Neuritic Transport of Herpes Simplex Virus in Rat Sensory Neurons *in vitro*. Effects of Substances Interacting with Microtubular Function and Axonal Flow [Nocodazole, Taxol and Erythro-9-3-(2-hydroxynonyl)adenine]

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SUMMARY

Herpes simplex virus type 1 and a fluorescein-labelled lectin (wheat germ agglutinin) were selectively transported to nerve cell bodies located in the inner compartment of a two-chamber tissue culture system after the application of virus or lectin to the neuritic processes in the outer culture compartment. Taxol, which stabilizes and alters intracellular arrangements of microtubules, and nocodazole, which disrupts microtubules, both inhibited this retrograde axonal transport of viral particles and lectin. The transport was also inhibited by erythro-9-3-(2-hydroxynonyl)adenine (EHNA), which blocks ATPases. However, EHNA was also an effective inhibitor of infection with the virus in non-neuronal cells (GMK AH-1). The nature of the action(s) of EHNA on neuritic transport of the virus is therefore less clear.

The pathogenesis of herpes simplex virus (HSV) infections involves axonal transport of virus and viral subunits by sensory neurons in the anterograde as well as the retrograde direction. Neurites of dissociated dorsal root ganglion cells have the capacity to internalize the virus (Ziegler & Herman, 1980), presumably by fusion of the viral envelope with the neuritic plasma membrane (Lycke *et al.*, 1984). Since nucleocapsids are detectable in neurites exposed to HSV and are seen around nuclear pores in HSV-infected cell lines (Batterson *et al.*, 1983), we assume that they are involved in retrograde axonal transport of the virus. The mechanisms of this axonal transport of HSV remain to be clarified.

We now report the effects on neuritic transport of HSV of three substances capable of inhibiting intracellular transport of endosomes, namely nocodazole, which causes disruption of microtubules, and taxol, which promotes assembly of microtubules (Herman & Albertini, 1984); the third drug, erythro-9-3-(2-hydroxynonyl)adenine (EHNA) (Bouchard *et al.*, 1981) is supposed to inhibit the function of the microtubule-associated ATPase dynein and can inhibit the retrograde movement of organelles in axons. EHNA is considered to have less effect on anterograde axonal transport (Forman *et al.*, 1983).

We have used a two-chamber culture system allowing us to infect the neuritic extensions of rat sensory neurons cultured *in vitro*, without exposing the cell bodies to the virus. For evaluation of the results the neuritic transfer of HSV was compared with the transport of wheat germ agglutinin (WGA).

The two-chamber system employed has been described previously (Ziegler & Herman, 1980). Briefly, 35 mm collagen-coated plates were scratched on the bottom, fitted in the centre with an 8 mm cloning cylinder kept in place by silicone high vacuum grease and seeded in the cloning cylinder with trypsin-dissociated dorsal root ganglion (DRG) cells. The cells were obtained by dissecting eight to ten embryos of 16 to 17 day pregnant Sprague-Dawley rats. Trypsinization was performed for 30 min at 37 °C using 0·25% trypsin (porcine pancreas, type 2, Sigma) in a Ca^{2+}- and Mg^{2+}-free Hanks' buffer pH 7·2. For the first 2 days, the cells were cultured in Eagle's

MEM with a mixture of horse and foetal calf serum, 10% of each. On days 3 and 4 this medium was replaced with Eagle's medium supplemented with 10% horse serum and 2·8 µg/ml cytosine arabinoside (Sigma). From then on the cells inside the cloning cylinder were supplied with medium containing 10% horse serum whereas the culture area outside the cloning cylinder received this medium supplemented with 1·4 µg each of uridine and fluorodeoxyuridine (Sigma). The latter two drugs and cytosine arabinoside were used to reduce mitotic activity and growth of Schwann cells and fibroblasts. Twenty-four h before experimental use, the outer culture chamber was washed and the medium was replaced with 10% horse serum medium devoid of antimitotic chemicals. A series of experiments have demonstrated that the cylinder attached to the collagen-coated plate provides an efficient diffusion-tight barrier (Lycke *et al.*, 1984).

