Ultrastructure of Mumps Virus Replication in Organotypic Cultures of Hamster Choroid Plexus

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SUMMARY

Organotypic cultures of newborn hamster choroid plexus were inoculated with equal titre doses of newly isolated or hamster adapted strains of mumps virus. The ultrastructure of virus replication in choroid epithelial cells of the cultures was compared. No qualitative differences were observed; however, the adapted strain produced significantly greater numbers of virions and earlier destruction of the cultures. These findings are consistent with previous in vivo observations of the ultrastructure of the replication of these strains in the newborn hamster central nervous system. This in vitro study lends further support to the hypothesis that differences in the in vivo biological effects of the virus strains are primarily the result of virus-cell rather than virus-host interactions.

INTRODUCTION

Mumps virus is a common human pathogen which frequently produces aseptic meningitis (Azimi, Cramblett & Haynes, 1969) and rarely causes severe encephalitis (Bang & Bang, 1943) as a result of central nervous system (CNS) invasion. In addition, mumps virus has been implicated in the aetiology of human aqueductal stenosis (Timmons & Johnson, 1970) and parainfectious encephalomyelitis (Harter & Choppin, 1971) and has recently been serologically associated with multiple sclerosis (Norrby et al. 1974). The hamster has provided a useful animal model for studying the pathogenesis of certain aspects of mumps virus infections.

After intracerebral inoculation virus strains recently isolated from man and attenuated vaccine strains of mumps virus replicate briefly in the choroid and ependyma of both newborn and adult hamsters but show little or no tendency to parenchymal invasion (Johnson & Johnson, 1968a, Ennis et al. 1969; Margolis, Kilham & Baringer, 1974; Wolinsky et al. 1974). Parenteral inoculation of these strains does not result in CNS invasion (Ennis et al. 1969). Alternatively, mumps virus adapted by serial passage through hamster brain produces encephalitis in newborn hamsters after either intracerebral (Kilham & Overman, 1953; Overman, Peers & Kilham, 1953; Johnson, 1968; Johnson & Johnson, 1968a; Margolis et al. 1974) or intraperitoneal inoculation (Wolinsky, Klassen & Baringer, 1975b), and causes transplacental infection of foetal animals (Kilham & Margolis, 1974). A previous ultrastructural study of mumps encephalitis in newborn hamsters suggested that differences between newly isolated, low tissue culture passage (wild) and hamster adapted strains were
due to variation in the replication cycles of virus rather than host immune responses (Wolinsky et al. 1974).

This study compares the replication of these two strains of mumps virus in organ cultures of hamster choroid plexus. These organotypic cultures provide a system in which specific host immune responses to the infection are eliminated (Hoorn & Tyrell, 1969) and in which the selection artifacts of electron microscopic techniques are minimized. The choroid plexus was chosen for organ cultures both because of the importance of local viral replication in the in vivo model and because it is the likely site of viral entry into the CNS of both the hamster and man after systemic mumps virus replication (Wolinsky et al. 1975b).

**METHODS**

*Virus.* The wild mumps virus was supplied by Dr Edwin Lennette, Berkeley, California and the hamster adapted strain by Dr Lawrence Kilham, Hanover, Vermont. The passage histories of both strains have been described previously (Wolinsky et al. 1974). The viruses were titred simultaneously before and after use on BSC 1 (Grivet monkey kidney cells) as described previously (Wolinsky et al. 1975b).

*Organized tissue cultures.* Organized tissue cultures of choroid plexus were prepared from neonatal Syrian hamsters obtained at less than 48 h of age from Simonsen Laboratories, Gilroy, California, using a modification of techniques previously described for cerebellar cultures (Bornstein & Murray, 1958; Seil et al. 1968; Seil & Herndon, 1970). The animals were anaesthetized with cold and the skin prepared with tincture of iodine followed by a 70 % ethyl alcohol rinse. After reflection of the skin from the head and neck, the bone overlying the parieto-occipital cerebral hemispheres and posterior fossa was removed. A portion of brainstem with the attached cerebellum was removed en bloc following transection of the medulla and midbrain at the level of the inferior colliculus. The fourth ventricular choroid plexus was freed from its lateral and mid-sagittal attachments to the brainstem using blunt dissection in a bath of Simm's X-7 balanced salt solution. The intact choroid plexus was then transected at its mid-point using a scalpel and transferred on the tip of the scalpel to a small quantity of nutrient medium. The choroid plexus explants were each pipetted onto a collagen-coated 22 mm round glass coverslip (Bornstein, 1958) with a drop of nutrient medium and incubated in sealed Maximow double-coverslip assemblies at 37 °C in the lying drop position. The nutrient medium consisted of two parts of 3 international units (i.u.)/ml low zinc insulin, one part of 20 % dextrose, four parts of bovine serum ultrafiltrate, four parts of Eagle's minimum essential medium with Hank's base and added L-glutamine, seven parts of Simm's X-7 balanced salt solution and 12 parts of foetal calf serum. The medium was further buffered with 0.1 M N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES). Cultures aged between one and two days in vitro were inoculated with 0.01 ml of either 10^4 TCD/ml of low passage mumps virus, 10^5 TCD/ml of adapted mumps virus or Hank's basic salt solution (HBSS). After adsorption at 20 °C for 1 h, the cultures were washed in HBSS, fed with maintenance media and returned to Maximow chambers. Maintenance media were replenished twice weekly.

