Cytopathic Effects in Mouse Neuroblastoma Cells during a Non-permissive Infection with a Mutant of Vesicular Stomatitis Virus

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SUMMARY

Morphological changes were extensive following infection of murine neuroblastoma N-18 cells with a temperature-sensitive (ts) mutant of vesicular stomatitis virus (VSV), G31 (complementation group III), and incubation at 39 °C, a non-permissive condition for virion maturation. Incubation for 24 h after infection resulted in extensive morphological degeneration of mitochondria with over 80% of the mitochondria having degenerated. Mitochondrial function, as determined by Janus green B supravital staining, was reduced by 81% from that in uninfected cells. Cellular ATP levels were reduced by 50% 12 h after infection. Mitochondrial degeneration still occurred in infected cells after the inactivation of lysosomes with chloroquine. Extensive cell fusion and cytoplasmic vacuole formation also occurred during the non-permissive infection with ts G31. Loss of plasma membrane integrity was not the cause of vacuole formation since 90% of the cells were able to exclude trypan blue 24 h after infection, nor were the vacuoles the result of inactivation of the mitochondria since cyanide-poisoned cells did not form vacuoles. The cytopathic alterations observed in N-18 cells during the non-permissive infection of N-18 cells with ts G31 did not occur during the non-permissive infection of N-18 cells with ts G11 (I), ts G41 (IV), or u.v.-inactivated ts G31. However, the non-permissive infection with ts O45(V) led to mitochondrial degeneration and cytoplasmic vacuole formation, but no cell fusion occurred. These results are discussed in light of the ultrastructural features previously observed in the central nervous system of mice infected with ts G31 and cells in culture infected with wild-type VSV.

INTRODUCTION

Certain temperature-sensitive (ts) mutants of vesicular stomatitis virus (VSV) have been shown to produce a status spongiosus in the central nervous system of mice (Dal Canto et al., 1976c; Rabinowitz et al., 1976), and the neuropathological features resulting from an intracerebral infection with these mutants include mitochondrial degeneration and the formation of cytoplasmic vacuoles (Dal Canto et al., 1976a). In many regards the ultrastructural changes with these mutants are even more extensive than those associated with wild-type (wt) VSV (Dal Canto et al., 1976b). Since the histopathological features in vivo were associated with a semi-permissive condition where some virus replication occurs (Rabinowitz et al., 1977), it was possible to determine if active viral replication was required to cause the histopathological changes in the central nervous system.
Although ultrastructural changes of cells in culture infected with wt VSV have been reported, most studies have been associated with the intracellular site of virus maturation (Reczko, 1961; Howatson & Whitmore, 1962; Coward et al., 1971; Faulkner et al., 1979). Several investigators have observed intracellular vacuoles that appeared during virus assembly (Mussgay & Weibel, 1963; David-West & Labzoffsky, 1968; Zajac & Hummeler, 1970; Zee et al., 1970). Schulze & Liebermann (1966) and Hackett et al. (1968) described both vacuole formation and mitochondrial swelling in cells infected with wt VSV. The growth of wt VSV in vivo in mouse brain and spinal cord results in intracytoplasmic inclusions and cellular degeneration particularly in the ependymal cells (Dal Canto et al., 1976b). VSV maturation is not a requisite to a cell cytopathic response, however, since cell killing and protein synthesis inhibition can be mediated by transcription of viral genes and the subsequent translational events in the absence of infectious virion production (Marvaldi et al., 1977).

This study was undertaken using ts G31 (complementation group III), a matrix (M) protein mutant (Hughes et al., 1979b) which induces status spongiosus in the central nervous system of mice (Dal Canto et al., 1976a, c; Rabinowitz et al., 1977), to compare cytopathic alterations which occur in vitro in cells derived from the nervous system with those changes previously observed in vivo with ts G31, and to use ts mutants from different genetic complementation groups and metabolic inhibitors, to determine if viral products were responsible for those alterations.