Neurons growing in the inner chamber, i.e. inside the cloning cylinder, extended neuritic processes. These grew parallel to the scratches in the collagen coat, penetrated the vacuum grease and appeared outside the diffusion barrier in the outer chamber. To prevent axonal transport after a certain fixed time, neuritic extensions in the outer chamber were destroyed by addition of 0·5 ml concentrated H_2SO_4 for 10 s. After thorough rinsing of the outer culture compartments the cells of the inner compartment could still be cultured, indicating that the diffusion-tight barrier had remained intact.

The McIntyre strain of HSV type 1 was used. Concentrations of infectious virus were assayed by plaquing on green monkey kidney (GMK) cells using standard procedures with 1% methyl cellulose in the overlay medium. Fluorescein-labelled WGA (FITC-WGA) (Vector Laboratories, Burlingame, Ca., U.S.A.) was added at a concentration of 200 µg/ml to medium containing 10% horse serum (fluorescein/protein ratio 2) in the outer compartment containing neuritic extensions. After 4 h at 4 °C, the outer compartments were rinsed three times, 10% horse serum medium was added and the cultures were incubated for a further 16 h at 37 °C. The cultures were then fixed in 4% buffered paraformaldehyde, mounted in glycerol : phosphate buffer (9 : 1) and examined in a fluorescence microscope equipped with incident light optics.

Taxol was obtained from Natural Products Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md., U.S.A. and nocodazole was purchased from Aldrich. The drugs were dissolved in DMSO at stock concentrations of 10 mM and stored in the dark at −20 °C. EHNA (Burroughs Wellcome) was dissolved at 200 to 500 µg/ml in heated cell culture medium and cooled to room temperature before use. Final concentrations of drugs in 10% horse serum medium ranged for taxol from 10 to 0·1 µM, for nocodazole 1 to 0·1 µM and for EHNA 4 to 0·1 mM.

Cultures with many neuritic extensions penetrating the diffusion barrier were selected for experimental use. FITC-WGA added to the outer chamber adsorbed to the neurites which thus became visible in the fluorescence microscope. After incubation of cultures, selective labelling of neurons was seen in the inner chamber. The fluorescent tracer had a granular distribution in the cytoplasm and the number of labelled neurons amounted to several hundreds (Fig. 1*a*). These findings are compatible with a neuritic transport of the lectin through the diffusion barrier.

Taxol was added to a concentration of 1 µM to the outer chamber 1 to 9 days before the lectin and was present while the lectin was allowed to adsorb to the neurites and during the following incubation period. A moderate effect on transport was seen 1 day after exposure, the inhibition was marked after 2 to 6 days and complete after 9 days. A concentration of 10 µM of taxol seemed to block the neuritic transport of the drug almost completely. Similar results were obtained with nocodazole and EHNA at the concentrations indicated in Fig. 2. The outer chamber was exposed to these drugs for 24 h and 30 min, respectively, before the lectin, and the drugs were present during the subsequent incubation period. No neuronal accumulation of FITC-WGA, added as described above to the outer chamber, was seen after exposing the inner chamber to the three drugs in a similar way.

