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Human Cytomegalovirus: Demonstration of Permissive Epithelial Cells and Nonpermissive Fibroblastic Cells in a Survey of Human Cell Lines

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To more clearly define the characteristics which render a cell permissive for human cytomegalovirus (HCMV), we screened a panel of human cell lines differing in morphology, ploidy, and extent of differentiation for the ability to sustain productive HCMV replication. Cells were exposed to HCMV at 5 to 20 PFU per cell and examined at 4 to 14 days postinfection to detect the production of infectious virus by a plaque assay and the assembly of progeny virions by electron microscopy. By these criteria, high-titer HCMV replication (10^6 to 10^7 PFU/ml) occurred in a well-differentiated, diploid, epithelial cell line, HCMC, which had been derived from normal human colonic mucosa. In contrast, all aneuploid human cell types proved to be nonpermissive, including a fibroblastic cell line designated HT-144. These results indicate that HCMV replication in cultures is not strictly limited to fibroblasts and conversely that not all human fibroblastic cells are permissive for HCMV. Nonpermissive cell types were further investigated by attempts to chemically induce HCMV replication. Treatment of nonpermissive cell types with 25 to 500 μg of 5-iodo-2′-deoxyuridine per ml prior to infection did not convert them to the permissive state. The implications of these findings for the possible mechanisms maintaining the nonpermissive state are discussed.

Human cytomegalovirus (HCMV) is a widespread and important human pathogen (19, 25), but it also has one of the most markedly restricted host ranges of the known herpesviruses. In vivo it replicates in a wide variety of epithelial cell types (29), while replication in cell cultures often appears limited to human diploid fibroblasts (13, 28). Exceptions to this latter generalization can be found in scattered reports of productive HCMV replication in certain human epithelial cell cultures (5, 8, 10, 12, 23, 24). Such reports indicate that the commonly accepted correlation between fibroblastic morphology and the HCMV-permissive state in human cell cultures is not a perfect one.

We report here on a survey of the susceptibility to HCMV of a panel of cultured human cell types. These studies demonstrate that a fibroblastic phenotype is neither necessary nor sufficient to ensure HCMV replication in human cells and suggest that other parameters, such as ploidy and the extent of differentiation, may be more useful predictors of cellular susceptibility to HCMV.

In addition, we report here on attempts to induce HCMV replication in nonpermissive cells by pretreatment with 5-iodo-2′-deoxyuridine (IDU). Other investigators have previously shown that HCMV replication can be induced in some nonpermissive cell types by treatment with this agent (1, 21).

MATERIALS AND METHODS

Virus. The AD-169 strain of HCMV was obtained from the American Type Culture Collection and plaque purified three times on MRC-5 cells. Cell-free stocks were prepared by infecting subconfluent monolayers of MRC-5 cells at a multiplicity of infection of 0.1 PFU per cell. When the cultures showed approximately 90% cytopathic effects, aliquots of medium were frozen in liquid nitrogen and subsequently titrated by a plaque assay under agarose (27).

Cells. The following human cell types were obtained from the American Type Culture Collection at the passage level indicated and used within 2 to 10 passages of receipt. MRC-5 (passage 18, ATCC CCL 171) is a fibroblastic cell line that has a normal diploid karyotype and was derived from male embryonic lung (9). These cells, which are known to be permissive for HCMV (17), served as positive controls. WI-38 VA13 subline 2RA (passage 263, ATCC CCL 75.1) is a hyperdiploid, continuous epithelial cell line (stem-line chromosome number, 73 to 78) that was derived by simian virus 40 transformation of the HCMV-permissive fibroblast cell line WI-38. The transformant no longer produces infectious simian virus 40. HCMC (passage 15, ATCC CCL 239) is an epithelial cell line that has a normal diploid karyotype (2.8 to 8.4% polyploid) and was derived from the normal colonic mucosa of an adult male (3). These cells show evidence of considerable morphological differentiation, including the presence of mucous droplets, tonofilaments, and junctional complexes. HT-144 (passage 81, ATCC HTB 63) is a hypo- to hypertriploid fibroblastlike cell line that was isolated from a malignant melanoma metastatic to the subcutaneous tissue in an adult male (6). RD (passage 37, ATCC CCL 136) is a near-diploid cell line (stem-line chromosome number, 49 to 50) that was derived from a malignant embryonal rhabdomyosarcoma in a juvenile female. The cultures contain spindle-shaped cells plus large multinucleated cells (11).

