CYTOMEGALOVIRUS CAUSES A LATENT INFECTION IN UNDIFFERENTIATED CELLS AND IS ACTIVATED BY INDUCTION OF CELL DIFFERENTIATION*

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Cytomegalovirus (CMV)[1] is a herpesvirus whose genome consists of double-stranded DNA of approximately 10^8 daltons. CMV can cause acute disease (1–10) as well as remain latent with subsequent activation (11–14). The answers to basic questions concerning the specific cells that harbor CMV in a latent state, the mechanisms of activation of the virus, and the reasons for restricted cell tropism in vivo and in vitro are not known.

Cytomegalovirus infection and latency can be studied in mice. From such studies, we have learned that a subset of B lymphocytes harvested from the spleen, peritoneal macrophages, and sperm cells can harbor latent murine cytomegalovirus (MCMV) (15–18). Virus can be activated by allogeneic cocultivation of B cells (15, 17), by procedures that activate macrophages (16), or by immunosuppression (19). In contrast, MCMV could not be detected in brain, kidney, thymus, or liver cells from latently infected mice by cocultivation assays or by DNA-DNA hybridization kinetics. Although the MCMV genome was detected in splenic B cells, peritoneal macrophages, and reproductive tissues by nucleic acid hybridization assay, the number of cells involved was few, and, hence, studies on the molecular basis of latency and activation are limited. For this reason, there is a need to develop an in vitro model of CMV latency. This would allow dissection of the molecular basis of cytomegalovirus latency and activation of virus and shed light on the restriction of cytomegalovirus to specific cell types. In this paper we present, to our knowledge, the first such model of CMV latency and evaluate several of its parameters.

Materials and Methods

Cells. PCC4 cells are a subclone of the azaguanine-resistant line PCC4 aza 1 (20, 21) and were obtained from W. Speers of the University of Colorado, Denver, Colo. (22). OTT6050AF1 BrdU cells are pluripotent cells, but remain undifferentiated in tissue culture (23) and were obtained from K. Huebner, Wistar Institute of Anatomy and Biology, Philadelphia, Pa.

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[1] *Abbreviations used in this paper:* CMV, cytomegalovirus; DMA, dimethylacetamide; FCS, fetal calf serum; FITC, fluorescein isothyocyanate; HSV, herpes simplex virus; LCMV, lymphocytic choriomeningitis virus; MCMV, murine cytomegalovirus; MEF, mouse embryo fibroblasts; MEM, minimal essential medium; MOI, multiplicity of infection; PBS, phosphate-buffered saline; PFU, plaque-forming units; PIPES, 1,4-piperazinediethane sulfonic acid; SDS, sodium dodecyl sulfate; TNE, 50 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA; TNM, 0.01 M Tris, 0.14 M NaCl, 0.0015 M $MgCl_2$, pH 7.4; VSV, vesicular stomatitis virus.

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OTTF12 cells are a differentiated line established from the same pluripotent stem cell line from which the OTT6050AF1 BrdU line was cloned (23) and were also obtained from K. Huebner. F9 cells (24) were obtained from G. Miller, Yale Medical School, New Haven, Conn., and were maintained in gelatin-coated flasks as described (25). The parietal yolk sac line (PSY2) was obtained from J. Lehman (University of Colorado Medical Center, Denver, Colo.). Mouse embryo fibroblasts were established by trypsinization of embryos from BALB/St mice.

PCC4 and PYS2 cells were propagated in Auto Pow minimal essential medium (MEM) (Flow Laboratories, Inc. Rockville, Md.) supplemented with 10% heat-inactivated (56°C for 30 min) fetal calf serum (FCS). OTT6050AF1 BrdU and OTTF12 cells were propagated in RPMI 1640 medium (Flow Laboratories, Inc.) supplemented with 10% heat-inactivated FCS. F9 cells were propagated in Dulbecco's modified Eagle's medium with 4.5 g glucose/liter and supplemented with 10% heat-inactivated FCS. Mouse embryo cells were propagated in MEM supplemented with 10% heat-inactivated calf serum. Monolayer cultures were grown in plastic flasks and dishes (Falcon Labware Co., Div. of Becton, Dickinson & Co., Oxnard, Calif.) at 37°C and 6% CO_2 in air. Cultures were subcultured after dispersion with 0.25% trypsin-0.02% EDTA. Suspension cultures of PCC4 cells were initiated with 5×10^6 cells in 50 ml MEM in 250-ml Erlenmeyer flasks with airtight rubber stoppers and were cultured at 37°C on a Gyrotory shaker, model G2 (New Brunswick Scientific Co., Inc., Edison, N. J.) rotating at 80 rpm. For experiments, monolayer cultures were seeded with 9×10^5 cells/75-cm^2 flask. To induce cell differentiation, 10 mM dimethylacetamide (DMA; Aldrich Chemical Co., Inc., Milwaukee, Wis., dissolved in MEM) was added to the culture medium.

