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Cytomegalovirus Infection of Human Teratocarcinoma Cells in Culture

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SUMMARY
Whereas human cytomegalovirus (HCMV) did not replicate in human embryonal carcinoma (EC) cells, it did replicate in some of the differentiated cells arising following the exposure of TERA-2-derived human EC cells to retinoic acid. On the other hand, retinoic acid did not induce a permissive state in several other diverse human cell lines, including an EC line, 2102Ep, which did not differentiate in response to this agent. Also, both TERA-2 and 2102Ep EC cells differentiated to a limited extent when grown at low cell density and a few of these cells became permissive for HCMV. Thus, susceptibility is the result of differentiation and not due to a direct effect of retinoic acid on viral replication. The nature of the block to HCMV replication in human EC cells is unknown, but viral DNA could be detected in the nucleus within an hour of infection and there was an increased anchorage-independent growth of undifferentiated and differentiated cells following HCMV infection. Viral replication is not subject to a general block in these cells, since another herpesvirus, herpes simplex virus type 1, replicated well.

INTRODUCTION
Human cytomegalovirus (HCMV) is endemic throughout the world and leads to abortion and to acute or chronic diseases of the foetus, newborn and young infants (Weller, 1971). The cellular and molecular mechanisms for the embryo-toxic and teratogenic effects of HCMV are unknown. To establish a model for the study of these phenomena in vitro, we have investigated the susceptibility of human embryonal carcinoma (EC) cells to infection with this virus. EC cells are the pluripotent stem cells of teratocarcinomas, tumours that often contain various disorganized mixtures of embryonic and adult tissues. In the mouse, EC cells share biochemical and developmental properties with cells of the early embryo and may take part in normal embryogenesis when inserted into a blastocyst by micromanipulation (for review, see Martin, 1980). Recent characterization of two human EC cell lines provides an opportunity to make observations that may be pertinent to the action of HCMV on the human embryo. Clones established from the human teratocarcinoma line 2102Ep undergo limited differentiation when cultured at low cell density, marked by changes in the morphology and surface-antigen phenotype of a proportion of the cells (Andrews et al., 1982; Andrews, 1982); their differentiation is not induced by retinoic acid (RA) (Matthaei et al., 1983). Clones (NT2/B9 and NT2/D1) derived from another human teratocarcinoma line, TERA-2, also undergo limited differentiation when cultured at low density (Andrews et al., 1984), but RA induces these EC cells to differentiate extensively into a variety of cell types, including neurons (Andrews, 1984).

Whereas the undifferentiated NT2/B9 EC cells do not permit HCMV gene expression or viral replication, their RA-induced differentiated derivatives are permissive (Göneczöl et al., 1984). We have now confirmed and extended these observations, especially to examine whether differentiation of human EC cells induced by growth at low cell density results in the appearance of HCMV-permissive cells.
**METHODS**

*Cells and treatment of the cells with RA.* The human EC clones derived from 2102Ep (cl. 2A6 and cl. 4D3) and from TERA-2 (clones NT2/B9 and NT2/D1) have been previously described (Andrews et al., 1982, 1984). Briefly, high density cultures (predominantly EC phenotypes) were seeded and maintained at >5 x 10^6 cells per 75 cm^2 flask, whereas low density cultures (mixed EC and differentiated phenotypes) were seeded at 10^6 cells per 75 cm^2 flask. Low to high density cultures were established by harvesting cells from low density cultures, seeded 7 days previously, and re-seeding them at high cell density (Andrews, 1982). All-trans-RA (Eastman Kodak) was added to the culture medium, at a final concentration of 10^{-5} M as required, by dilution from a 10^{-2} M stock solution in DMSO; 0.1% DMSO, the consequent concentration in medium containing 10^{-5} M RA, has no observable effect on the cells (Andrews, 1984). Other cells used included the gestational choriocarcinoma lines Bewo and Jar (Pattillo & Gey, 1968; Pattillo et al., 1971); the HeLa derivative Hep 2-5 (Nelson-Rees & Flandermeyer, 1976), human embryonic kidney cells (obtained from M. A. Bioproducts), and MRC-5 human embryo lung fibroblasts, between the 21st and 40th passage level (obtained from the Medical Research Council, U.K.).

*Virus preparation.* The Towne strain, an established laboratory strain of HCMV, was plaque-purified and propagated on MRC-5 cells as previously described (Furukawa et al., 1973). Two to 3 days after complete c.p.e. was observed, the culture medium was collected, clarified and stored in 1 ml aliquots in liquid nitrogen. The infectivity of stock virus, assayed by the viral plaque method (Wentworth & French, 1970), was 5 x 10^6 to 8 x 10^6 p.f.u./ml.

