Observations on Cultures of Rabbit Retina Infected with Vaccinia and Herpes Simplex Virus

By K. KRISTENSSON, H.-A. HANSSON AND P. SOURANDER

Neuropathological Laboratory, Department of Pathology I and Department of Histology, University of Göteborg, Sweden

(Accepted 15 August 1969)

SUMMARY

Retinal cultures from newborn rabbits were infected with herpes simplex virus and neuro- and non-neuroadapted strains of vaccinia virus. Herpes simplex virus gave rise to characteristic nuclear changes of mesenchymal cells, and rod cells and a rapid destruction of neurons. The vaccinia virus strains all caused development of cytoplasmic inclusion bodies in the mesenchymal cells, which then degenerated. Changes in neuroectodermal cells were observed relatively late and might be secondary to the deterioration of mesenchymal cells. The implication of these findings for the study of the problem of selective cellular susceptibility of the central nervous system to viruses is discussed.

INTRODUCTION

The different types of cells in the central nervous system display a varied degree of susceptibility to infection with different viruses. In mice, for instance, as shown by fluorescent antibody studies, herpes simplex appears to infect all types of cell, while rabies infects only specific neurons and pox-viruses only leptomeningeal and ependymal cells (cf. Johnson & Mims, 1968). The sensitivity of a particular cell to infection with a particular virus is determined by many factors. Experiments in vitro should provide certain advantages in studying this phenomenon compared with in vivo studies, especially concerning the process of virus penetration through cellular surfaces. However, it has been shown that cells in visceral organs which are insensitive to a virus in vivo may be susceptible in vitro and conversely (cf. Kunin, 1964). Therefore, the first question to be examined is whether similar differences in the reaction between various kinds of cells of nervous tissue in culture are produced by infection with viruses which attack different types of cells in the central nervous system in vivo.

For this purpose we have used explants of retina (Hansson & Sourander, 1964; Hansson, 1966a), which can grow directly on coverslips without any collagen or plasma clot and without any preceding treatment with trypsin or other agents which will modify the cellular surfaces. The viruses used were different strains of vaccinia and herpes simplex virus, which produce contrasting clinical pictures and morphological changes in animals following intracerebral inoculation (cf. Kristensson & Sourander, 1969).
METHODS

Viruses. One strain of a non-neuroadapted vaccinia virus (SBL E46) and two mouse-adapted neurotropic strains of vaccinia virus, known as WR and IHD, were used. The former strain had been serially passaged on chorioallantoic membrane. The latter strains, which were obtained from Professor C. Kaplan, Lister Institute of Preventive Medicine, England, were both passaged once in mouse brain and twice in green monkey kidney (GMK) cell-cultures before use. The titres of the virus suspensions were determined at the Department of Virology, Institute of Medical Microbiology, University of Göteborg. Using GMK-cells there were $1.1 \times 10^8$, $6.3 \times 10^7$ and $5.5 \times 10^7$ p.f.u./ml. for the SBL E46, WR and IHD strains respectively. One LD50 unit, as determined after intracerebral inoculation of three-week-old mice, corresponded to 4 and 3 p.f.u. for the neuro-adapted strains WR and IHD respectively and to $6.9 \times 10^5$ p.f.u. for the non-neuroadapted SBL E46 strain.

One strain of herpes simplex virus (st 66 gbg 8) was used. This strain had a titre of $1.1 \times 10^8$ p.f.u./ml. determined on GMK-cells.

Tissue culture. Explants of retina from newborn rabbits were cultured using the method described by Hansson & Sourander (1964), modified by Hansson (1966a). Briefly, small pieces of retinal tissue were explanted directly on clean cover glasses in Leighton tubes. The nutrient medium consisted of a modified Hanks's solution with high glucose concentration (about 500 mg./100 ml.) and 20 % calf serum with addition of penicillin (50 i.u./ml.) and streptomycin (50 μg./ml.). For ultrastructural examination explants grown in Petri dishes were used.