Viruses. MCMV source, handling, and characteristics have been reported (15–18). Virus stocks were maintained by passage in BALB/St mouse embryo fibroblasts, and the plaque assay was performed in mouse embryo cells propagated in 60-mm dishes. Lymphocytic choriomeningitis virus (LCMV), strain Armstrong, was passaged in baby hamster kidney (BHK 21) cells, and infectious virus was plaqued in Vero cells as described (26). Vesicular stomatitis virus (VSV), strain Indiana (Mudd Summers), was obtained from J. Holland, University of California, San Diego, Calif. Virus stocks were prepared in BHK21 cells, and infectious virus was plaqued in Vero cells. Herpes simplex virus type 1 (HSV-1) was obtained from A. Notkins, National Institutes of Health, Bethesda, Md. HSV-1 was passaged and infectious virus was plaqued in human foreskin fibroblasts.

Microcytotoxicity Absorption. PCC4 cells propagated with or without dimethylacetamide (10 mM) were dispersed with phosphate-buffered saline (PBS) containing 0.02% EDTA. As controls, spleen cells from 129/J (H-2^b) or C3H/St (H-2^k) mice were included. Various numbers of cells were used to absorb for 30 min at 4°C, a concentration of congenic mouse antiserum to H-2^b that would ordinarily lyse 90% of target cells expressing H-2^b antigens (EL4 cells). The absorbed sera were then examined for guinea pig complement-dependent lysis of EL4 cells as described (27).

Binding of [^{3}H]MCMV to Cells. ^{3}H-labeled MCMV was produced by adding [^{3}H]thymidine (New England Nuclear, Boston, Mass.) to MCMV-infected mouse embryo fibroblasts. Virus was purified by equilibrium centrifugation in a 20–60% (vol/vol) Renografin (E. R. Squibb & Sons, Princeton, N. J.) in TNE (50 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA) gradients. The purified preparation of ^{3}H-MCMV contained approximately 10^6 cpm per 10^6 plaque-forming units (PFU) of virus. 2×10^6 cells (PCC4 cells propagated in the presence and absence of 10 mM DMA for 4 d, or mouse embryo fibroblasts) were exposed for various lengths of time at 37°C to 50,000 cpm of [^{3}H]MCMV. The cells were centrifuge-washed five times, lysed by resuspension in 1% sodium dodecyl sulfate (SDS), and added to a scintillation cocktail. The radioactivity was determined in a scintillation counter.

Transport of MCMV DNA to the Nucleus. [^{3}H]Thymidine-labeled MCMV was produced and purified as described above. Undifferentiated or differentiated PCC4 cells were infected with 20,000 cpm of [^{3}H]MCMV at a multiplicity of infection (MOI) of 5. At various times after infection, the cells were dispersed (and virions attached to the cell surface were removed) by treatment with 0.25% trypsin in PBS for 5 min at 37°C. The cells were pelleted and washed twice with TNM buffer (0.01 M Tris, 0.14 M NaCl, 0.0015 M $MgCl_2$, pH 7.4). The cells were disrupted by resuspension in 0.1% Nonidet P-40 nonionic detergent (Shell Oil Co.) in TNM buffer. After 10 min at 4°C, the nuclei were pelleted at 1,500 *g* for 5 min. The trichloroacetic

acid-precipitable radioactivity in the supernatant (cytoplasm) and the pellet (nuclei) was determined.

Indirect Immunofluorescence Assay. Cells grown on cover slips were washed with PBS and then fixed with ether-ethanol (1:1) for 10 min. After being washed in 95% ethanol for 20 min and airdryed, the cells on the cover slips were treated first at 37°C for 30 min with monospecific mouse antibodies to MCMV and then with fluorescein isothiocyanate (FITC)-conjugated monospecific rabbit anti-mouse IgG. After five washes with PBS, the cells were mounted with PBS-glycerol (9:1) and examined with a fluorescence microscope (Carl Zeiss, Inc., New York). Negative controls consisted of uninfected cells treated first with mouse antibodies to MCMV and then with FITC-conjugated rabbit anti-mouse IgG, as well as of infected cells treated first with nonimmune mouse serum and then with FITC-conjugated rabbit anti-mouse IgG. The specificity of the antibodies used and their production and conjugation to FITC have been detailed (15, 28).