No morphological changes of the neuritic extensions were observed at the lower concentrations of nocodazole and EHNA, but treatment with 4 mM-EHNA was associated with degeneration and partial loss of neurites. Loss of neurites was noticed at 10 µM-taxol, but not at 1 µM. However, at this concentration neurites sometimes appeared deformed, with an inwards bending of the distal parts 6 days after exposure (Fig. 1*b* to *d*).
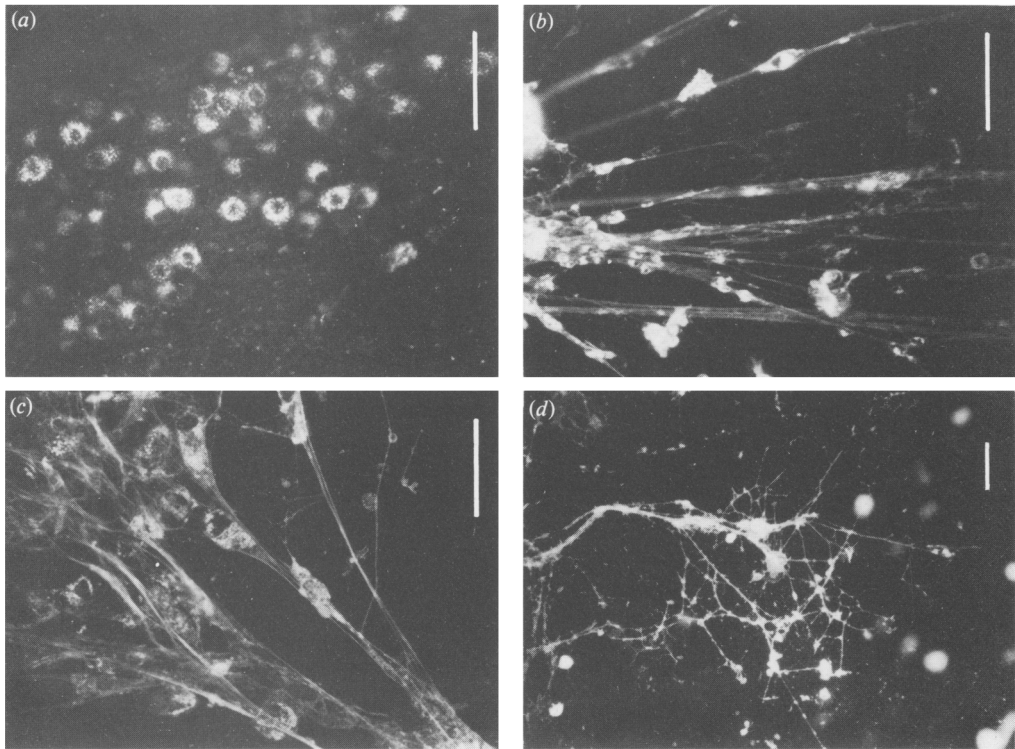
Fig. 1. (a) Accumulation of FITC-WGA in several nerve cell bodies in the inner chamber after exposing the neurites in the outer chamber to the lectin 16 h previously. (b,c,d) Attachment of FITC-WGA to neurites in the outer chamber: (b) control, (c) 1 μM-taxol, (d) 1 mM-EHNA. In the drug-exposed cultures the neurites were still present but may have been somewhat distorted. Bar markers represent 100 μm.
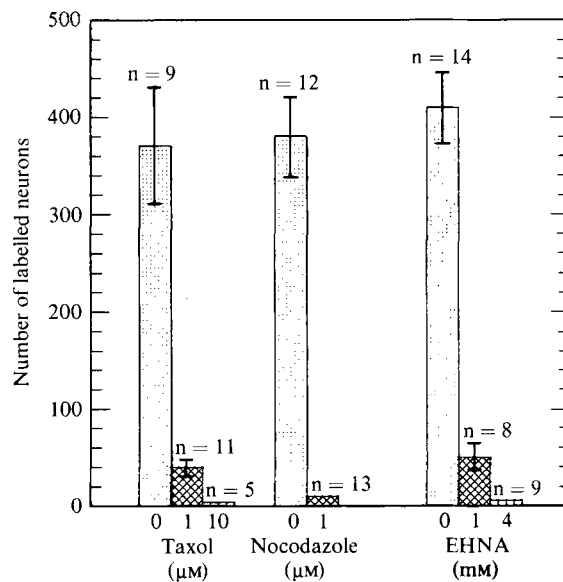

Fig. 2. Number of neurons in the inner tissue culture chamber showing incorporation of FITC-WGA following exposure of the neurites to the different drugs at various concentrations. The exposure time for taxol was 6 days, nocodazole 24 h and EHNA 30 min before addition of FITC-WGA to the outer chamber.