All cell types were grown in minimum essential medium with Earle salts and containing 20 mM sodium bicarbonate, 2 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% heat-inactivated newborn bovine serum; this medium is hereafter referred to as MEM 10. Monolayer cultures were grown at 37°C in 5% CO_2 and passaged at the recommended split ratios and subculture intervals with trypsin-EDTA. All cell lines were tested for mycoplasmas and found negative by growth in modified

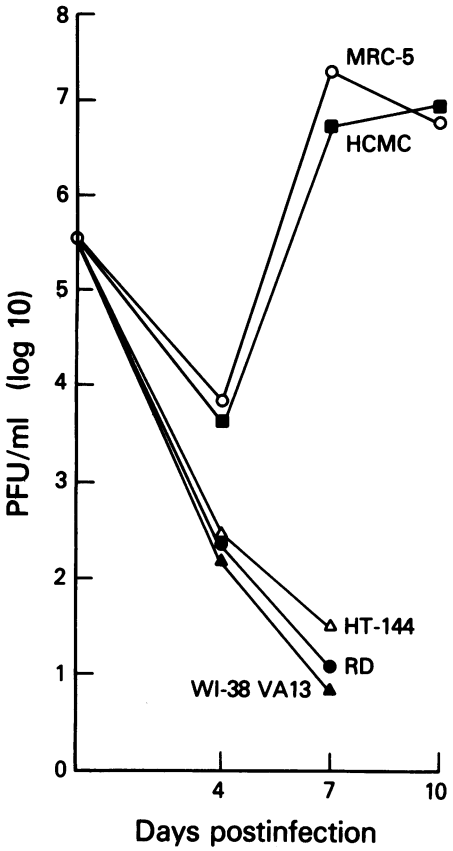

FIG. 1. Titers of culture medium from five different human cell lines sampled at intervals after exposure to HCMV (multiplicity of infection, 5 PFU per cell). Titers were determined by a plaque assay on MRC-5 cells. Only MRC-5 and HCMC cells showed evidence of productive HCMV replication.

liquid medium, growth on modified solid medium (Flow Laboratories, Inc.), and staining of cover slip preparations with Hoechst 33258 (2).

Infection. Subconfluent monolayers of each cell type were exposed to HCMV at a multiplicity of infection of 5 PFU per cell unless otherwise stated. The inocula were adsorbed for 90 min, after which the monolayers were washed and incubated in MEM 10 at 37°C. At intervals postinfection (p.i.), the culture medium was sampled for titration by a plaque assay (27) on MRC-5 cells. All flasks received a medium change after the appropriate samples were taken on day 7 p.i.

Electron microscopy. Cells were trypsinized, pelleted, and fixed for electron microscopy by standard methods (17) with 2.5% glutaraldehyde, 1% osmium tetroxide, and 0.5% uranyl acetate in successsion. The samples were dehydrated and embedded in Epon 812. Thin sections of polymerized blocks were stained with uranyl acetate followed by lead citrate.

IDU pretreatment. Subconfluent monolayers of nonpermissive cells were pretreated as previously described (21, 22) with 25 to 500 μg of IDU per ml for 4 days prior to infection. The cells were then washed with MEM 10, infected, and grown in MEM 10 lacking IDU. On days 4, 7, and 10 p.i., the culture medium was sampled for a plaque assay, and the cells from the same flask were trypsinized for viable cell counts and electron microscopy.