MCMV DNA-Cellular DNA Hybridization Analysis. DNA was isolated from cells by the 8 M urea-phosphate method as described (16). In brief, cells were lysed in 0.24 M phosphate buffer with 2% SDS, and proteins were digested with proteinase K (E. M. Laboratories, Fullerton, Calif.; 500 μg/ml). Nucleic acids were extracted with phenol and adsorbed to hydroxylapatite (Bio-Rad Laboratories, Richmond, Calif.). DNA was eluted with 0.48 M phosphate buffer, dialyzed against 10^{-3} M EDTA (pH 8), lyophilized to a 1.0-ml volume, and sonicated to a fragment size of 6–8S. DNA samples were analyzed for MCMV genome equivalents per cell by a phenol emulsion hybridization technique (18, 29). In brief, 0.1 ml samples containing 50 μg DNA/ml (optical density of 1.0 at 260 nm), 0.48 M phosphate buffer (pH 7), MCMV [^{3}H]DNA (nick-translated to 10^7 cpm/μg DNA), and 9% (vol/vol) phenol were denatured at 110°C for 1 min. Samples were cooled in an ice bath and then mixed at room temperature on a Vortex mixer (American Scientific Products, McGaw Park, Ill.). 10-μl samples were removed at various times, and the radioactivity in single- and double-stranded DNA was assayed by hydroxylapatite chromatography (15).

MCMV DNA-Cellular RNA Hybridization Analysis. Cytoplasmic RNA was prepared from cells by Triton N101 (Sigma Chemical Co., St. Louis, Mo.) lysis and extraction with phenol-chloroform-sodium dodecyl sulfate, as described (30). RNA was precipitated with ethanol at −20°C. Cellular RNA samples were analyzed for MCMV RNA by MCMV [^{3}H]DNA-cellular RNA hybridization kinetics. The conditions of hybridization (70% formamide, 0.4 M NaCl, 0.01 M 1,4-piperazinediethane sulfonic acid (PIPES), pH 7.4, 46°C, 500 μg RNA/ml [optical density at 260 nm, 10]) were chosen to facilitate RNA-DNA hybridization while minimizing DNA-DNA hybridization (31, 32). 0.1-ml samples were denatured at 110°C for 1 min and then cooled in an ice bath. At various times after incubation at 46°C, 10-μl samples were removed, and the radioactivity in single-stranded nucleic acid and DNA-RNA duplexes was determined by hydroxylapatite chromatography. The buffer used for elution of single-stranded nucleic acid was 0.14 M phosphate buffer (pH 7), 0.1% SDS and 0.1 M NaCl, and the buffer used for elution of DNA-RNA hybrids was 0.3 M phosphate buffer (pH 7) and 0.1 M NaCl.

MCMV Nuclear RNA-MCMV DNA Hybridization. At 18 h after infection with MCMV, cells were pulse-labeled for 45 min with [^{3}H]uridine (New England Nuclear). The cells were washed with PBS, dispersed by trypsinization, and then washed with TNM buffer. The cells were disrupted by resuspension in 0.1% Nonidet P-40 in TNM buffer. After 10 min at 4°C, the nuclei were pelleted at 1,500 *g* for 5 min and resuspended in a high-salt buffer (0.5 M NaCl, 10 mM Tris, 50 mM $MgCl_2$, pH 7.4). The nuclei were digested with DNase (100 μg/10^8 cells at 37°C for 30 min) that had been treated with iodoacetate to remove RNase (33). Three volumes of sodium acetate (pH 5) and EDTA were added to the digested nuclei to a final concentration of 100 and 10 mM, respectively. The nuclei were disrupted by the addition of SDS to 0.5%, and the RNA was extracted twice with phenol at 65°C and once with chloroform-isoamyl alcohol (24:1). The RNA was precipitated with ethanol overnight at −20°C, dissolved in 10 mM Tris, 10 mM EDTA, pH 7.4 buffer, and precipitated in 2 M LiCl overnight at 4°C. The RNA was fragmented by dissolving in 10 mM Tris-10 mM EDTA buffer, adding NaOH to 0.2 N, and incubating 30 min at 4°C. The RNA solution was neutralized by the addition of HEPES buffer to 0.4 M.

