Increased Accumulation of a Lipophilic Cation (Tetraphenylphosphonium) in Human Embryo Fibroblasts after Infection with Cytomegalovirus

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(Accepted 3 August 1984)

SUMMARY

The distribution of a lipophilic cation, tetraphenylphosphonium (TPP+), has been used to monitor changes in mitochondrial and plasma membrane potentials within human embryo fibroblasts infected with human cytomegalovirus. An increase in TPP+ accumulation was observed throughout the whole viral replication cycle, beginning 6 h after infection. This effect requires an active viral DNA and seems to be dependent on the early transcription of the viral genome. In the early phase of viral replication, the increase in TPP+ accumulation is insensitive to mitochondrial inhibitors and sensitive to ouabain, and could be due to a hyperpolarization of the plasma membrane. Later during the infectious cycle, enhanced accumulation of the lipophilic cation is sensitive to mitochondrial inhibitors.

When human cytomegalovirus (HCMV) infects human diploid fibroblast cells, it can lead to an overall stimulation of the host cell RNA, DNA (both nuclear and mitochondrial) and protein synthesis (St. Jeor et al., 1974; Tanaka et al., 1975; Estes & Huang, 1977; Furukawa et al., 1976) which begins at and is dependent on an early stage of the viral genome transcription. The ability to stimulate host cell macromolecular syntheses is one of the differences between HCMV and other members of the Herpesviridae family and it supports the view that HCMV is potentially oncogenic (for review, see Spector & Spector, 1984).

The enhanced cellular metabolism probably leads to an increased need for energy-yielding reactions. A selective increase in glucose uptake has recently been reported to depend on the early HCMV genome transcription (Landini, 1984). In the present communication we discuss some experiments designed to verify whether HCMV-infected cells (human embryo fibroblasts, HEF) show a modified polarization with respect to mock-infected cells.

For this purpose the transmembrane distribution of the lipophilic cation tetraphenylphosphonium (TPP+) has been studied during the HCMV replication cycle. TPP+ and other lipophilic cations have been used for isotopic determinations of plasma and mitochondrial potentials within hepatocytes (Hoek et al., 1980), synaptosomes (Scott & Nicholls, 1980), fat cells (Davis et al., 1981) and lymphocytes (Felber & Brand, 1982). TPP+ is accumulated across both the plasma and the mitochondrial membranes and therefore its distribution is a function of both potentials. However, in the presence of the uncoupler, carbonyl cyanide p-trifluoromethoxyphenyl hydrzone (FCCP), TPP+ uptake into intracellular mitochondria is abolished and the contribution of the plasma membrane to total TPP+ accumulation can be resolved (Felber & Brand, 1982).

HEF (obtained from the Istituto Zooprofilattico, Brescia, Italy) were grown in MEM with Eagle's salts and 10% (v/v) foetal calf serum (FCS). The Towne strain of HCMV (obtained through the courtesy of M. Stinski, University of Iowa, Iowa City, Iowa U.S.A.) was used in all the experiments. The virus was propagated as described by Stinski (1977) and the virus titre was determined using standard procedures. In all the experiments confluent HEF, grown in Costar cluster multiwells, were infected at a multiplicity of infection of 1 to 2 p.f.u./cell. After 1 h adsorption at 37 °C in a humidified 5% CO2 incubator, the inoculum was removed and replaced.
with fresh MEM with 10% FCS. At each experimental point, cells were washed with 2 ml/well of Na⁺ buffer (130 mM-NaCl, 3 mM-KCl, 1 mM-NaHPO₄, 1.3 mM-CaCl₂, 1.5 mM-MgSO₄, 10 mM-D-glucose, 20 mM-HEPES pH 7.4) and then preincubated in 2 ml/well of the same buffer in a 37 °C water bath. After 10 min the buffer was shaken from the wells and 0.25 ml of the incubation medium containing 0.8 μM-[¹⁴C]TPP⁺ (0.2 μCi/ml, Amersham CFA.651) was added to each well using the cluster tray method described by Gazzola et al. (1981).