METHODS

Cell culture. Murine neuroblastoma N-18 cells obtained from B. Spooner (Kansas State University, Manhattan, U.S.A.) were grown in nutrient mixture F-12 as described by Hughes et al. (1979b). Baby hamster kidney (BHK-21) cells were grown in medium 199 as described by Rabinowitz et al. (1976). All cell culture medium components were purchased from Grand Island Biological Company, Grand Island, N.Y., U.S.A. Cells were grown at 37 °C in humidified 5% CO₂; 95% air, water-jacketed incubators.

Virus stocks. VSV mutants ts G11 (I), ts G31 (III), ts G41 (IV) and ts O45 (V), and wild-type (wt) Glasgow were provided by M. Reichmann (University of Illinois, Urbana, Ill., U.S.A.) and stocks were prepared by infecting BHK-21 cells as described by Hughes et al. (1979b). Briefly, BHK-21 cells were infected with a multiplicity of infection (m.o.i.) of 0.1 p.f.u./cell, allowed to adsorb for 30 min at 25 °C, incubated at 31 °C for 48 h, centrifuged to remove cellular debris, and stored at −85 °C. The virus was purified by sucrose gradient centrifugation as described previously (Rabinowitz et al., 1977), and p.f.u. were determined as described by Holland & McClaren (1959). Virus growth assays were performed as previously described (Hughes et al., 1979b), and the increase in infectious virus yield at 12 h post-infection was determined.

Infection of neuroblastoma cells. N-18 cells (1 x 10⁶ cells) were seeded into culture dishes (35 x 10 mm) with 2 ml F-12 medium and incubated for 2 days at 37 °C. The medium was removed and the cells were infected with the virus at a m.o.i. of 10 and allowed to adsorb for 30 min at 25 °C, incubated at 31 °C for 48 h, centrifuged to remove cellular debris, and stored at −85 °C. The virus was purified by sucrose gradient centrifugation as described previously (Rabinowitz et al., 1977), and p.f.u. were determined as described by Holland & McClaren (1959). Virus growth assays were performed as previously described (Hughes et al., 1979b), and the increase in infectious virus yield at 12 h post-infection was determined.

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Microscopy. For the light microscopy, cells were examined in the culture dish by phase contrast with a Zeiss photomicroscope II. For the electron microscopy, the medium was removed by aspiration and the cells were washed with 0.07 M-Sorenson's phosphate buffer (SPB; 0.05 M-Na₂HPO₄, 0.02 M-NaH₂PO₄). The cells were then fixed in the culture dish for 30 min with 2% glutaraldehyde (Pelco, Costa Mesa, Ca., U.S.A.) in SPB at 25 °C, washed in SPB, and post-fixed for 30 min with 1% osmium tetroxide in SPB. The cells were then washed and dehydrated in a graded series of ethanol, and then embedded in the culture dish with epon–araldite (Mollenhauer, 1964) and cured at 60 °C for 48 h. The plastic culture dish was separated from the layer of resin containing the cells, and the cells were mounted on resin
blocks. Thin sections were made with a Reichert OM-2 ultramicrotome, stained with uranyl acetate and lead citrate, and observed with a Philips 201 electron microscope.