Nitrocellulose filters containing MCMV DNA were prepared using MCMV purified from

the supernatant medium of infected mouse embryo cells (16). In brief, MCMV was pelleted by centrifugation at 30,000 rpm for 1 h in a Type-35 rotor (Beckman Instruments, Inc., Palo Alto, Calif.) and purified by equilibrium centrifugation at 44,000 rpm for 2 d in a CsCl gradient in a Type-65 rotor. The purified virions were disrupted by adding *N*-lauryl sarcosinate to 0.5% and heating to 70°C. MCMV DNA was purified from the disrupted virions by equilibrium centrifugation at 44,000 rpm for 2 d in a CsCl gradient in a Type-65 rotor. MCMV DNA prepared in this manner did not contain any detectable cellular DNA, since nick translated probes did not react with uninfected cell DNA in either solution or in Southern blot hybridization experiments (data not shown). The concentration of MCMV DNA was determined by optical density at 260 nm. Approximately 30 μg of MCMV DNA was affixed to each nitrocellulose filter (25 mm in diameter, 0.45 pore size, Millipore Corp., Bedford, Mass.) by alkali denaturation of the DNA in 0.1 N NaOH, dilution with 14 vol of 2 M NaCl, and slow suction of this solution through the filters. The filters were washed with six times concentrated saline sodium citrate (SSC) and baked for 2 h in a vacuum oven at 80°C. After prehybridization in buffer (0.3 M NaCl, 10 mM EDTA, 10 mM PIPES, pH 6.5, 0.2% SDS, and 100 μg of yeast RNA/ml) for 2 h at 65°C, the buffer was withdrawn and replaced with 0.5 ml of the fragmented nuclear RNA in the same hybridization buffer. After incubation for 40 h at 65°C, the filters were washed twice with twice concentrated SSC at 65°C for 30 min, treated with RNase (5 μg of pancreatic RNase and 5 U of RNase T_1/ml of 2 × SSC) for 30 min at 37°C, and washed again with twice concentrated SSC. After drying, the filters were added to vials containing Scintiverse (Fisher Scientific Co., Pittsburgh, Pa.) and counted in a scintillation counter.

Results

MCMV Fails to Productively Infect Undifferentiated but Does Infect Differentiated Cell Lines. We examined the ability of several undifferentiated and differentiated cell lines to replicate MCMV. Undifferentiated cultures of OTT6050AF1 BrdU, F9, and PCC4 cells, as well as differentiated cultures of PYS2, OTTF12, and mouse embryo fibroblasts (MEF) were infected with MCMV and assayed for virus replication by plaque assay and immunofluorescence. The results presented in Table I show that after MCMV infection undifferentiated cells did not produce a significant increase in the amount of infectious virus in the culture fluids, and very few of the >200 cells

TABLE I

*Infection of Undifferentiated and Differentiated Cells with MCMV**

Cell line	Differentiation state	0 h	48 h	Cells showing viral antigens at 48 h‡
		PFU/ml		%
OTT6050AF1 BrdU	Undifferentiated	2.3×10^3	2.6×10^3	<1
F9	Undifferentiated	5.3×10^3	4.7×10^3	<1
PCC4	Undifferentiated	5.0×10^3	4.1×10^3	<1
PYS2	Differentiated	1.3×10^4	1.4×10^7	>90
OTTF12	Differentiated	3.6×10^3	8.9×10^6	>90
MEF	Differentiated	4.1×10^3	2.1×10^7	>90

* Cells were infected with MCMV at an MOI of 5. After a 1.5-h adsorption period at 37°C, nonadsorbed virus was removed by washing the cells three times with medium. A supernatant sample was taken immediately after washing (0 h) and after 48 h of incubation of the infected cells at 37°C. Infectious virus was measured by plaque assay on monolayers of BALB/St mouse embryo fibroblasts. The presence or absence of MCMV cytoplasmic antigens in cells was determined by indirect immunofluorescence assay as described in the text. Similar results were seen in a repeated experiment.

‡ 200 cells counted.

examined from each line expressed viral antigens. In contrast, the differentiated cell lines produced infectious virus in the culture fluids, and a majority (>90%) of the cells from each differentiated line produced viral antigens. The results show that undifferentiated cells are refractory to MCMV infection, whereas differentiated cells are permissive to MCMV and make infectious MCMV.

Differentiation of PCC4 Cells by DMA. We examined the response to MCMV infection of a pluripotent cell line in both an undifferentiated and a differentiated state. For these studies, we used the PCC4 cell line that can be induced to differentiate by treatment with DMA (22). First, we examined PCC4 cells propagated in the presence and absence of DMA for their content of H-2^b antigens as a marker for cell differentiation (other undifferentiated cells lack H-2 antigens) (34, 35). Fig. 1 shows that PCC4 cells propagated in the absence of DMA do not express H-2^b antigens. In contrast, PCC4 cells grown in the presence of 10 mM DMA for 3 d expressed H-2^b antigens. Specificity controls included the use of monospecific antibodies to H-2^b and H-2^k, appropriate H-2^b and H-2^k targets for study, and experiments showing that absorption with H-2^k spleen cells did not change the cytotoxic activity, whereas absorption with H-2^b spleen cells significantly removed cytotoxicity activity. For all subsequent experiments we used the presence of H-2^b antigens on the cell's surface to confirm the state of cell differentiation. As seen in Fig. 2, MCMV replicated in differentiated PCC4 cells but failed to make infectious virus in these cells when they were in the undifferentiated state.