*Electron microscopy.* Organ cultures on coverslips were removed from the chambers and immediately fixed in situ by immersion in cold, quarter strength Karnofsky solution (Karnofsky, 1965). These were stored in cold fixative for 1 to 7 days, post-fixed in 2 % osmium tetroxide, dehydrated in graded ethanol solutions, cleared in propylene oxide and infiltrated with epon. Then, the organ cultures and collagen base were removed from the glass coverslips with a razor blade and placed in moulds for curing. One micron thick sections were taken
Fig. 1. A portion of a control choroid plexus organ culture 9 days after explant. The polygonal cells with large nuclei are the choroid epithelium, the flattened cells are stroma mesenchymal cells.

Fig. 2. A portion of the free surface of a choroid cell from a 3 day old control culture reveals multiple microvilli and cilia.
for orientation and adjacent 10 μm sections re-embedded for thin sectioning by techniques previously described (Wolinsky, Gilden & Rorke, 1975a). Thin sections were mounted on 75 × 300 mesh copper grids.

For quantitative analysis the total number of choroid ependymal cells identified in a standard area between two grid bar rectangles was counted at a scanning magnification of 7000. The same area was then again scanned at 12 600 and the number of infected cells and virions counted. Viral nucleocapsids and virus particles were inspected at a magnification of 120 000 for confirmation in each instance. Statistical comparisons were done by the Chi-square method for evaluating fourfold contingency tables (Mainland, 1963).

RESULTS

Control cultures revealed intact cytoarchitecture throughout the two week observation period (Fig. 1). Choroidal epithelial cells maintained their cilia (Fig. 2) which could be observed to beat using a phase microscope. The ultrastructure of these cells resembled those of the choroid of newborn hamsters fixed in vivo by perfusion techniques (Wolinsky et al. 1974). However, glycogen was not as prominent in the cytoplasm of organotypic choroidal cells. The underlying stroma and endothelial cells also retained their structure. The architecture of the choroid was unaltered except at the edges of the explant where flattened fibroblast like cells formed a monolayer outgrowth. Circulating cells trapped in vessels at the time of explant underwent progressive degeneration.

Infected cultures did not differ from control cultures over the first two days of the infection. Virus particles were not detected in cultures sampled 1 h after infection. Ultrastructural evidence of replication of both viral strains was first evident on day 3 post-inoculation (p.i.) and was limited to the choroidal epithelial cells. In infected cells there were no qualitative differences in the replication cycles of the two viral strains. Nucleocapsids accumulated in abundance in the cytoplasm of infected cells (Fig. 3). The cytoplasm of infected cells was more electron lucent than that of uninfected choroidal cells. Individual nucleocapsids appeared as smooth, 16 to 18 nm diam. flexed tubules of variable length (Fig. 3 inset). They aligned along stretches of altered host plasma membrane where surface projections could be seen (Fig. 4). At such sites the inner leaflet of the trilaminar membrane was characteristically thickened. Various stages of evagination of these membranes and virus particle formation were encountered. Pleomorphic virus particles frequently collected between apposed choroidal cells (Fig. 5). Nucleocapsids within such virus particles frequently appeared to have separated from the envelope which no longer enclosed elements of choroidal cytoplasm.

The cytoplasm of some of the infected choroidal cells contained abundant smooth endoplasmic reticulum, frequently in complex, concentrically laminated whorls (Fig. 6). There was no evidence of virus-specific intranuclear changes in infected cells. Degeneration of infected cells was encountered with both viral strains (Fig. 7). Nucleocapsids within such cells were characteristically compacted, straightened and highly osmophilic (Fig. 8). Cell death was most evident with the adapted viral infection. Because of widespread necrosis the adapted viral infection could not be followed past day 5.

Quantitative changes emerged between the two infections (Table 1). On day 3 p.i. less than one percent of the choroidal cells infected with the wild strain contained viral nucleocapsid. By day 5 the percent of infected cells increased to nearly 10 %. In contrast, 5 to 10 % of the choroidal cells inoculated with the adapted strain became infected at all times after day 2 p.i. The cultures infected with wild virus produced few budding or complete virus
Fig. 3. A portion of a choroid cell 4 days after inoculation with the adapted mumps viral strain shows a cytoplasmic inclusion (arrows) composed of viral nucleocapsids. Inset: detail of nucleocapsids from a similarly infected cell 5 days after inoculation.