To prepare radiolabelled HCMV, confluent cultures of MRC-5 cells were infected at a m.o.i. of 0.4 to 0.5 p.f.u./cell, and incubated with 10 μCi/ml methyl[^3]H]thymidine (80-1 Ci/μmol; New England Nuclear) from the 2nd day after infection; fresh medium with the same amount of isotope was added on the 5th day. Medium, harvested on the 8th to 9th day, when the c.p.e. reached 100%, was centrifuged at 6000 r.p.m. for 15 min to remove cell debris, after which the virus was pelleted by centrifugation at 15000 r.p.m. for 2 h (Beckman rotor number 19). The virus was suspended in Tris-buffered saline (0.14 M-NaCl, 0.052 M-Tris-HCl pH 7.2) and purified by centrifugation on a linear gradient of 20 to 60% (w/w) sucrose for 1 h at 25000 r.p.m. in an SW-41 rotor. A sharp band of virus observed at approximately 38% sucrose was removed, washed, and resuspended in the Tris-buffered saline. The purified virus suspension contained about 500 μg protein/ml and a specific activity of 1-2 x 10^7 c.p.m./μg protein.

Herpes simplex virus type 1 (HSV-1), strain NS, was isolated by Dr H. Friedman, Children's Hospital, Philadelphia, Pa., U.S.A. The virus was passaged 10 times in MRC-5 cells before it was used in these experiments. HSV-1 infectivity of the culture medium was determined by a viral plaque assay on MRC-5 cells.

*Virus susceptibility and virus uptake.* To test their sensitivity to virus, cell cultures were infected at an m.o.i. of 5 to 10 (7 days in the case of low density cultures) after seeding. After a 2 h incubation, unadsorbed virus was washed and the cultures re-fed. Subsequent expression of viral antigens was assayed by immunofluorescence on acetone-fixed cells, as described previously (Vaczi & Goncezol, 1973), using four HCMV human immune sera (No. 1, 2, 3, 4) at a dilution of 1:10, and two monoclonal antibodies directed against early HCMV proteins [19-21], Biotech Research Laboratory and E-13 (Colimon et al., 1984) kindly provided by Dr R. Colimon, Laboratoire Central de Bactériologie-Virologie, Paris, France at a dilution of 1:20. The human immune sera stained early and late HCMV antigens (Vaczi & Goncezol, 1973) in separate experiments. The percentage of cells containing viral antigens was calculated based on direct counts of 3000 to 5000 cells using Leitz microscope equipped for epilumination.

The number of cells harbouring infectious HCMV was determined by an infectious centre assay: monolayers of infected cells were extensively washed, harvested by treatment with trypsin-EDTA, and incubated in 1 ml of a mixture of HCMV immune serum and complement at 37°C for 1 h to neutralize viruses still attached to the cell surface. These cells were then plated onto MRC-5 monolayers. Cultures were overlaid with culture medium containing 0.3% agarose 6 h after plating and plaques were counted after 2 weeks.

To test the penetration of radiolabelled HCMV, cells were seeded at a density of 4 x 10^5 per 25 cm^2 tissue culture flask, and infected with purified methyl[^3]H]thymidine-labelled HCMV particles (m.o.i. = 1 to 2) 1 day later. Samples of the inoculum immediately after infection, and 1 and 2 h later, were assayed for unadsorbed virus by determining residual radioactivity. At these time intervals, cells were harvested with trypsin-EDTA, following three washes with phosphate-buffered saline (PBS), washed again three times with PBS and lysed by suspension in a hypotonic solution (0-02 mM-NaCl, 2 mM-Tris, 1.5 mM-MgCl_2), containing 0-35% NP40. Nuclei were pelleted by centrifugation at 1000 g for 5 min and washed once with PBS. The supernatant was taken as the cytoplasmic and nuclear fraction. A portion of each fraction was acid-precipitated to determine the amount of viral DNA associated with the cytoplasmic and nuclear fraction.