Incubations were carried out at 37 °C for 40 min. At the end of the incubation time, aliquots of the incubation medium were transferred to 6 ml of Instagel for scintillation counting. The reaction was then stopped by quickly shaking the medium and washing the plates twice with 0.85% NaCl as described by Gazzola et al. (1981). The cluster tray was then drained and the cells dissolved in 0.3 ml of 0.2 M-NaOH (10 min at 50 °C). 0.15 ml of the cell lysate was then mixed with 6 ml of 0.5 M-HCl in Instagel and the radioactivity was determined by scintillation counting. Protein content was determined directly in the wells by the Bio-Rad protein assay method using bovine serum albumin as standard. All the data are expressed as the pmol incorporated per mg of protein. Protein contents below 80 or above 120 μg/well were not taken into consideration.

As shown in Fig. 1, the increase in total TPP⁺ uptake in HCMV-infected cells begins very early after infection: at 6 h the infected cells showed a TPP⁺ accumulation 47% higher than uninfected cells. This increase is more evident during the late phase of the viral replication cycle (42 to 65 h) when more than 50% of the infected cells showed the presence of HCMV-specific late nuclear antigens as detected by indirect immunofluorescence using a human serum (HB-153) with an antibody titre of 1/320 against HCMV-specific late antigens and negative for early antigens (data not shown). When the uncoupler FCCP is present in the incubation medium (Fig. 1), and therefore TPP⁺ accumulation is a function only of the plasma membrane potential, the early increase is very evident while late enhancement cannot be observed.

From these results it seems that the enhanced TPP⁺ accumulation observed occurs in two ways: the first occurs during the initial 24 h of the viral replication cycle, is insensitive to FCCP and seems to be due to an alteration of the plasma membrane potential; the second occurs later in the viral replication cycle, is sensitive to FCCP and therefore is probably due to a
Table 1. **TPP+ accumulation in HEF infected with u.v.-inactivated HCMV or in the presence of Ara-C**

<table>
<thead>
<tr>
<th></th>
<th>17 h</th>
<th>42 h</th>
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<tbody>
<tr>
<td><strong>TPP+ accumulation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ FCCP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mock-infected cells</td>
<td>51.2 ± 4</td>
<td>20.9 ± 2.5</td>
</tr>
<tr>
<td>HCMV-infected cells</td>
<td>84.9 ± 11</td>
<td>141.1 ± 10</td>
</tr>
<tr>
<td>U.v.-HCMV-infected cells†</td>
<td>ND</td>
<td>28.0 ± 9</td>
</tr>
<tr>
<td>Mock-infected cells + Ara-C§</td>
<td>ND</td>
<td>85.6 ± 5</td>
</tr>
<tr>
<td>HCMV-infected cells + Ara-C</td>
<td>ND</td>
<td>130.0 ± 14</td>
</tr>
<tr>
<td>+ FCCP and ouabain‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mock-infected cells</td>
<td>12.9 ± 2</td>
<td>30.6 ± 3</td>
</tr>
<tr>
<td>HCMV-infected cells</td>
<td>13.0 ± 3</td>
<td>21.7 ± 3</td>
</tr>
<tr>
<td>U.v.-HCMV-infected cells</td>
<td>ND</td>
<td>13.0 ± 6</td>
</tr>
<tr>
<td>Mock-infected cells + Ara-C</td>
<td>ND</td>
<td>88.0 ± 6</td>
</tr>
<tr>
<td>HCMV-infected cells + Ara-C</td>
<td>ND</td>
<td>141.1 ± 10</td>
</tr>
</tbody>
</table>

* Data are expressed as pmol/mg protein (mean values of at least three different determinations ± standard deviation).

† When present, 5 μM-FCCP and 0.1 mM-ouabain.

‡ The virus stock was spread on a Petri dish and left 15 min with constant shaking under a germicidal lamp (NIS G30 T8 bulb) at 750 J/m²s. Under these experimental conditions the virus titre was reduced > 99%.

§ Ara-C was used at a concentration of 50 μg/ml in MEM with 2% (v/v) FCS.

‖ND, Not done.

