjects. Our selection of the hind foot site permitted injection without undue extravasation of virus suspension, and the delivery of an appropriate volume, the importance of which has been noted by others. The present rat model of human cutaneous infection had a number of characteristics. Infection was initiated by subcutaneous injection of $6.4 \times 10^6$ TCID50 of HSV-1 in 20 μl into the dorsum of a hind foot. HSV was detectable at the injection site up to 4 days after injection. Data from immunohistological studies and a sciatic nerve transection experiment indicated that HSV migrated centripetally within the sciatic nerve, causing lytic infection, primarily in the neuronal cell bodies of small unmyelinated fibres in lumbar sensory ganglia. The number of cells infected was maximal 3-4 days after inoculation (1-20 cells/section). In 25% of animals, paralysis of the ipsilateral leg developed and 30% died, presumably due to encephalitis caused by cephalad HSV migration along the central neuraxis. Virus also migrated centrifugally into the contralateral lumbar sensory ganglia but not into the skin of the contralateral hind foot, HSV complement-fixing antibody was detected in all rats—in some as early as the second day after infection. By the sixth day, HSV antigen was not detected in lumbar sensory ganglia but demonstration of lytic HSV infection in these ganglia at day 28 by co-cultivation indicated that infection had become latent.

Immunohistological staining of acutely infected rat ganglia (within 2-5 days) showed that primarily small-type unmyelinated neurones were displaying HSV antigens, as reported previously by Cook and Stevens in mice. However, they also reported infection of satellite cells and found that Schwann cells associated with myelinated axons were free of virus while those associated with non-myelinated axons were infected. Differences in virus susceptibility between neurones and supporting cells have also been seen by others and may be due to species differences.
Lytic infection in sensory ganglia of mice and rats was evident up to 8 days and 5 days, respectively. However, it is unclear whether this occurs in all human cutaneous HSV infection. In all animal models, an apparently asymptomatic latent infection of sensory ganglia follows the productive phase. This has been characterised by the appearance of HSV CPE in monolayer substrates on which ganglia are co-cultivated, at a longer interval than is required during cultivation of productively infected ganglia. In our system, the mean interval for HSV CPE to appear was 63% greater around ganglia removed 28 days after HSV inoculation than around those removed after 4 days.3

Immunohistological staining of latently infected ganglia associated with CPE in co-culture demonstrated an apparent central role, once again, for small-type unmyelinated neurones in amplification of HSV antigen in rat ganglia. However, staining primarily of small-type neurones for HSV antigens was not observed in studies in mice.10 Although there is little doubt that the virus recovered in cell culture originated from the co-cultivated ganglia, we cannot be certain whether the neurones staining positive for HSV antigen represented cells that were displaying reactivated virus or were cells that had been re-infected from the infected monolayer. However, ganglia removed from infected monolayers either immediately or 24 h after recognition of CPE exhibited similar types of neurones stained positive for HSV antigens.

The present immunohistological observations of acute, latent and reactivated virus in vitro suggest that mainly small unmyelinated neurones are involved in the replication of HSV infection during acute and reactivated stages of infection of rats in vitro. However, we were unable to establish whether the small-type neurones are those in which latent virus resides. Small neurones could allow virus replication (permissive) while another cell type(s) harbours the latent virus (non-permissive). Permissive and non-permissive cells may either release virus to permissive cells during reactivation or they may become permissive after receiving signals from the skin, following the application of an activating or triggering stimulus.

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References