Table 1. *Inhibition of neuritic transport of HSV in rat sensory neuron cultures* in vitro

Compound	Concentration	Virus yield	(p.f.u./ml)	%*
Taxol	1·0 μM	1·8 × 10⁶†	(6·25 ± 0·12)	9·4 (P < 0·05)‡
	0·1 μM	4·3 × 10⁶	(6·63 ± 0·58)	22·6
	Control	1·9 × 10⁷	(7·25 ± 0·08)	100
Nocodazole	1·0 μM	6·8 × 10³	(3·83 ± 0·27)	0·95 (P < 0·01)‡
	0·1 μM	2·0 × 10⁵	(5·30 ± 0·13)	28·1
	Control	7·1 × 10⁵	(5·85 ± 0·21)	100
EHNA	1·0 mM	1·0 × 10⁴	(4·0 ± 0·41)	2·2 (P < 0·05)‡
	0·1 mM	2·3 × 10⁵	(5·36 ± 0·09)	51·1
	Control	4·5 × 10⁵	(5·65 ± 0·07)	100

* Percentage of mock-treated controls.

† The virus yields after treatment of neurites with nocodazole and EHNA refer to assays at 24 h post-infection, when the cells were frozen and thawed, scraped into the medium and tested for virus production by plaque titrations. Means of p.f.u. from three to six experiments. Values in parentheses are mean $\log_{10}$ values ± S.E.M.

‡ Significance of reduced virus yields in relation to mock-treated controls. Group comparison was made with Student's *t*-test.

Table 2. *Yields of HSV in GMK cells treated with taxol, nocodazole or EHNA*

		Virus yield (p.f.u./ml)*			
Compound	Concn.	0·1 p.f.u./cell	%	1·0 p.f.u./cell	%
Taxol	1·0 μM	5·0 × 10⁶ (6·70 ± 0·07)‡	96·2§	4·1 × 10⁷ (7·61 ± 0·03)	95·3§ (N.S.)‖
	0·1 μM	6·6 × 10⁶ (6·82 ± 0·06)	100	4·6 × 10⁷ (7·66 ± 0·08)	100
	Control	5·2 × 10⁶ (6·72 ± 0·02)	100	4·3 × 10⁷ (7·63 ± 0·04)	100
Nocodazole	1·0 μM	1·2 × 10⁶ (6·08 ± 0·06)	23·1	1·6 × 10⁷ (7·20 ± 0·05)	37·2
	0·1 μM	4·3 × 10⁶ (6·63 ± 0·09)	82·7	4·3 × 10⁷ (6·63 ± 0·07)	100
	Control	5·2 × 10⁶ (6·72 ± 0·02)	100	4·3 × 10⁷ (7·63 ± 0·04)	100
EHNA	1·0 mM	< 10³	< 0·1	< 10³	< 0·01 (P < 0·01)
	0·1 mM	2·5 × 10⁶ (6·40 ± 0·07)	48·1	2·8 × 10⁷ (7·45 ± 0·04)	65·1
	Control	5·2 × 10⁶ (6·72 ± 0·02)	100	100 (7·63 × 0·04)	100

* Monolayer cultures of GMK cells were treated with taxol, nocodazole or EHNA and infected with HSV at 0·1 or 1·0 p.f.u./cell. After 1 h adsorption the cells were washed and re-incubated at 37 °C for 23 h. Twenty-four h post-infection, the cells were frozen and thawed, scraped into the medium and tested for virus production by plaque titrations.

‡ Within parentheses, $\log_{10}$ number p.f.u. ± S.E.M.; n = 4.

§ Percentage of mock-treated controls.

‖N.S., Non-significant reduction of virus yields in relation to mock-treated controls.

For neuritic transport of HSV two concentrations of taxol and nocodazole were tested, 1 and 0·1 μM. The treatment with taxol lasted for 6 days and that with nocodazole for 24 h. EHNA was added to the neurites 30 min before the virus, at 1 or 0·1 mM. The drugs were not present during the virus adsorption period. HSV at a concentration of 10⁷ p.f.u./plate was inoculated into the outer culture chambers and after 6 h for adsorption, the neurites were destroyed by treatment with H_2SO_4. At this time the amounts of infectious virus in the inner compartments were < 10² p.f.u./ml. After destruction of the neurites, cells in the inner culture compartments were incubated for a further 18 h and then concentrations of virus were assayed. The amounts of infectious virus found are listed in Table 1.