RESULTS

Viral growth curves in untreated cells. Figure 1 shows the viral titers recovered from the various cell types by a plaque assay of the culture medium sampled at 4, 7, and 10 days p.i. with HCMV. One-step growth curves indicative of productive HCMV replication were observed for the known permissive MRC-5 cells and also for the diploid epithelial cell line HCMC. HCMC cells yielded titers comparable to or exceeding those from MRC-5 cells. In contrast, there was no evidence of productive infection in the aneuploid fibroblastic cell line HT-144, in the near-diploid RD cells, or in the aneuploid epithelial cell line WI-38 VA13. The viral titers from these three cell types (Fig. 1) decreased steadily over the course of the experiment and reached undetectable levels by 10 days p.i. The low levels of infectious virus present on days 4 and 7 p.i. presumably represented residual inoculum which eventually underwent inactivation. These data therefore indicate that HCMC cells are permissive for HCMV, while RD, HT-144, and WI-38 VA13 cells are nonpermissive.

Light microscopy. By phase-contrast microscopy, HCMC cells showed a moderate rounding within 24 h p.i. By 4 days p.i. they contained typical skein-like nuclear inclusions (Fig. 2) as well as cytoplasmic inclusions indistinguishable from those in infected permissive fibroblasts (15, 17). Small num-

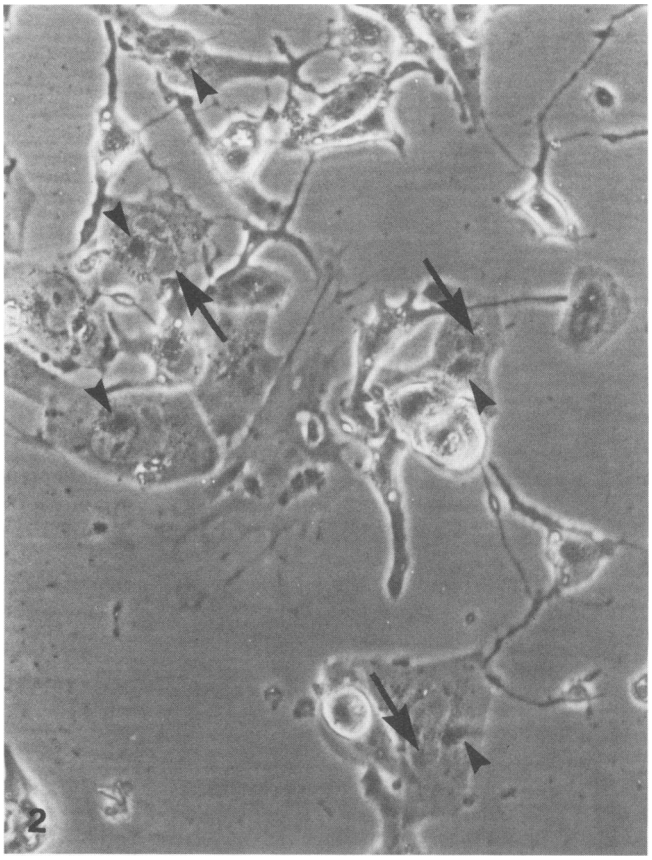

FIG. 2. HCMV-infected HCMC cells 7 days p.i. Several cells contain dark cytoplasmic inclusions (arrowheads) and skeinlike nuclear inclusions (arrows) identical to those normally seen in productively infected fibroblasts. The nuclear inclusions are characteristically separated from the nuclear membrane by a clear euchromatic halo and often appear to be divided into two separate segments by the nucleolus. ×525.