Fig. 4. Virus particles budding from a portion of the plasma membrane of a choroid cell 5 days after inoculation with the adapted mumps virus strain. A pinocytotic vesicle at the base of the triangular shaped developing virus particle reflects the functional integrity of the plasma membrane lacking underlying nucleocapsids or surface projections.

Fig. 5. Abundant complete viral particles lie between portions of two choroidal cells five days after inoculation with the neuroadapted viral strain.
Fig. 6. A portion of an infected choroidal cell showing a concentrically laminated smooth endoplasmic reticulum whorl formation.

Fig. 7. Portions of several infected choroidal cells are shown undergoing various stages of necrosis on day five of the adapted mumps strain infection.

Fig. 8. Detail of nucleocapsids from a portion of a necrotic choroidal cell described in Fig. 7. The nucleocapsids are straightened, compacted and intensely osmophilic.
Table 1. *Hamster-adapted and low passage, non-adapted (wild) mumps virus replication in organotypic choroid plexus cultures*

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<th>Day</th>
<th>Virus strain*</th>
<th>Total choroid epithelial cells</th>
<th>Total cells with nucleocapsids</th>
<th>Total virus particles</th>
<th>Cells with nucleocapsids per total cells</th>
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* Each culture infected with 0.03 ml virus suspension contained 10^5 TCD_{50}/ml.
† NS, not significant.
‡ Cultures infected with adapted strains highly necrotic.

particles throughout the observation period. By day 5 p.i. significantly greater numbers of virus particles were noted in cultures infected with the adapted strain, both per total choroidal cells sampled and per infected choroidal cells.

**DISCUSSION**

In a prior *in vivo* study of adapted and non-adapted (wild) mumps virus in newborn hamster CNS the apparent inability of the wild virus strain to align along and bud from infected ependymal cell membranes was correlated with the limited infection seen in the non-adapted illness (Wolinsky *et al.* 1974). The present study represents an attempt to eliminate host immune factors that might contribute to this difference, and to reduce errors resulting from the selection artifact inherent to electron microscopy.

This study confirms that nucleocapsid replication by infected choroidal cells is ultrastructurally identical for both strains. Further, it supports the observation that cells of the CNS infected with mumps virus display a marked proliferation of smooth endoplasmic reticulum. Such smooth endoplasmic reticulum proliferation and whorl formation is a likely accompaniment of mumps viral infection of man as seen in ependymal cells obtained from the cerebrospinal fluid of humans with mumps meningitis (Herndon *et al.* 1974).

Nucleocapsid alignment at, and virus particle formation from the surfaces of choroidal and ependymal cells was not seen in the *in vivo* wild strain infection of newborn hamsters (Wolinsky *et al.* 1974). In the present study virus was produced in choroid plexus organ cultures similarly infected. This suggests that the failure to observe virus production in the *in vivo* study was related, in part, to selection artifact. However, significantly fewer virus particles are produced by organ cultures infected by the wild mumps viral strain as...
compared with the adapted strain. This observation in organotypic culture may be of importance in considering pathogenetic mechanisms in the hamster encephalitis model.

In organ culture, host-specific cellular and humoral immune responses are not present. The relative inability of the wild mumps virus strain to produce structurally complete virus in this system is, therefore, a reflection of virus-cell interactions. In the same manner, the production of progeny wild mumps virus in vivo may be relatively limited by virus-cell interaction in choroid and ependymal epithelium. Host-specific immune responses might then better contain and eliminate this infection. In contrast, the replication of the hamster-adapted strain by choroid, which appears to be more efficient in organ culture, might be expected to overwhelm early host responses in vivo. Such appears to obtain in the adapted mumps virus encephalitis model.

The occurrence of straightened, highly osmophilic viral nucleocapsids in necrotic choroidal ependymal cells has been previously described in macrophages during experimental mumps virus encephalitis (Wolinsky et al. 1974). Examination of several different paramyxoviruses from disrupted cells after pre-treatment with enzymes such as trypsin, chymotrypsin and ficin in vitro reveals nucleocapsids composed of smaller protein subunits than found with standard preparations (Mountcastle et al. 1970, 1974). These nucleocapsids appeared more tightly wound and less flexible in negative stained preparations (Mountcastle, 1970). The osmophilic and 'rigid' nucleocapsids of mumps virus seen in degenerating choroid cells in this study and within membrane bound macrophages in the previous study may represent a similar intracellular enzymatic process. A similar process was shown for parainfluenza I (6/9) virus in vivo (Wolinsky et al. 1975a).

The choroid plexus is one of the sites of viral entry into the CNS (Wolinsky et al. 1975b). A number of paramyxoviruses in addition to mumps virus have a propensity to replication in the choroid (Johnson & Johnson, 1968b). Organotypic choroid plexus cultures provide a new tool for the evaluation of such viruses.

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Mumps virus in organotypic choroid culture


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