**Isolation of mitochondria.** N-18 cells were scraped off dishes and pelleted at 1600 g for 2 min. The cells were then resuspended in NET buffer (10 mM-NaCl, 1 mM-EDTA, 10 mM-tris-HCl pH 7.4) and homogenized. The nuclei were pelleted at 1600 g for 6 min, and the supernatant fluid was centrifuged at 6000 g for 15 min to pellet mitochondria. The pellet was resuspended in NET buffer with 0.25 M-sucrose and layered on a 0.8 M- to 1.2 M-sucrose gradient in NET buffer, and centrifuged at 80000 g for 25 min. The resulting pellet was resuspended in NET buffer plus 0.25 M-sucrose and layered on a 0.5 M- to 2 M-sucrose gradient in NET buffer, centrifuged at 160000 g for 2 h, and fractions were collected on an Isco density-gradient fractionation, model 640 (Instrumentation Specialties Company, Lincoln, Nebr., U.S.A.) with the absorbance at 254 nm or 280 nm monitored continuously with an Isco model UA-5 absorbance monitor. Fractions were assayed for cytochrome oxidase activity (Cooperstein & Lazarow, 1951) and gave a single peak of activity which corresponded to the single absorbance peak at a density of 1.19 g/ml. The cytochrome oxidase activity per µg of cellular protein showed greater than 100 times enrichment of mitochondria. Cellular protein was measured by the technique of Lowry et al. (1951) using crystalline bovine serum albumin as a protein standard.

**Trypan blue exclusion and Janus green B staining.** At various times after infection N-18 cells were scraped off the dish and divided into two aliquots. Trypan blue (0.2 ml) (Grand Island Biological Company) was added to one aliquot, and after 5 min incubation at 25 °C, the percentage of the cells excluding the dye was determined in a haemocytometer. A 0.1 ml amount of 100 µg/ml Janus green B (C.I. No. 11050, Sigma) in 0.15 M-NaCl was added to the second aliquot of cells and incubated at 39 °C for 15 min. Janus green B stains mitochondria with functioning electron transport chains green, while non-functioning mitochondria are colourless (Novilloff, 1961). The percentage of the cells positive for Janus green B was calculated by counting the cells with green mitochondria in a haemocytometer. In each case, two measurements were made from each of four separate samples.

**Cellular ATP assay.** A TCA-soluble cellular fraction was prepared and assayed for ATP by the procedure of Adams (1963). The change in absorbance of the NADH at 340 nm was monitored on a Gilford spectrophotometer, model 240. Each assay was performed in duplicate with three assays per time point.

**Treatment of infected cells with chloroquine.** A 100 µM solution of chloroquine diphosphate (Sigma) in F-12 medium was added to ts G31-infected N-18 cells 2 h after infection or to uninfected N-18 cells and the cells were incubated at 39 °C for 22 h. The cells were then observed by light microscopy or fixed and examined by electron microscopy.

**Viral protein synthesis.** N-18 cells (2 × 10^6 cells) were infected with either ts G31 or ts G41 at a m.o.i. of 10, the virus allowed to adsorb for 30 min at 25 °C, and then incubated at 31 °C or 39 °C in labelling medium (Hanks' balanced salt solution, MEM vitamins, antibiotics, and BME amino acids without methionine) with 5 µg/ml actinomycin D (Sigma). After 6 h of incubation, the cells were pelleted and resuspended in labelling medium with 5 µg/ml actinomycin D and 100 µCi/ml [35S]methionine (985 Ci/mmol, New England Nuclear) and reincubated for 15 min at the respective temperatures. The cells were then washed with phosphate-buffered saline, suspended in RSB (10 mM-tris–HCl pH 7.4, 10 mM-NaCl, 1.5 mM-MgCl₂) and 1% Nonidet P40 (Bethesda Research Laboratories, Rockville, Md., U.S.A.) for 10 min on ice to lyse the cells, and the nuclei were pelleted at 1600 g for 6 min. The proteins in the supernatant were precipitated three times with acetone at −20 °C, and then electrophoresed on 8 to 17.5% gradient SDS–polyacrylamide gels (Laemmli, 1970). The polyacrylamide gels were then dried and the radiolabelled proteins were identified by autoradiography. The amount of protein synthesized was determined by scanning the autoradiogram with a Joyce-Loebl densitometer.
Fig. 1. (a) Electron micrograph of a normal, uninfected neuroblastoma N-18 cell grown at 37 °C then shifted to 39 °C for 24 h. The mitochondria (M) in these cells were distinct, with double membranes and cristae (arrows) readily visible. (b) Electron micrograph of an N-18 cell 24 h after infection in an advanced state of degeneration. Double membranes and the remnants of cristae (arrows) could be seen. Bar markers represent 0.5 μm.
**Table 1. Kinetics of mitochondrial degeneration**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Loss of Morphology (%)*</th>
<th>Loss of Janus green B staining (%)†</th>
<th>Loss of cellular ATP (%)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>19 ± 0.2</td>
<td>54 ± 1</td>
<td>34 ± 1</td>
</tr>
<tr>
<td>12</td>
<td>53 ± 0.1</td>
<td>57 ± 1</td>
<td>50 ± 1</td>
</tr>
<tr>
<td>18</td>
<td>78 ± 0.1</td>
<td>75 ± 1</td>
<td>51 ± 1</td>
</tr>
<tr>
<td>24</td>
<td>81 ± 0.1</td>
<td>81 ± 2</td>
<td>50 ± 0.4</td>
</tr>
</tbody>
</table>