Experimental procedure. The cultures were infected 9 to 11 days after their establishment. The inoculum was for the three vaccinia virus strains $5.0 \times 10^5$ p.f.u. and for the herpes simplex virus strain $1.0 \times 10^5$ p.f.u. in 0.5 ml. nutrient medium. After 6 to 12 hr and then regularly at 12 hr intervals until the 4th or 5th day after infection, four to six cultures were fixed in 10 % formalin. Half the number was stained with Giemsa, the other impregnated with silver according to Holmes (as described by Margolis & Pickett, 1956) for neurofibrils. For each virus strain three series of infected cultures were examined.

A preliminary ultrastructural examination was made on cultures infected with herpes simplex virus and the neuroadapted vaccinia strains on the 2nd and 3rd day after inoculation. For this purpose infected retinal cultures were fixed in 1 % purified glutaraldehyde in 0.1 M-cacodylate buffer at pH 7.4. The cultures were post-fixed in osmium tetroxide. The specimens were dehydrated in a graded series of ethanol, hydroxypropylmethacrylate and embedded in Epon 812. Selected parts were sectioned in a LKB Ultrotome III ultramicrotome and examined in a Siemens IA electron microscope after staining with uranyl acetate and lead citrate.

RESULTS

Uninfected cultures

Three types of cells could be identified with the light microscope: mesenchymal cells, rod cells and neurons. Large mesenchymal cells formed a layer and grew out from the explants at the periphery. These cells were rich in cytoplasm and had a large variably shaped, often round or oval, nucleus containing several nucleoli. Mitosis was
Fig. 1. Uninfected retinal culture. (a) Layer of mesenchymal cells at the periphery of the explant, one cell in mitosis. Giemsa. (b) Silver impregnated culture with one large nerve cell and numerous rod cells. Holmes.

Fig. 2. Rod cells arranged in a rosette-like structure. Holmes.

Fig. 3. Herpes simplex virus-infected culture. (a) Mesenchymal cells showing nuclear changes, 48 hr after inoculation. Giemsa. (b) Nerve cells showing large central vacuoles, 48 hr after inoculation. Holmes.
common and some multinuclear cells were seen (Fig. 1a). These cells have been shown to form extra-cellular precollagen fibres (Hansson, 1965). Among these cells macrophages and mast cells have been demonstrated (Olsson, Hansson & Sourander, 1965). Upon and intermingled with the mesenchymal cells, neurons with silver-impregnated neurofibrils were seen (Fig. 1b). Histochemically these cells display activity of acetylcholinesterase (Hansson, 1966b). In addition, numerous rod cells were discernible, possessing a small round nucleus with dense chromatin granules and with only a thin rim of cytoplasm. These cells occurred generally in large aggregates, sometimes forming rosette-like structures (Fig. 2). During the infection period, most of the nerve cells in the uninfected cultures looked well preserved, though, a small proportion of them, especially towards the periphery, showed some irregular swellings of the processes and occasionally small vacuoles.

**Cultures infected with herpes simplex virus**

Twelve hours after infection some and after 24 h many of the mesenchymal cells showed changes in their nuclei. These appeared larger than normal and had a slight eosinophilia obscuring the normal pattern of chromatin and nucleoli. Many of these nuclei had a honey-comb like appearance with small vacuoles separated by thin cords. Numerous small basophilic masses, probably constituting condensed chromatins were also seen (Fig. 3a). The cytoplasm was little affected until cellular degeneration occurred. After 48 to 60 h most of the mesenchymal cells were involved, some of them revealed an increased basophilia and began to round-up and became detached. No definite retraction space, 'halo', in the nuclei was seen. After 3 days most of the mesenchymal cells were degenerated and after 4 to 5 days there was an almost total destruction of these cells.