The results obtained with nerve cell cultures revealed some minor changes in the morphology of neurites at 1 μM-taxol. These changes were similar to those previously seen at this concentration of the drug and described above. Moreover, virus yields at 24 h post-infection demonstrated that all three drugs efficiently blocked neuritic transport of HSV to the inner culture chamber and that this inhibition was related to the drug concentration.

The effect of the three drugs on HSV replication in GMK cells is illustrated by the results summarized in Table 2, concentrations of the drugs and duration of the treatments being the same as those mentioned above. No significant inhibition of virus production was observed with taxol, whereas nocodazole at 1.0 µM reduced the virus yields to approximately one-third of those seen with the untreated controls. EHNA at 1 mM efficiently blocked production of infectious virus and when applied at 0·1 mM reduced the yield by about 50%. The results thus supported the assumption that taxol, and probably also nocodazole, were specifically inhibiting neuritic transport of HSV, whereas the inhibiting effect of EHNA might have resulted from more generalized reactions not specific to HSV–neural cell interactions.

Lectins have been widely used for studies of axonal transport (Gonatas, 1979) since they specifically bind to distinct and defined neuronal structures and when labelled can be traced optically. WGA, the lectin used in the present study, binds to sialic acid when the acid is present at certain specific positions of the oligosaccharide (Månsson & Olofsson, 1983). When the effects of three compounds (taxol, nocodazole and EHNA) on the neuritic transport of WGA and HSV in rat sensory neurons were compared, all three were found to have an equal blocking capacity in the lectin and the virus systems. Since the lectin in contrast to the virus is internalized into neurites by endocytosis (Gonatas, 1979) the drugs presumably interfere with mechanisms of general importance for moving particulate matter with the axonal flow. Taxol is an anti-tumour agent which binds to polymerized tubulin and promotes assembly of microtubules. It causes the formation of abundant microtubules clustered in multiple bundles in various cell types (Schiff & Horwitz, 1980; Herman *et al.*, 1983) including neurons (Masurovsky *et al.*, 1981; Röyttä *et al.*, 1984) and their axons. In contrast to the microtubule-stabilizing effect of taxol, a disruption of microtubules is achieved with nocodazole. Both drugs demonstrate inhibition of endosome movements, but their effects on movements and distribution of lysosomes might vary (Herman & Albertini, 1984). EHNA can arrest sperm mobility, probably by inhibiting the microtubule-associated ATPase dynein (Bouchard *et al.*, 1981). The drug also blocks axoplasmic transport in neurons of *Aplysia californica* (Goldberg, 1982) and selectively inhibits retrograde transport of microscopically visible organelles in lobster axons (Forman *et al.*, 1983). Our observations that EHNA also inhibited HSV infection of an established cell line (GMK AH-1) suggest that its ATPase blocking effects might cause a more general arrest of cellular activities.

Axonal microtubules have been repeatedly suggested to be involved in axoplasmic transport (see e.g. Thoenen & Kreutzberg, 1981). However, earlier data from experiments with inhibitors of cell mitosis such as colchicine and vinblastine have sometimes been difficult to interpret, since the drug-induced morphological changes might encompass a variety of degenerative changes up to total destruction of the axon. Recently, some elegant experiments by Schnapp *et al.* (1985) have clearly demonstrated that axoplasmic organelles move in both the retrograde and anterograde directions in preparations of single microtubules from the squid giant axon.

Our observations suggest that the movements of endosomes as well as the 'foreign' nucleocapsids in the neuritic plasma might be mediated by microtubular transport. That the transport machinery of cells and axons is able to induce directed movements also of foreign organelles and particles has been shown after intracellular injection of synaptic vesicles from different species and inert beads with charged surfaces (Adams & Bray, 1983; Beckerle, 1984; Schroer *et al.*, 1985). From studies on organelle movements along microtubules in systems reconstituted from squid axons it appears that such organelle movements are dependent upon reversibly bound soluble ATPase generating translocating forces and affecting the microtubules (Vale *et al.*, 1985). This energy requirement might explain some of the inhibitory effects of EHNA on neuritic transport.

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