* The extent of mitochondrial degeneration was determined at 6-h intervals after infection and compared to uninfected cells to determine the percentage lost. Morphological degeneration was determined by electron microscopy by counting normal and near-normal appearing mitochondria in several 130 μm² cytoplasmic areas (5 to 20).

† Physiological degeneration was determined by Janus green B supravital staining as described in Methods and done in quadruplicate for each time point.

‡ Cellular ATP levels were measured in triplicate for each time point as described in Methods. One mM-KCN-treated cells had a maximum loss of Janus green B staining of 100% after 15 min, and had a maximum loss of cellular ATP of 50% ± 1 by 30 min. All values are the averages (±S.E.M.) for each determination.

**RESULTS**

**Effects of a non-permissive infection on neuroblastoma cells**

When uninfected cultures of N-18 cells, grown at 37 °C, were shifted to 39 °C for 24 h, the ultrastructure of the mitochondria appeared normal (Fig. 1a) with distinct double membranes and cristae.

In order to eliminate any possible contribution that virus assembly and budding from the cell may have had on the cytopathology, N-18 cells were infected with ts G31 and incubated at 39 °C which is non-permissive for virus maturation (Hughes et al., 1979b). Despite the lack of virus maturation at 39 °C, within 24 h after infection extensive mitochondrial degeneration was observed (Fig. 1b). By this period of time most mitochondria could be identified only by their double membranes and the remnants of cristae. In addition to ultrastructural studies the remarkable mitochondrial changes were evident by their inability to fractionate with normal mitochondria on sucrose gradients (data not shown).

Since most of the mitochondria appeared to have degenerated by 24 h post-infection, the kinetics of the mitochondrial degeneration was determined by examining N-18 cells at 6 h intervals after their infection with ts G31 and the incubation of the cells at 39 °C. The proportion of the mitochondria that appeared abnormal was determined by electron microscopy (Table 1). By 6 h post-infection, extensive alterations were evident in 19% of the mitochondria, and the degeneration progressed with 50% of the mitochondria appearing to have degenerated by about 12 h post-infection. By 24 h, over 80% of the mitochondria appeared swollen and possessed only remnants of cristae.

Due to the marked alterations in the morphology of the mitochondria in the non-permissively infected cells, it seemed likely that the physiological function of the mitochondria would also be affected. To determine if this was the case, Janus green B was used to assay mitochondrial electron transport chain activity. The loss of physiological function was more rapid than the loss in morphology, with over 50% loss of the Janus green B staining by 6 h after infection. The loss of physiological function progressed through 12 h and 18 h, similar to the loss in morphology, and by 24 h post-infection 81% of the cells no longer had functional mitochondria (Table 1). By 12 h post-infection cellular ATP was reduced to a level comparable to that measured in cells treated with 1 mM-KCN (Table 1).