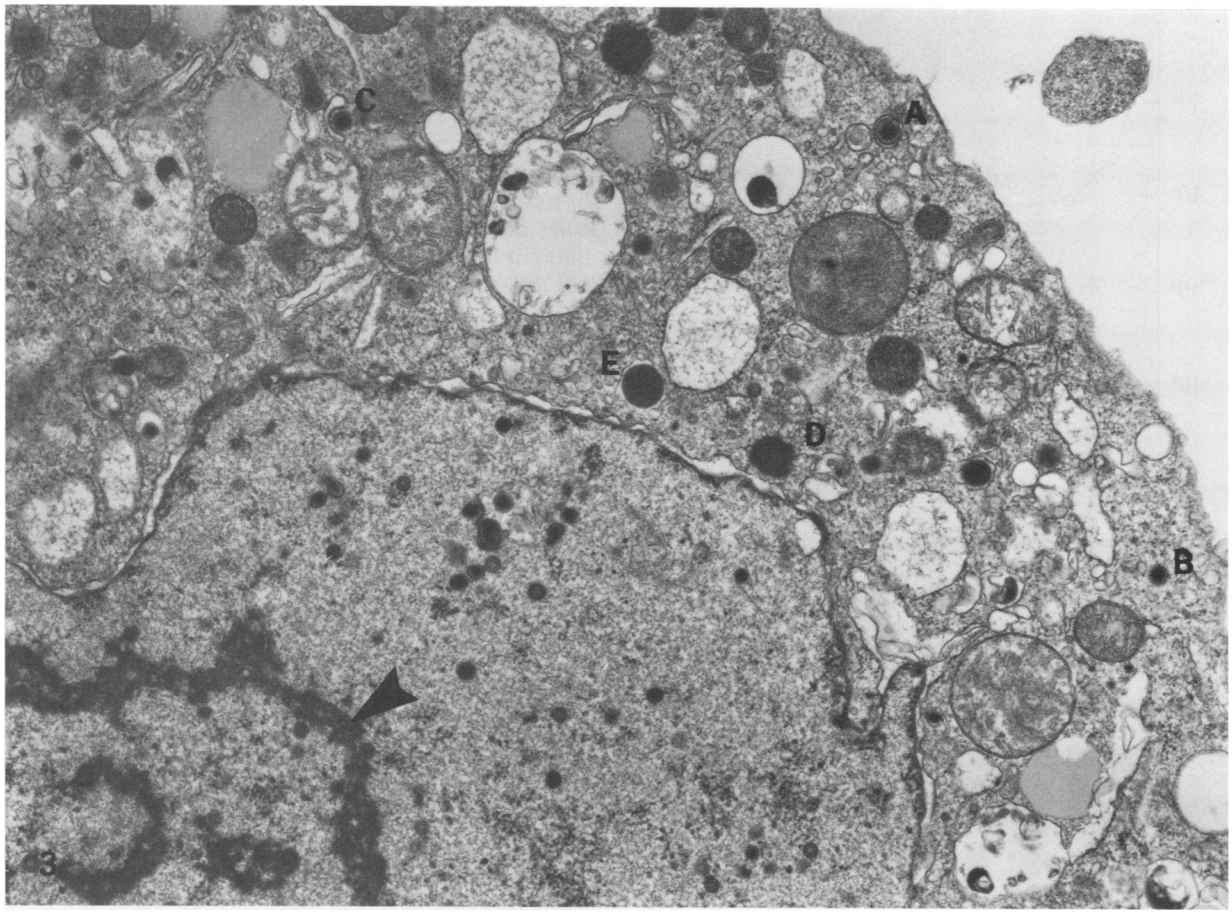

FIG. 3. HCMC cell productively infected with HCMV (10 days p.i.). The nucleus contains unenveloped viral capsids representing newly assembled progeny virions as well as a skeinlike inclusion (arrowhead). In the cytoplasm are enveloped virions (A), unenveloped capsids (B), unenveloped capsids budding into cytoplasmic vacuoles (C), unenveloped dense bodies (D), and enveloped dense bodies (E). ×28,000.

bers of multinucleated cells were also present, presumably as a result of cell fusion.