Several Viruses Other Than MCMV Replicate in Undifferentiated and Differentiated PCC4 Cells. Next, we examined the ability of undifferentiated and differentiated PCC4 cells to replicate a variety of viruses. PCC4 cells either undifferentiated or differentiated with DMA were permissive to VSV, LCMV, and herpes simplex virus type 1. Fig. 2 shows that VSV, LCMV, and herpes simplex virus replicated to an equivalent extent in undifferentiated and differentiated PCC4 cells. In contrast, undifferentiated PCC4 cells infected with MCMV did not produce infectious MCMV in the supernatant fluids, whereas differentiated PCC4 cells produced almost 10^7 PFU/ml. These

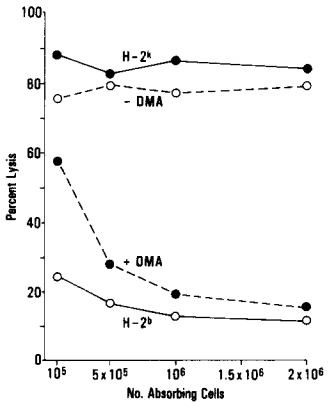

FIG. 1. Microcytotoxicity absorption of PCC4 cells propagated with and without DMA. Various numbers of PCC4 cells propagated in monolayers with (●- - -●) or without (○- - -○) DMA (10 mM), or spleen cells from 129/J (H-2^b) (○——○) or C3H/St (H-2^k) (●——●) mice were used to absorb for 30 min at 4°C a concentration of mouse antiserum to H-2^b that would lyse 90% of target cells expressing H-2^b antigens (EL4 cell). The absorbed sera were then examined for anti-H-2^b antibody activity by guinea pig complement-dependent lysis of EL4 cells.

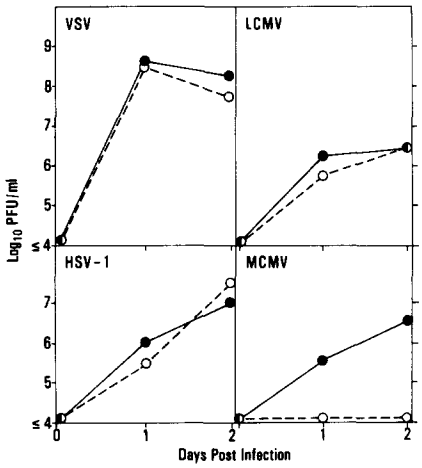

FIG. 2. PCC4 cells were propagated for 4 d at 37°C in the presence (differentiated cells, ●——●) or absence (undifferentiated cells, O- - -O) of 10 mM DMA. The state of differentiation was determined by the presence of H-2^b antigens on the cell's surface (see Fig. 1). Cultures were infected with VSV, LCMV, HSV-1, or MCMV at an MOI of 1. Supernatant samples were removed immediately after adsorption (0 d) and at 1 and 2 d after infection. The titers of infectious virus were determined by plaque assays on Vero cells (VSV and LCMV), human fibroblasts (HSV-1), and mouse embryo fibroblasts (MCMV).

undifferentiated cells inoculated with MCMV did not produce a significant increase in the amount of infectious virus during the 4 d of observation (data not shown for days 3 and 4 postinfection).

Block in the MCMV Infection of Undifferentiated Cells Is in Viral RNA Transcription. To determine the stage at which virus infection was blocked in undifferentiated cells, we examined the binding of radiolabeled MCMV to cells (adsorption), the transport of MCMV DNA to the cell nucleus and the presence of MCMV DNA, MCMV RNA (transcription), and MCMV proteins (translation) in undifferentiated cells after viral inoculation. The results presented in Fig. 3 show that [^{3}H]MCMV bound equally well to undifferentiated PCC4 cells, differentiated PCC4 cells, and mouse embryo fibroblasts. Thus, MCMV adsorbs to the nonpermissive undifferentiated PCC4 cells as efficiently as to the permissive differentiated PCC4 cells or to the permissive mouse embryo fibroblasts.

The results in Table II show that the majority of the [^{3}H]thymidine-labeled MCMV DNA was detected in the nuclei of undifferentiated or differentiated PCC4 cells at 6 h after infection. These data suggest that MCMV can adsorb, penetrate, and be transported to the nucleus of undifferentiated PCC4 cells and that the block in the MCMV infection of undifferentiated cells is not at any of these early stages of infection.