In addition to the mitochondrial changes, the non-permissive infection of N-18 cells also resulted in marked alterations in cell morphology. Cells infected with ts G31 and incubated at 39 °C for 24 h underwent cell fusion resulting in the formation of large polykaryocytes.
Fig. 2. Light micrograph of N-18 cells 24 h after infection with \( ts \) G31 and incubation at 39 °C. Compared to a single cell (S), the polykaryocytes (P) were quite large and contained several large intracytoplasmic vacuoles (arrows). Bar marker represents 25 μm.

Table 2. *Kinetics of cell fusion and cytoplasmic vacuole formation*

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Cells fused (%)</th>
<th>Vacuole formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1 ± 0.4</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>26 ± 3</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>73 ± 4</td>
<td>++</td>
</tr>
<tr>
<td>18</td>
<td>88 ± 1</td>
<td>++++</td>
</tr>
<tr>
<td>24</td>
<td>89 ± 2</td>
<td>++++</td>
</tr>
</tbody>
</table>

* At 6-h intervals after infection, cells grown on 22 mm² coverslips were fixed in acetone : methanol (2 : 1), stained with haematoxylin and eosin Y, and observed by light microscopy. The percentage of cells fused was calculated by counting the number of nuclei present in polykaryocytes versus the total number of nuclei present. About 1000 nuclei were counted per coverslip and each point is the average (±S.E.M.) for triplicate determinations.

† The extent of vacuole formation was estimated by light microscopy of fixed and stained cells.

(Hughes et al., 1979a) and large intracytoplasmic vacuoles (Fig. 2); about 90% of the cells had fused by 18 h after infection (Table 2). Cytoplasmic vacuole formation was also extensive by 18 h and the kinetics of vacuole formation appeared to follow closely the kinetics of fusion. However, cells infected with \( ts \) G31 and incubated at 39 °C as suspension cultures, which do
not undergo fusion, still formed large intracytoplasmic vacuoles. Ultrastructural examination of these vacuoles revealed that they were membrane bound, devoid of cellular organelles (Fig. 3), and often reached diameters of 15 to 20 μm.

Since vacuole formation did not appear to be directly related to cell fusion, it was possible that the loss of cellular ATP may have led to a loss of plasma membrane integrity causing an ion imbalance between the cell cytoplasm and the culture medium. However, through 18 h of infection there was no difference in the trypan blue exclusion between infected and uninfected cells, and only 10% of the infected cells failed to exclude the dye after 24 h. We reasoned that the loss of functional mitochondria may have led to the formation of vacuoles. However, uninfected N-18 cells treated with 1 mM-KCN and incubated at 39 °C for 24 h did not form cytoplasmic vacuoles.

Effect of various metabolic inhibitors on ts G31-induced cytopathic changes

Three inhibitors of different cellular functions were examined for their effects on the morphological changes in the N-18 cells during the infection with ts G31 (Table 3). The inhibition of host cell-directed RNA and/or protein synthesis in infected cells with 5 μg/ml actinomycin D, which does not affect virus RNA or protein synthesis, did not prevent the cytopathic alterations from occurring (Table 3). Actinomycin D itself was not responsible for the cytopathology since uninfected cells treated with the inhibitor did not undergo mitochondrial degeneration, cytoplasmic vacuole formation or cell fusion. Virus protein or
Table 3. Cytopathic effects in N-18 cells after infection with \(ts\) G31 in the presence of various metabolic inhibitors*

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Mitochondrial degeneration (%)</th>
<th>Cell fusion (%)</th>
<th>Cytoplasmic vacuoles (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>80</td>
<td>90</td>
<td>80</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>80</td>
<td>75</td>
<td>70</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>80</td>
<td>70</td>
<td>70</td>
</tr>
</tbody>
</table>

\*N-18 cells were infected with \(ts\) G31 and either F-12 medium, F-12 medium with 5 \(\mu\)g/ml actinomycin D or F-12 medium with 100 \(\mu\)g/ml cycloheximide was added to the cells after a 30 min adsorption period at 25 °C. Two h after infection some of the cells which received F-12 only medium were treated with 100 \(\mu\)M-chloroquine in F-12 medium. All of the infected cells were incubated at 39 °C for 24 h after infection. Mitochondrial degeneration was examined by electron microscopy, and cell fusion and cytoplasmic vacuole formation were observed by light microscopy as described in Methods. The extent of the cytopathic effects was compared to uninfected N-18 cells.