*Cell surface antigens.* Cell surface antigen expression was assayed by flow cytofluorimetry as previously described (Andrews et al., 1982, 1984). Fluorescence-activated cell sorting (FACS) was performed using an Ortho Cytofluorograf to separate viable cells according to their expression of surface antigens SSEA-1 and SSEA-3 (Andrews, 1982). Monoclonal antibodies to SSEA-1 (Solter & Knowles, 1978), and to SSEA-3 (Shevinsky et al., 1982) were provided as ascites fluids by Drs B. Knowles and D. Solter, The Wistar Institute.
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Growth in soft agar. NT2/D1 cells pretreated with RA for 7 days and the same cells without RA pretreatment were infected at an m.o.i. of 5 to 10 or mock-infected. After 2 h, the cells were washed with PBS, trypsinized and tested for anchorage-independent growth as follows. A bed of 1 ml of 0.6% (w/v) agar (Bacto-Agar, Difco) containing MEM and 10% foetal bovine serum (FBS) was prepared in 3.5 cm Petri dishes. Subsequently, 2 ml of a suspension containing 5 × 10⁶ cells/ml MEM, 10% FBS and 0.3% agar was added. The cells were fed every 4th day with 0.5 ml of 0.3% agar/MEM/10% FBS. After 21 days, colonies greater than 10 cells were counted. The size of the colonies of infected undifferentiated cells was usually larger in diameter (0.3 to 0.4 mm) than that of the infected differentiated cells (0.1 to 0.2 mm).

RESULTS

Induction of HCMV-permissive cells by treatment of non-permissive cells with RA

Extensive differentiation of NT2/B9 EC cells into a variety of cell types, including neurons, is induced by RA (Andrews, 1984) and this is accompanied by the appearance of many cells that are fully permissive for HCMV replication (Gönczöl et al., 1984). We tested the susceptibility of several additional human cell lines (2102Ep, Bewo, Jar, HeLa, human embryonic kidney cells and NT2/D1) and found that none except for NT2/D1 was permissive for HCMV, whether or not pretreated with RA. Of these cells, 2102Ep is a human EC cell line that does not differentiate in response to RA (Matthaei et al., 1983). By contrast, NT2/D1, a second clone of TERA-2-derived EC cells which differentiates similarly to NT2/B9, generated many HCMV-permissive cells. This indicates that RA does not have a general ability to induce an HCMV-permissive state and that the effect of RA on NT2/B9 and NT2/D1 cells appears to depend upon its ability to induce their differentiation into a variety of distinct cell types, one or more of which are permissive for HCMV replication.

In our previous experiments, a single human convalescent serum (serum No. 1) was used to detect viral antigens. To confirm the absence of viral gene products in the HCMV-infected EC cells, we have repeated these studies using three additional human convalescent sera (serum No. 2, No. 3, No. 4) and two monoclonal antibodies (91-21 and E-13) specific for early viral proteins. Again, none of these reagents was found to detect viral antigens in the infected EC cells, although they reacted specifically with HCMV-infected MRC-5 cells or the differentiated derivatives of NT2/B9 and NT2/D1 (data not shown).

Uptake of HCMV by undifferentiated EC cells

We have previously shown, using an infectious centre assay, that HCMV is able to penetrate NT2/B9 cells. To confirm this and to assess whether the viral DNA can reach the nucleus, we followed the uptake of HCMV radiolabelled with [³H]thymidine (Table 1). The results indicate that NT2/D1 EC cells, their differentiated derivatives and MRC-5 fibroblasts all take up the virus similarly and even 1 h after infection a significant fraction of the virus was found in the nucleus of each cell type. Similar observations were made for NT2/B9 (not shown).

Replication of HSV-1 in NT2/B9, NT2/D1 and 2102Ep cl. 2A6 EC cells

To ascertain whether the block in HCMV replication in human EC cells reflects a general block in viral replication, we tested the susceptibility of the cells to infection by another herpesvirus, HSV-1. NT2/B9, NT2/D1 and 2102Ep cl. 4D3 cells, whether or not treated with RA, did support replication of this virus, and the yield of virus from non-RA-treated and RA-treated cultures and from MRC-5 cultures was similar (2 × 10⁷ to 5 × 10⁷ p.f.u./ml).