We then examined MCMV-infected undifferentiated PCC4 cells for the presence of viral gene products. Fig. 4 shows that undifferentiated PCC4 cells infected with MCMV did not produce detectable amounts of viral antigens. On the other hand, differentiated PCC4 cells infected with MCMV produced viral antigens.

Next, we investigated whether undifferentiated PCC4 cells infected with MCMV contained viral DNA or viral RNA. We separately analyzed DNA and RNA harvested from undifferentiated PCC4 cells infected with MCMV for hybridization to a MCMV

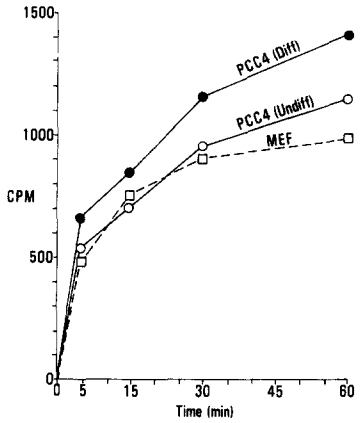

FIG. 3. 2 million cells (●, differentiated cells propagated in the presence of 10 mM DMA for 4 d; ○, undifferentiated cells propagated in the absence of DMA; □, mouse embryo fibroblasts) were exposed for various times at 37°C to [^{3}H]thymidine-labeled mouse cytomegalovirus. The cells were centrifuge-washed five times, lysed by resuspension in 1% SDS, added to scintillation cocktail, and counted in a scintillation counter. The counts per minute minus the background was plotted vs. the time at 37°C. State of differentiation of PCC4 cells was confirmed by analyzing the expression of H-2^b antigens on the cell's surface (see Fig. 1).

TABLE II

*Cellular Fractionation of Undifferentiated and Differentiated PCC4 Cells Infected with [^{3}H]MCMV**

PCC4 Cells	Hours after infection			
	1 h		6 h	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
	cpm			
Undifferentiated	150	613	904	113
Differentiated	201	757	781	212

* PCC4 cells were grown on 60-mm culture dishes either without DMA (undifferentiated) or with 10 mM DMA for 3 d (differentiated). Cells were infected with 20,000 cpm of [^{3}H]thymidine-labeled MCMV at an MOI of 5. At 1 and 6 h after infection, the cells were resuspended in 0.1% Nonidet P-40 and separated by centrifugation into cytoplasmic and nuclear components. The trichloroacetic acid-precipitable radioactivity in the supernatant (cytoplasm) and the pellet (nuclei) was determined. The state of PCC4 differentiation was confirmed by assaying expression of H-2^b antigens on the cell's surface (see Fig. 1).

DNA probe. Table III shows that infected undifferentiated PCC4 cells contained detectable MCMV DNA but did not possess detectable MCMV RNA in the cell cytoplasm. In contrast, both differentiated PCC4 cells and mouse embryo fibroblasts infected with MCMV contained viral DNA and viral RNA.

To determine whether MCMV-infected undifferentiated cells contained any detectable MCMV RNA in the nucleus, we pulse-labeled cells with [^{3}H]uridine, isolated the nuclear RNA fraction, and hybridized this RNA to nitrocellulose filters that contained MCMV DNA. The results presented in Table IV show that MCMV-infected undifferentiated PCC4 cells did produce detectable MCMV RNA at ~17% of the amount of MCMV RNA found in MCMV-infected differentiated cells. These