Cultures of the nonpermissive cell types (RD, HT-144, and WI-38 VA13) also contained rounded-up cells within 24 h p.i., but no cytoplasmic or nuclear inclusions typical of HCMV-infected cells were observed even when cultures were passaged and maintained for up to 21 days p.i. Experiments at higher multiplicities of infection (20 PFU per cell) resulted in the death of cells by 4 days p.i., as judged by vital staining, followed shortly by the disruption of the monolayer. In such cultures the cells showed extreme rounding, and balloon cells were present, but the nuclear or cytoplasmic inclusions characteristic of productively infected cells were not observed.

Electron microscopy. Transmission electron microscopy of HCMC cells fixed 7 and 10 days p.i. revealed the presence of intranuclear capsids (Fig. 3). These capsids must represent progeny virions, since viral particles from the inoculum become uncoated in the cytoplasm and do not enter the nucleus intact (16). No qualitative or quantitative differences were observed in the events of viral morphogenesis between HCMC and MRC-5 cells. At 7 and 10 days p.i., both cell types contained intranuclear capsids budding through the inner nuclear membrane, enveloped virions within cytoplasmic vacuoles, and unenveloped cytoplasmic capsids coated by a fine fibrillar material (15). Some of the unenveloped cytoplasmic capsids appeared to be acquiring an envelope by budding into vacuoles in the Golgi region. Also present in the cytoplasm of both cell types were unenveloped and enveloped dense bodies, which are known to be composed at least in part of unassembled viral structural proteins (14). The number of dense bodies and their average size were comparable in both cell types. Finally, the size and morphology of viral capsids and cores were identical in both HCMC and MRC-5 cells.

In contrast, HT-144, RD, and WI-38 VA13 cells fixed at 7 and 10 days p.i. with HCMV showed no evidence of progeny virion morphogenesis. For each cell type 300 cells were examined by using sections which passed through a substantial portion of the nucleus. No normal or abnormal viral particles or subcomponents were observed, nor were cytoplasmic dense bodies seen. These results confirm the nonpermissive nature of these three cell lines, including the fibroblastic cell line HT-144, and indicate that the HCMV replicative cycle is interrupted at some point prior to progeny virion assembly.

In two of the three nonpermissive cell types, exposure to HCMV resulted in characteristic changes in the chromatin distribution pattern. In HT-144 cells, numerous small heterochromatin aggregates were distributed evenly within the nucleus at both 7 and 10 days p.i., producing a distinctive spotted appearance in thin sections. In RD cells, larger aggregates of heterochromatin were present and tended to be marginated along the inner surface of the nuclear membrane