Spontaneous differentiation of human EC cells

In addition to the effects of RA on NT2/B9 EC cells, these, as well as 2102Ep cl. 2A6, EC cells also differentiate spontaneously, although at a rather lower frequency. This differentiation is most marked in cultures seeded at low cell densities and is accompanied by a variety of changes including altered cell morphology and surface antigen phenotype (Andrews et al., 1982, 1984; Andrews, 1982; Damjanov & Andrews, 1983). As shown in Table 2, growth at low density also promoted the appearance of a small population of HCMV-permissive cells in cultures of both NT2/B9 and 2102Ep cl. 2A6. To confirm that these permissive cells were part of the differentiated subpopulation and not amongst the persisting EC cells, cells from low density
Table 1. Adsorption and penetration of radiolabelled HCMV into NT2/D1 EC cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Time post-infection (h)</th>
<th>C.p.m. in culture medium (× 10^{-2})</th>
<th>Adsorbed* (%)</th>
<th>C.p.m. in cytoplasm (× 10^{-2})</th>
<th>C.p.m. in nucleus (× 10^{-2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT2/D1</td>
<td>0</td>
<td>2285</td>
<td>0</td>
<td>36</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1682</td>
<td>26</td>
<td>229</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1305</td>
<td>43</td>
<td>380</td>
<td>155</td>
</tr>
<tr>
<td>No RA</td>
<td>0</td>
<td>2348</td>
<td>0</td>
<td>39</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1582</td>
<td>33</td>
<td>385</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1338</td>
<td>43</td>
<td>588</td>
<td>205</td>
</tr>
<tr>
<td>NT2/D1 + RA</td>
<td>0</td>
<td>1940</td>
<td>0</td>
<td>34</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1045</td>
<td>46</td>
<td>342</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1005</td>
<td>48</td>
<td>606</td>
<td>210</td>
</tr>
</tbody>
</table>

* Percent decrease in c.p.m. as compared to c.p.m. of the 0 time inoculum.

Table 2. Effect of culture conditions of NT2/B9 and 2102Ep cl. 2A6 cells on HCMV replication

<table>
<thead>
<tr>
<th>Cells</th>
<th>Cells containing viral antigen (%) on day post-infection</th>
<th>Infectious virus production on 7th day post-infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT2/B9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High density</td>
<td>0.1   0.1  0.2</td>
<td>0.01</td>
</tr>
<tr>
<td>Low density</td>
<td>3.5  3.9  3.8</td>
<td>0.2</td>
</tr>
<tr>
<td>Low to high density</td>
<td>4.2  3.8  4.0</td>
<td>0.1</td>
</tr>
<tr>
<td>2102Ep cl. 2A6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High density</td>
<td>0.05  0.1  0.1</td>
<td>0.02</td>
</tr>
<tr>
<td>Low density</td>
<td>1.8  1.7  2.2</td>
<td>0.1</td>
</tr>
<tr>
<td>Low to high density</td>
<td>1.7  2.1  2.0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

* Assayed using human convalescent serum No. 1.
† Fifteen ml medium per 75 cm² flask seeded initially with 5 × 10⁶ cells (high density and low to high density) or 10⁵ cells (low density).

Table 3. Appearance of HCMV antigens after infection of cells from subpopulation of NT2/B9 cultures expressing different cell surface antigen phenotypes

<table>
<thead>
<tr>
<th>NT2/B9 cells*</th>
<th>Cells containing viral antigen (%) on day post-infection</th>
<th>Cells expressing SSEA-3 or SSEA-1 at the time of seeding (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSEA-3-positive cells</td>
<td>0.2  0.1  0.1</td>
<td>90  ND§</td>
</tr>
<tr>
<td>SSEA-3-negative cells</td>
<td>6.1  7.2  7.5</td>
<td>0.6  ND</td>
</tr>
<tr>
<td>Unsored cells</td>
<td>1.5  1.8  1.5</td>
<td>69  ND</td>
</tr>
<tr>
<td>SSEA-1-positive cells</td>
<td>5.8  6.2  6.5</td>
<td>ND  95</td>
</tr>
<tr>
<td>SSEA-1-negative cells</td>
<td>0.1  0.1  0.2</td>
<td>ND  0.7</td>
</tr>
<tr>
<td>Unsored cells</td>
<td>1.2  1.5  1.6</td>
<td>ND  32</td>
</tr>
</tbody>
</table>

* The cells were harvested from low density cultures, and fractionated according to their expression of SSEA-3 and SSEA-1 by FACS.
† Assayed using human convalescent serum No. 1.
‡ Immediately after sorting, each subpopulation was re-analysed for expression of SSEA-3 or SSEA-1, seeded on coverslips, and infected the following day.
§ ND. Not determined.
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Table 4. Growth characteristics of virus-infected undifferentiated and differentiated NT2/DA cells in soft agar

<table>
<thead>
<tr>
<th>Cells</th>
<th>Colony formation (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected, undifferentiated</td>
<td>8</td>
</tr>
<tr>
<td>Infected, undifferentiated</td>
<td>65</td>
</tr>
<tr>
<td>Uninfected, differentiated</td>
<td>0.05</td>
</tr>
<tr>
<td>Infected, differentiated</td>
<td>6</td>
</tr>
</tbody>
</table>

* Percentage of cells forming colonies (mean from two separate experiments each performed in five Petri dishes).

cultures of NT2/B9 were fractioned, using the fluorescence activated cell sorter, according to their expression of the surface antigens SSEA-1 and SSEA-3 and then infected with HCMV. EC cells are SSEA-1-negative, SSEA-3-positive. We observed that, indeed, the HCMV-permissive cells were largely found amongst the SSEA-1-positive and the SSEA-3-negative subpopulations (Table 3). Nevertheless, many of the differentiated cells, according to these criteria, remained non-permissive.