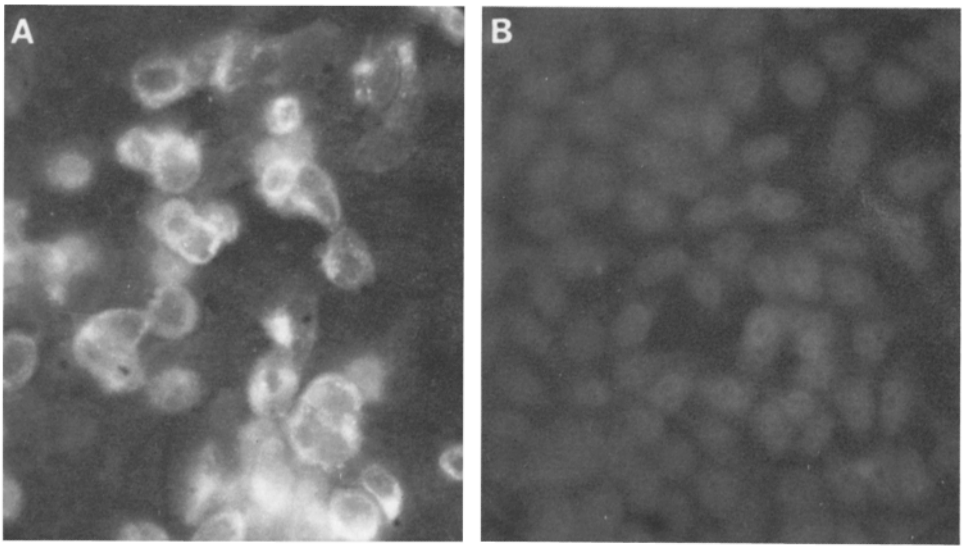

FIG. 4. Detection of MCMV antigens in infected differentiated or undifferentiated PCC4 cells. PCC4 cells were either induced to differentiate by propagation for 3 d with 10 mM DMA (A) or maintained as undifferentiated cells by propagation without DMA (B). The cells were infected with MCMV at an MOI of 1. At 24 h after infection, the cells were examined for cytoplasmic MCMV antigens by indirect immunofluorescence. × 360.

results demonstrate that some MCMV RNA is transcribed but that a significant block in the MCMV infection of undifferentiated PCC4 cells occurs at the level of RNA transcription.

Establishment of an MCMV Latent Infection in Undifferentiated Cells and Activation of MCMV during PCC4 Cell Differentiation. Undifferentiated PCC4 cells adsorbed MCMV and contained viral DNA but did not produce infectious virus, whereas differentiated PCC4 cells made infectious virus. Thus, we determined whether a model of latent infection with subsequent virus activation and infectious virus production could be established by inducing undifferentiated cells infected with MCMV to differentiate. Undifferentiated PCC4 cells were infected with MCMV, and any residual infectious virus was inactivated with antibody to MCMV and guinea pig complement. These infected undifferentiated PCC4 cells were then propagated under subconfluent conditions that maintained the undifferentiated state. No infectious virus was detected in the culture fluids. 1 d later, the cells were subcultured either without DMA to maintain the cells as undifferentiated or with DMA to induce the cells to differentiate. Uninfected PCC4 cells were cultured in a similar manner. At 0, 2, 3, and 4 d after infection, the cells were analyzed for their content of MCMV DNA, for the presence of MCMV antigens, and, for the production of infectious virus. Table V shows the data from one of three representative experiments. When MCMV-infected PCC4 cells were cultured without DMA, infectious virus was not detectable until 3 and 4 d after infection. The presence of a small amount (3.3×10^1 PFU/ml at day 3 postinfection) of infectious virus correlated with a small increase in the MCMV DNA genome equivalent per cell from 0.02 (day 2) to 0.12 (day 3), as well as with an increase in the number of cells positive for MCMV antigens from 0.7% (day 2) to 2.4% (day 3). Inasmuch as spontaneous differentiation occured in ~3% of these cells

TABLE III

*Detection of MCMV DNA in the Nucleus or MCMV RNA in the Cytoplasm of MCMV-infected Undifferentiated and Differentiated PCC4 Cells**

To detect MCMV	Culture	State of differentiation	Double-stranded nucleic acid at hour of hybridization			
			0 h	1 h	2 h	3 h
				%		
DNA	Uninfected	Undifferentiated	0.0	4.8	13.0	20.0
	Uninfected	Differentiated	0.0	3.5	12.3	18.1
	Infected	Undifferentiated	0.0	37.5	50.0	58.3
	Infected	Differentiated	0.0	51.2	64.3	73.3
			0 h	20 h		96 h
				%		
RNA	Uninfected	Undifferentiated	7.5	7.8		8.1
	Uninfected	Differentiated	7.6	7.7		8.3
	Infected	Undifferentiated	7.9	7.9		8.5
	Infected	Differentiated	7.8	23.6		25.1

* PCC4 cells were incubated with or without 10 mM DMA for 3 d at 37°C and then infected with MCMV at an MOI of 5. After adsorption for 1.5 h at 37°C, the cells were washed and then incubated at 37°C with MEM. 16 h later, DNA was isolated from one group and RNA from another group of cells. The DNA samples were analyzed for MCMV DNA by the phenol emulsion reassociation technique with MCMV [^{3}H]DNA probe, and the RNA samples were analyzed for MCMV RNA by MCMV [^{3}H]DNA-RNA hybridization in the presence of formamide as described in the text. The amount of probe (MCMV [^{3}H]DNA) in double-stranded form was determined by hydroxylapatite chromatography. Equivalent results were obtained in three repeat experiments. The state of PCC4 differentiation was monitored by assaying H-2^b antigen expression on the cell's surface.