The early increase in TPP+ accumulation was abolished in the presence of the Na+/K+ ATPase inhibitor, ouabain (Table 1) confirming its dependence upon alterations of the plasma membrane potential alone, and by the fact that other mitochondrial inhibitors (such as a combination of the mitochondrial respiratory chain inhibitor rotenone with the inhibitor of the mitochondrial ATPase, oligomycin) gave results similar to those obtained with FCCP (data not shown). As shown in Table 1, a u.v.-inactivated virus was unable to induce the modifications of cell membrane potentials that were responsible for the increased TPP+ accumulation, which supports its dependence upon the functioning of all the viral DNA regions (or of a large part of them) transcribed during the early phases of infection. In order to investigate whether the increase in TPP+ accumulation was dependent only on the early functions expressed by the input viral genome as suggested by the early occurrence of this event during the viral replication cycle, HEF were infected after 5 h incubation in the presence of cytosine arabinoside (Ara-C) and kept in the presence of the drug for 42 h after infection, when the assay was carried out. Ara-C is a well known inhibitor of DNA replication and in the presence of this drug the virus does not go through the late phase of its replication cycle (The \textit{et al.}, 1974). As shown in Table 1, the presence of Ara-C during viral growth did not affect the level of total TPP+ uptake, but did increase the level of FCCP-insensitive TPP+ uptake in infected cells. This result clearly indicates that the early increased TPP+ accumulation is only dependent on the early transcription of the HCMV genome. Further investigations are required to understand the effect of the drug on the late TPP+ uptake, in view of the possibility that the concentration of Ara-C used in the present study inhibits mitochondrial DNA synthesis.

A few other plasma membrane alterations have been described in HCMV-infected cells which depend on early viral genome transcription. HCMV-specific early membrane antigens are detectable in infected cells starting from 12 h after infection (Boldogh \textit{et al.}, 1977; Tanaka \textit{et al.}, 1981; Landini \textit{et al.}, 1984) and may be composed of at least the two glycoproteins of 100K and 134K mol. wt. (Stinski, 1977). Glucose uptake is greatly increased early after HCMV infection both in permissive and non-permissive cell systems (Landini, 1984) and the ability of infected cells to accumulate high levels of intracellular Ca²⁺ has also been shown (Garnett, 1981). Early virus-induced alterations in the cellular membrane can be of great interest as it has been shown (Durham, 1978) that many metabolic changes occurring early in virus infection may be due to altered ion fluxes through the plasma membrane.

Our finding that early after HCMV infection there is an enhanced accumulation of a lipophilic cation, insensitive to mitochondrial inhibitors, may be the consequence of an increased
plasma membrane potential in infected compared to mock-infected cells. This hyperpolarization is sensitive to ouabain and is presumably due to a virus-induced stimulation of the membrane Na⁺/K⁺ ATPase or to the presence of a new virus-specific enzyme (M. P. Landini et al., unpublished results). On the other hand, we cannot exclude that this hyperpolarization is concomitant with other virus-induced modifications in membrane permeability leading to altered ion fluxes.

We have also shown that later in the viral replication cycle the enhanced accumulation of the lipophilic cation is sensitive to mitochondrial inhibitors. This could be due either to an enhanced mitochondrial membrane potential or, more likely, to an increased number of mitochondria within infected cells. The latter hypothesis is supported by the finding (Furukawa et al., 1976) that HCMV infection stimulates the synthesis of mitochondrial DNA before the replication of the viral genome and before the stimulation of host cell DNA synthesis. Therefore HCMV could act as a trigger for the replication of mitochondria, leading to a higher level of ATP, which is also in agreement with the previously reported HCMV-induced enhancement in glucose uptake. The events described in this report require a functioning viral genome and seem related to early viral functions expressed by the input viral genome.

This work was partially supported by The Italian Ministry of Education (40%) and Consiglio Nazionale delle Ricerche, Progetto Finalizzato 'Controllo delle Malattie da Infezione', grants no. 83.00662 and 83.02677.04. We thank Dr Maura Ferrari (Istituto Zooprofilattico, Brescia, Italy) for providing HEF, and Mrs Valeria Zagnoli for typing the manuscript.

REFERENCES


(Received 29 May 1984)