Effect of HCMV infection on anchorage-independent growth of differentiated and undifferentiated NT2/D1 cells

It has been observed that HCMV promotes the formation of colonies of both permissive human fibroblasts (Lang et al., 1974) and non-permissive BALB/c 3T3 cells (E. Gencz, unpublished observation) in soft agar. We therefore tested the ability of NT2/D1 cells to grow in soft agar following HCMV infection. As shown in Table 4, prior infection of both NT2/D1 EC cells and the RA-induced differentiated derivatives promoted their growth in soft agar. This is the first evidence that we have obtained indicating that HCMV is not totally passive in human EC cells and influences their behaviour.

DISCUSSION

Our results confirm that human EC cells are non-permissive for HCMV replication, while being fully permissive for the replication of another herpesvirus, HSV-1. In addition to our previous demonstration that RA-induced differentiation of pluripotent EC cells derived from TERA-2 results in the appearance of cells permissive for HCMV replication, we have now observed that spontaneous differentiation of both TERA-2 and 2102Ep-derived EC cell lines grown at low density also results in the appearance of a small number of permissive cells. In this case, whereas the permissive cells are found amongst the SSEA-1-positive, SSEA-3-negative subpopulation [i.e. cells exhibiting a surface antigen phenotype distinct from human EC cells (Andrews et al., 1982, 1984)] they constitute only a small fraction of these differentiated cells.

To understand the mechanism of HCMV restriction in human EC cells it is first essential to establish whether the virus can penetrate the cells and reach the nucleus. We therefore studied the uptake of radiolabelled virus. In agreement with our previous results using the infectious centre assay (Gencz et al., 1984) the virus uptake by NT2/B9 and NT2/D1 cells was similar whether or not the cells were from EC or differentiated cultures, although the adsorption and penetration seemed to be slower than in the fully permissive MRC-5 cells. Further, the present experiments show that about 21 to 24% of the DNA present in virions that had adsorbed to and penetrated the cells by 2 h was associated with the nuclear fraction of NT2/B9 and NT2/D1 cells. Accordingly, the restriction to HCMV gene expression in human EC cells occurs after penetration of the virus. Murine EC cells are similarly resistant to infection with a number of viruses [e.g. simian virus 40, polyoma (Schwartzendruber & Lehman, 1975); murine C-type viruses (Teich et al., 1977); murine CMV (Dutko & Oldstone, 1981); adenovirus type 5 (Cheng & Praszkier, 1982)] whereas the differentiated derivatives of the same cells may be permissive. The nature of the block to the virus expression in murine EC cells is controversial, but it may be that replication is restricted at several different levels of virus replication. Thus, blocks to transcription (Katinka et al., 1981; Dutko & Oldstone, 1981; Gautsch & Wilson, 1983), post-transcriptional mRNA processing (Segal et al., 1979) or translation (Linnenbach et al., 1981)
may be involved. However, human EC cells differ in many ways from their murine counterparts (Andrews et al., 1980, 1982, 1984; Cotte et al., 1982; Damjanov & Andrews, 1983), and conclusions drawn from the murine EC cell–virus interactions might not be valid for the virus-infected human EC cell systems.

The only effect of HCMV infection on the behaviour of pluripotent EC cells we have so far been able to detect is the promotion of anchorage-independent growth of these cells following virus infection. Although it has been suggested that changes at the cellular surface mediate the ability to initiate cell division (Montagnier, 1968), we cannot rule out the possibility that an interaction of the viral DNA with the cellular genome or the production of a viral product, not so far detectable by the immunological reagents available, may also be involved.

Our observations reported here indicate that the partial or full expression of the HCMV genome can be a useful marker for following the differentiation of human EC cells, and that investigations to pinpoint the stage of differentiation at which these teratocarcinoma cells become susceptible to HCMV infection might also reveal information about the mechanisms of restricted or productive infections and the cell type(s) in which replication can occur.

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