The first changes of the nerve cells were noted after 24 h. Many of these showed condensation of the neurofibrils and varicose swellings of the processes. After 36 to 48 h all nerve cells showed severe changes. They often had large vacuoles in the region of the nucleus (Fig. 3b) and bizarre forms with irregular swelling of the processes were frequent (Fig. 4). With progress of time they were broken up into fragments and became detached. After 3 to 4 days only fragments of condensed neurofibrils were left. The nuclei of the neurons appeared to be enlarged, but as it was impossible to identify the neurons in Giemsa stain, the nuclear changes could not further be evaluated. Also the rods showed severe changes with fragmentation of the nuclei with onset after 36 to 48 hr.

**Cultures infected with vaccinia virus strains**

The changes were qualitatively similar in cultures infected with both the two neuro-adapted strains and the non-neuroadapted strain. These changes appeared in a similar time sequence. Eight to 12 h after infection some of the mesenchymal cells showed large eosinophilic inclusion-bodies in their cytoplasm, while after 24 h, these inclusion bodies appeared in most of the cells. The cells began to retract from each other and the layer of mesenchymal cells was split. No definite nuclear changes were seen until the cells began to round up and became detached. After 48 h a large proportion of the mesenchymal cells were destroyed (Fig. 5a) and after 3 days there was an almost total destruction of these cells.

As compared to the uninfected cultures, no definite changes of the neurons could be seen during the first 36 h after infection. After 3 days, when there was an almost total
Fig. 4. Herpes simplex virus-infected culture with bizarre, irregular swellings of nerve cells. Three days after inoculation. Holmes.

Fig. 5. Cultures infected with neurovaccinia strain 1110. (a) Mesenchymal cell layer with degeneration of several cells and cytoplasmic inclusion bodies in others. 48 hr after inoculation. Giemsa. (b) Two well preserved nerve cells with surrounding rod cells arranged in rosette-like structures. Three days after inoculation. Holmes.

Fig. 6. Culture infected with the non-neuroadapted SBL E46 vaccinia strain showing two well-preserved nerve-cells bordering to totally destructed mesenchymal cells. Four days after inoculation. Holmes.
Fig. 7. Electron-micrograph of transected rod cells forming a rosette. Note the dense chromatin in the nuclei and the inner segments (arrows), with numerous mitochondria, radiating towards the centre.

Fig. 8. Group of rod cells with intranuclear herpes like particles. Some of the nuclei are swollen with aggregates of chromatin at the nuclear membrane. Inserted, higher magnification of virus particles.
Vaccinia and herpes infected retinal cultures

destruction of the mesenchymal cells, many of the neurons showed changes with swellings and fragmentations of the processes. Still, many nerve cells appeared to be intact (Fig. 5b) and even after 4 and 5 days some neurons remained well preserved (Fig. 6). The rod cells showed no changes and the rosette-like structures were well preserved until 3 to 4 days after infection when they began to deteriorate.

The ultrastructural characteristics of retinal cultures have been described by Hild & Callas (1967). In our infected cultures the rod cells could be identified on account of their arrangement and their small dense nuclei with characteristic margination of chromatin surrounded by a thin rim of cytoplasm (Fig. 7). In cultures infected with herpes simplex virus particles were seen in the nuclei of these cells, which later showed marked nuclear swelling (Fig. 8). In vaccinia infected cultures no virions could be recognized in these cells. The other cellular types could not readily be identified in the infected cultures and were not evaluated in this study.