Table 4. Cytopathic effects induced by \(ts\) mutants of VSV during a non-permissive infection*

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Mitochondrial degeneration</th>
<th>Cell fusion</th>
<th>Vacuole formation</th>
<th>Virus replication at 39 °C</th>
<th>31 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt VSV</td>
<td>++++</td>
<td>0</td>
<td>++++</td>
<td>(9 \times 10^6)</td>
<td>(1.5 \times 10^7)</td>
</tr>
<tr>
<td>(ts) G31</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>&lt;10</td>
<td>(2 \times 10^6)</td>
</tr>
<tr>
<td>u.v. (ts) G31</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>&lt;100</td>
<td>&lt;100</td>
</tr>
<tr>
<td>(ts) G11</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>&lt;10</td>
<td>(8 \times 10^6)</td>
</tr>
<tr>
<td>(ts) G41</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>&lt;10</td>
<td>(1 \times 10^7)</td>
</tr>
<tr>
<td>(ts) O45</td>
<td>++++</td>
<td>0</td>
<td>++++</td>
<td>&lt;10</td>
<td>(1.7 \times 10^7)</td>
</tr>
</tbody>
</table>

\*The cytopathic effects induced in N-18 cells 6 h post-infection with wt VSV or 24 h post-infection with \(ts\) G31, \(ts\) G11, \(ts\) G41, \(ts\) O45 and u.v.-inactivated \(ts\) G31 (u.v. \(ts\) G31) at 39 °C are summarized. Mitochondrial degeneration was determined by electron microscopy as described in Methods, and cell fusion and vacuole formation were determined by light microscopy as described in Table 2. The increase in virus titre is expressed as p.f.u./ml 12 h post-infection.

RNA synthesis appeared necessary for these cytopathic changes since N-18 cells infected with \(ts\) G31, and incubated at 39 °C for 24 h in the presence of 100 \(\mu\)g/ml cycloheximide, which blocks both viral and host cell protein synthesis, did not have any cytopathological alterations.

The effect of chloroquine, a lysosomotropic agent (Ohkuma & Poole, 1978), was also examined. A previous report using chloroquine during VSV infection suggested that lysosomes were required for uncoating of the virus, and that only 50% of the viral RNA was made when chloroquine was added 1.5 h after infection (Miller & Lenard, 1980). Therefore, we added chloroquine to \(ts\) G31-infected N-18 cells 2 h after infection to allow initiation of infection but to block subsequent lysosomal enzyme activity. After 24 h of infection with \(ts\) G31 at 39 °C, the N-18 cells had extensive mitochondrial degeneration, cell fusion and cytoplasmic vacuole formation (Table 3).

Cytopathic effects induced by non-permissive infections with other VSV \(ts\) mutants