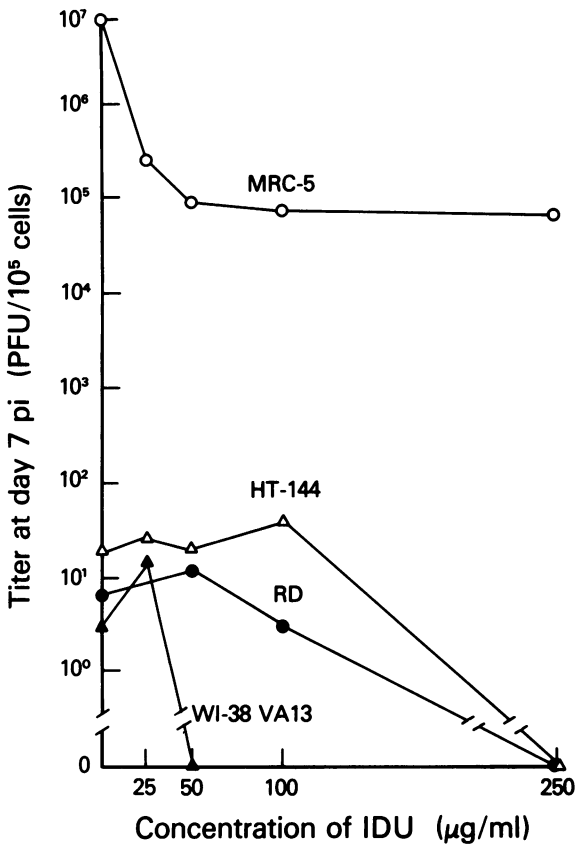

FIG. 4. Effects of IDU pretreatment on HCMV replication. Four human cell lines were treated with IDU for 4 days prior to exposure to HCMV and then infected and incubated without IDU. Shown here are the titers of culture medium sampled 7 days p.i. and plotted as a function of the concentration of IDU used in pretreatment. At a pretreatment dose of 500 μg/ml (data not shown), titers for all cell types were equivalent to those at 250 μg/ml.

in a pattern similar to that observed in herpes simplex virus-infected cells (15). Numerous perichromatin granules were also present. In contrast, WI-38 VA13 cells exposed to HCMV retained the normal chromatin pattern of uninfected cells. Beyond these changes in chromatin pattern, no other morphological differences between control and virus-exposed cells were observed with any of the nonpermissive cell types.

IDU pretreatment. Treatment with IDU at doses of 25 to 500 μg/ml for 4 days prior to infection did not significantly decrease the viability of any cell type but did result in a dose-dependent decrease in cell number for all cell types (data not shown), presumably through an inhibition of DNA synthesis (21). The results of all plaque assays are therefore reported as PFU/10^5 cells.

The titers of culture medium from IDU-treated cells sampled 7 days p.i. are shown in Fig. 4. These results and the similar findings at 10 days p.i. (data not shown) indicate that a 4-day pretreatment of nonpermissive cells with 25 to 500 μg of IDU per ml did not induce detectable replication of HCMV. In fact, increasing the dose of IDU to 50 μg/ml (WI-38 VA13) or 250 μg/ml (RD and HT-144) caused viral titers to fall to undetectable levels by 7 days p.i. In addition, IDU pretreatment of MRC-5 cells resulted in viral titers which were approximately 1 to 2 logs lower than that of the untreated control (Fig. 4). Therefore, under these experimental conditions, IDU pretreatment failed to convert nonpermissive cells to the permissive state and also inhibited viral replication in permissive cells.

DISCUSSION

HCMV was originally isolated from clinical specimens by using fibroblastic cell cultures derived from a variety of different tissues (13, 18, 26). Soon afterward, it was shown that the virus did not replicate in cultured epithelial cell lines such as HeLa or KB (13, 20). These and other similar reports led to the widely accepted generalization that HCMV replication in cultures was limited to human fibroblasts. This situation was paradoxical in that HCMV replication in vivo most often involved epithelial cell types such as proximal tubule cells of the kidney, bronchiolar epithelium, the lining of lung alveoli, and hepatocytes (29).

In succeeding years, the concept that HCMV replication in cultures occurred exclusively in human fibroblasts was challenged by occasional reports of productive HCMV infection in a cultured epithelial cell type. Permissive epithelial cells included primary cultures of lung epithelium (12) or endocervical cells (23), secondary or tertiary cultures of thyroid epithelial cells (10), a cell line derived from amnionic cells (24), and first-passage cultures of human amnion cells (5). In all cases in which the comparison was made, HCMV replication in epithelial cells was slower than that in fibroblasts, and peak titers were at least 1 log lower in the epithelial cells (5, 10, 24). The original hypothesis was therefore modified to suggest that human fibroblastic cultures are more sensitive to the virus but that less efficient HCMV replication can also occur in at least some cultured epithelial cell types.