DISCUSSION

There are only a few reports on the effect of viral infection on nervous tissue in vitro. Hogue et al. (1955) found that poliovirus produced relatively rapid cytopathogenic effects on nerve cells from explants of human brains, while other cells as astrocytes, oligodendroglia and macrophages appeared to survive longer. In a careful study of rabies-infected explants of cerebellum and Ammons horn from kitten and puppies, Fernandes & Pomerat (1961) observed shrinkage of the nerve cells accompanied by development of inclusion-like masses in the cytoplasm 24 to 36 h after infection while after 72 h all nerve cells were destroyed. Glial cells appeared to be more resistant; only after 8 days slight changes were noted and the mesenchymal cells or fibroblast-like elements were intact during the first passages of virus in vitro. Yoshida & Hotta (1966) described changes in nerve and glial cells in canine cerebellum cultures after infection with Japanese encephalitis virus and they also considered that the viral antigen was localized to these cells using immuno-fluorescent technique. Storts, Koestner & Dennis (1968) noted cytopathogenic effects of predominantly astroglial and mesenchymal cells in canine cerebellum explants infected with canine distemper virus. Degeneration of Purkinje cells was also noted. In vivo, these viruses have been shown to infect nerve cells (e.g. Bodian, 1964; Johnson, 1965; Hamashima et al. 1959; Coffin & Liu, 1957) and in this respect there seems to be a good correlation between in vitro and in vivo conditions.

It is difficult to decide whether the nerve cell changes observed in the present study are a direct effect of virus multiplication within these cells or secondary to deterioration of the layer of mesenchymal cells. Herpes simplex virus caused severe changes of all the nerve cells at an early stage when mesenchymal cells first began to degenerate; and particles with the characteristic morphology of herpes simplex virus were seen in the nuclei of one type of neuroectodermal cells, the rod cells. But after infection with the vaccinia virus strains, the first changes in nerve cells appeared when there was severe degeneration of the mesenchymal cells, and only when there was an almost total destruction of these did most of the nerve cells show degenerative changes. It therefore appears as if herpes simplex virus caused direct cytopathogenic changes in neuroectodermal cells, while the changes of these cells observed after infection with the vaccinia virus strains might well be secondary to the destruction of the mesenchymal cells. These findings in
herpes simplex virus infected cultures accord with those recently reported by Mannweiler & Palacios (1969). In an ultrastructural study, they found that in tissue culture, herpes simplex virus can multiply in Schwann's cells from spinal ganglia of the rabbit and in tumour cells from different human cerebral tumours. They also found that the cytopathogenic changes appeared somewhat earlier in these cells than in the mesenchymal cells of the explants. Further, Feldman, Sheppard & Bornstein (1968) described nucleolar enlargement, rarefactions and development of dense particulate matter in the nuclei of nerve cells in explants from newborn rat cerebellum and embryonic rat dorsal root ganglion infected with herpes simplex virus. Ultrastructurally they described formation of herpes simplex virus particles in all nervous tissue cells of newborn rat cerebellum, although 'the identification of the various cell types presented a problem since the cells were too immature to permit identification of any single cell by means of established ultrastructural criteria (Leestma et al. 1969)'.

In vivo, herpes simplex virus has been shown to infect nerve cells, astrocytes, Schwann cells, leptomeningeal and ependymal cells and probably oligodendroglia (e.g. Johnson, 1964; Yamamoto, Otani & Shiraki, 1965), while both neuroadapted and non-neuroadapted strains of vaccinia virus do not seem to attack nerve cells. These viral strains appear to multiply predominantly in leptomeningeal and ependymal cells and the difference in neurovirulence between the strains may be associated with a strong capacity of the neuroadapted strains to cause changes in vascular permeability with accompanying brain oedema (Kristensson & Sourander, 1969). It therefore seems as if infection with herpes simplex virus and vaccinia virus produces cytopathogenic effects \textit{in vitro} comparable with conditions \textit{in vivo}. Consequently cultures of nervous tissue should be convenient for further, ultra-structural, studies concerning the problem of selective cellular susceptibility of the central nervous system to viruses.

The authors are indebted to Professor C. Kaplan, Department of Microbiology, University of Reading for valuable advice and supply of the neurovaccinia virus strains and to Assistant Professor E. Lycke for criticism and for placing his laboratory resources at our disposal. This study was supported by a grant from the Swedish Medical Research Council (B69-12 X-82-05 A) and a grant from the Medical Faculty, Göteborg, Sweden.

\textbf{REFERENCES}


(Received 9 July 1969)