As expected from previous reports (Schulze & Liebermann, 1966; Hackett et al., 1968), infection of N-18 cells with wt VSV resulted in mitochondrial degeneration and cytoplasmic vacuoles by 6 h post-infection (Table 4). It was possible to eliminate the contribution of virus assembly and budding as well as some of the viral proteins to these cytopathic changes by using various VSV \(ts\) mutants during non-permissive infections. Three VSV \(ts\) mutants from other genetic complementation groups, as well as u.v.-inactivated \(ts\) G31 (u.v. \(ts\) G31) were
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examined. Consistent with the lack of cytopathology in the presence of cycloheximide, infection of N-18 cells with u.v. ts G31 resulted in no cytopathic responses (Table 4). This was supported by a lack of cytopathology when cells were infected, at 39 °C, with ts G11, a RNA− mutant that is unable to transcribe RNA at the non-permissive temperature (Pringle & Duncan, 1971). Ts G41, a mutant associated with a temperature-labile nucleocapsid (N) protein (Knipe et al., 1977) was somewhat puzzling since it did not cause mitochondrial degeneration, cell fusion or cytoplasmic vacuole formation at the non-permissive temperature. However, subsequent analysis of this mutant showed that there was only 10% of viral protein synthesis at 39 °C compared to a permissive infection at 31 °C or to the infection with ts G31 at 39 °C (data not shown). Although mitochondrial degeneration and vacuole formation were not directly coupled, both cytoplastic features require virus protein synthesis. Infection of N-18 cells at 39 °C with ts O45, a mutant that does not completely glycosylate the glycoprotein (G) at the non-permissive temperature (Schnitzer et al., 1979), led to both mitochondrial degeneration and cytoplasmic vacuole formation (Table 4). Consistent with the role of the G protein in cell fusion (Hughes et al., 1979a), no polykaryocyte formation occurred with ts O45-infected cells incubated at the non-permissive temperature.

DISCUSSION

An interesting relationship exists between the non-productive infection of neuroblastoma N-18 cells with ts G31 and the semi-permissive infection with ts G31 in the central nervous system of mice. Intracerebral infection of mice leads to a dramatic alteration in cell ultrastructure accompanied by a low level of infectious virus production (Dal Canto et al., 1976c; Rabinowitz et al., 1977). Five days after infection with ts G31, an advanced status spongiosus develops in the grey matter of the central nervous system that includes mitochondrial degeneration and cytoplasmic vacuole formation (Dal Canto et al., 1976a). The ultrastructural features of the mitochondria of infected neuroblastoma cells described in this study (Fig. 1) are similar to those previously seen in vivo including the advanced degeneration of mitochondrial cristae (Dal Canto et al., 1976a). Cytoplasmic vacuole formation in the infected neuroblastoma cells (Fig. 3) also appeared similar to the neuronal vacuoles seen in the grey matter of the central nervous system of ts G31-infected mice.

Mitochondrial degeneration and cytoplasmic vacuole formation have also been observed following a permissive infection with wt VSV in vitro in pig kidney cells and chick embryo fibroblasts (Hackett et al., 1968), as well as in the semi-permissive infection with the ts G31 in vivo (Dal Canto et al., 1976a). However, these studies did not allow conclusions regarding the relationship between cytopathic features and virus maturation. We have shown that, in contrast to normal uninfected N-18 cells (Fig. 1a), extensive mitochondrial degeneration and cytoplasmic vacuole formation occur in the infected cells by 24 h even though the cells were incubated at 39 °C, a non-permissive condition for virus maturation (Hughes et al., 1979a, b). The mitochondria appeared to lose physiological function well before they appeared to physically degenerate (Table 1). Cellular ATP levels were also reduced to 50% of that in uninfected cells, which is in contrast to cell killing by simian virus 40 where mitochondrial degeneration was observed, but ATP levels remained normal (Norkin, 1977). Interestingly, N-18 cells treated with 1 mM-KCN gave a maximum reduction of cellular ATP levels to 50% of untreated cells. It is likely that the assay is measuring not only mitochondria-synthesized ATP, but the total cellular nucleoside triphosphate level. Therefore, 50% of the 'energy' of the cell remained even after 24 h of infection when less than 20% of the mitochondria were functional, which explains how protein synthesis can continue in these infected cells.

Mitochondrial degeneration and cytoplasmic vacuole formation may have been the result of hydrolytic destruction of the cell by its own lysosomal enzymes. Therefore, we added chloroquine to ts G31-infected cells (Table 3). In the presence of the lysosomotrophic agent
cytopathological alterations still occurred which supports the concept that the cytopathology results directly from virus-directed events.