The present study has added the following information. HCMV replication is not always slower or less efficient in epithelial cells than in fibroblasts. The growth curves obtained for HCMV replication in epithelial HCMC cells and MRC-5 fibroblasts (Fig. 1) indicated no detectable differences in the speed or efficiency of replication in the two cell types. The eclipse periods and the peak viral titers were comparable in both HCMC and MRC-5 cells. Electron microscopy showed that the events of viral morphogenesis and the viral particles themselves were indistinguishable in HCMC and MRC-5 cells and that HCMC cells did not contain either larger or more numerous cytoplasmic dense bodies than did MRC-5 cells. Since dense bodies are thought to represent, at least in part, unassembled viral structural proteins (14), any increase in dense body size or number might have indicated a relatively less efficient assembly of progeny virions. This does not appear to be the case.

In addition to demonstrating high-titer HCMV replication in an epithelial cell culture, this study has also shown that not all human fibroblastic cells are permissive for HCMV. The aneuploid fibroblastic cell line HT-144, derived from a metastatic malignant melanoma, was unable to support the production of infectious HCMV and showed no evidence of normal or abortive viral morphogenesis when examined by electron microscopy. To our knowledge, this is the first report of a fibroblastic human cell which is nonpermissive for HCMV in cultures.

The data suggest that factors other than cellular morphology, such as ploidy or extent of differentiation, may be more valid predictors of susceptibility to HCMV. Consistent with this hypothesis is the observation that productive replication occurred in HCMC, a diploid cell line with morphological

evidence of considerable cellular differentiation (3), while the cell types which proved to be nonpermissive were all aneuploid and morphologically unspecialized. The potential importance of the differentiated state is also suggested by the results of Gönczöl and co-workers, who reported that undifferentiated human embryonal carcinoma cells were nonpermissive for HCMV but that the virus did replicate in human embryonal carcinoma cells whose differentiation had been induced by retinoic acid (8). Similarly, Dutko and Oldstone (4) reported that undifferentiated murine teratocarcinoma cells (PCC4) were nonpermissive for murine cytomegalovirus but that PCC4 cells induced to differentiate by treatment with dimethylacetamide were permissive for the virus.

Electron microscopy of the nonpermissive cell types showed that in HT-144 and RD cells, exposure to HCMV resulted in changes in the chromatin pattern and in the abundance of perichromatin granules which were characteristic for each cell type. Changes in chromatin distribution are also among the earliest morphological events observable in productive HCMV infections (15). The observation of such changes in HT-144 and RD cells suggests that at least some of the early events of HCMV replication may also occur in these nonpermissive cells.

The mechanisms by which HCMV replication is restricted in nonpermissive cells are as yet unknown and may differ in different cell types. We observed that when permissive and nonpermissive cells were cocultivated prior to infection, both phenotypes were still expressed in the infected cultures (data not shown). It would therefore appear that neither the permissive nor the nonpermissive cells release soluble mediators capable of reversing the HCMV susceptibility of the other cell type.

Previous investigators have reported that the treatment of cells with 20 to 100 μg of IDU per ml induced the replication of several different herpesviruses, including HCMV (1, 7, 21, 22). It was postulated that IDU might act by suppressing the synthesis of one or more viral inhibitors produced by nonpermissive cells (21). In the case of HCMV, pretreatment for 3 to 4 days with 100 μg of IDU per ml enhanced the titers recovered from permissive fibroblasts (22) and converted nonpermissive human embryonic kidney (HEK) cells to the permissive state (21). IDU also induced HCMV replication in nonpermissive somatic cell hybrids in which one parent had been latently infected prior to cell fusion (1). However, in the present study, HCMV replication did not occur in any of three nonpermissive cell lines pretreated for 4 days with up to 500 μg of IDU per ml. Since this maximal dose of IDU was considerably higher than those previously found effective in inducing HCMV synthesis, these findings suggest that even within a single species, quantitatively and perhaps qualitatively different mechanisms may underlie the nonpermissiveness of different cell types. To evaluate this hypothesis we are currently conducting experiments with cloned permissive-nonpermissive somatic cell hybrids which should allow us to map the cellular genes which regulate HCMV replication in different cell types.

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