Since the cytopathic features occurred during a non-permissive infection with ts G31, the maturation and release of virus particles from the cell were not responsible for the structural alterations. The virus also did not carry any factor, either RNA or protein, into the cell which was directly responsible for the cytopathology since the introduction of either u.v. ts G31 or ts G11 did not lead to cytopathic changes (Table 4). The inhibition of the cytopathology with cycloheximide further supported that viral RNA and/or protein synthesis was required for the cytopathic effects.

The ts G31 is a M protein mutant (Hughes et al., 1979b) and ts O45 is a G protein mutant (Schnitzer et al., 1979), and each is able to synthesize the remaining four viral proteins at the non-permissive temperature. It is apparent that mitochondrial degeneration and cytoplasmic vacuole formation do not require the synthesis of mature M or G proteins (Table 4). Cell fusion, however, required the synthesis of a mature G protein which further supports our earlier work on ts G31-induced cell fusion (Hughes et al., 1979a).

The ts G31 is a M protein mutant (Hughes et al., 1979b) and ts O45 is a G protein mutant (Schnitzer et al., 1979), and each is able to synthesize the remaining four viral proteins at the non-permissive temperature. It is apparent that mitochondrial degeneration and cytoplasmic vacuole formation do not require the synthesis of mature M or G proteins (Table 4). Cell fusion, however, required the synthesis of a mature G protein which further supports our earlier work on ts G31-induced cell fusion (Hughes et al., 1979a).

The N protein has been implicated in VSV-induced cell killing (Marvaldi et al., 1977) and is synthesized by both ts G31 and ts O45 at the non-permissive temperature. However, it has been reported that ts G41 has a thermally labile N protein which rapidly degrades at the non-permissive temperature (Knipe et al., 1977). A non-permissive infection of N-18 cells with ts G41 did not induce any cytopathic alterations in the cells (Table 4). These results must be interpreted with caution, however, since we have found that ts G41 synthesized only 10% of its proteins at 39 °C compared to those synthesized at 31 °C or the level synthesized by ts G31 at 39 °C. The lack of cytopathology during the ts G41 infection, however, did conform to the absence of cell killing observed with ts G41 (Marvaldi et al., 1977).

Although mitochondrial degeneration and the formation of cytoplasmic vacuoles appeared to be closely coupled, the loss of mitochondrial function did not induce vacuole formation. Cells poisoned with KCN did not form vacuoles. Therefore, cytoplasmic vacuole formation and mitochondrial degeneration appeared to be independent events and both were induced by virus RNA and/or protein synthesis.

Status spongiosus and cell fusion have been observed in the central nervous system of victims of slow virus diseases such as kuru and Creutzfeldt-Jakob which are caused by 'atypical viruses' (Klatzo et al., 1959), and recently cell fusion has been induced in cell cultures by homogenates of brain tissue infected with these atypical infectious agents (Kidson et al., 1978; Moreau-Dubois et al., 1979). However, no infectious agent for these diseases has yet been purified, making biochemical analyses virtually impossible. In light of the similar ultrastructural changes in ts G31-infected mice and ts G31-infected neuroblastoma cells in culture, we may have a valuable model system which will aid in our understanding of cytopathological responses associated with slow and/or persistent virus–host responses and diseases. Whether or not infection with ts O45, which causes both mitochondrial degeneration and cytoplasmic vacuole formation at the non-permissive incubation temperature (Table 4), also results in status spongiosus is not known. It is apparent from the ultrastructural changes induced during the non-permissive infection of neuroblastoma cells with ts G31 that virus-directed RNA and/or protein synthesis are necessary, although the formation of infectious virus is not needed to produce marked cyopathic changes. This concept may be an important feature of the host–parasite interaction of persistent or chronic viral infections that lead to severe clinical diseases without a significant amount of virion maturation.

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