TABLE IV

*Detection of MCMV RNA in the Nucleus of MCMV-infected Undifferentiated and Differentiated PCC4 Cells**

Culture	State of differentiation	cpm bound	cpm (infected) — cpm (uninfected)
Uninfected	Undifferentiated	1,284	—
Uninfected	Differentiated	1,466	—
Infected	Undifferentiated	3,144	1,860
Infected	Differentiated	12,652	11,186

* Culture dishes (175 cm^2) of PCC4 cells propagated without (undifferentiated) or with 10 mM DMA for 3 d (differentiated) were either mock infected or infected with MCMV at an MOI of 5. At 18 h after infection, the medium was decanted and replaced with 10 ml of medium containing 3 mCi of [^{3}H]uridine per dish. After 45 min of incubation at 37°C, the nuclear RNA fraction was isolated. MCMV RNA was detected by hybridization to nitrocellulose filters containing either purified MCMV DNA or no DNA (blank filters). The hybridization conditions were 40 h at 65°C in a buffer consisting of 0.3 M NaCl, 10 mM EDTA, 10 mM PIPES, pH 6.5, 0.2% SDS, and 100 μg of yeast RNA/ml. After hybridization, the filters were treated with RNase, dried, and counted in a scintillation counter. Counts per minute (cpm) bound is the average of the cpm bound to duplicate filters that contained MCMV DNA minus the cpm bound to duplicate blank filters. Similar results were obtained in three repeat experiments. The state of differentiation was monitored by assaying H-2^b antigens expressed on the cell's surface.

TABLE V

*Activation of Latent MCMV from Undifferentiated PCC4 Cells Occurs during Cell Differentiation**

Culture	Day postinfection	MCMV DNA genome equivalents per cell	Cells positive for MCMV antigen	Plaque-forming units per milliliter
			%	
−DMA	0	<0.01	0	<10
−DMA	2	0.02	0.7	<10
−DMA	3	0.12	2.4	3.3×10^1
−DMA	4	0.11	2.1	4.0×10^2
+DMA	2	0.01	3.4	1.6×10^2
+DMA	3	0.37	17.2	1.0×10^2
+DMA	4	1.20	75.0	6.0×10^5

* Undifferentiated PCC4 cells were infected for 1.5 h at 37°C at an MOI of 10. Residual infectivity was removed by incubating cells for 30 min at 37°C with mouse antisera to MCMV and guinea pig complement. A sample of cells and the supernatant fluid was removed at this time (day 0 postinfection). The infected undifferentiated cells were propagated for 24 h under conditions that maintained the majority of the cells in an undifferentiated state. At 1 d after infection, 5×10^6 cells were seeded into each of several 250-ml Erlenmeyer flasks containing 50 ml of medium, with or without 10 mM DMA. At 2, 3, and 4 d after infection, samples of both supernatant fluids and cells were taken. Supernatants were analyzed for infectious virus (plaque-forming units) by plaque assay on MEF. The cells were analyzed for MCMV DNA by the hybridization technique described in the text. In addition, the cells were pelleted onto glass slides and fixed with ether-alcohol, and 200 cells were examined for MCMV antigens by indirect immunofluorescence. The state of PCC4 differentiation was monitored by assaying the expression of $H\text{-}2^b$ antigens on the cell's surface.

by day 3, the infectious virus detected in undifferentiated cell cultures probably came from cells that spontaneously differentiated as the cultures became overconfluent. In contrast, when MCMV-infected PCC4 cells were cultured with DMA to induce cell differentiation, infectious virus was detectable at 2 d after infection (1.6×10^2 PFU/ml) and reached 6.0×10^5 PFU/ml at 4 d after infection, the majority of the cells (>75%) contained MCMV antigens (Fig. 5), and the cells contained 1.2 MCMV DNA genome equivalents/cell.

Discussion

In this paper we report two new observations. First, MCMV cannot productively infect several undifferentiated cell lines tested. Although virus can adsorb to undifferentiated cells and viral DNA is detected in the cell nucleus, complete viral RNA transcription, viral translation, or production of infectious virus does not occur. Second, this observation can be extended to develop a model of MCMV latency and activation. Upon induction of cell differentiation in previously MCMV-infected undifferentiated cells, viral transcription and release of infectious virus occur.

We found that MCMV could not productively infect undifferentiated cells. This finding was generalized to include several undifferentiated cell lines such as OTT6050AF1 BrdU, F9, and PCC4. In contrast, MCMV did productively infect differentiated teratocarcinoma cell lines such as PYS2 and OTTF12. Although these findings were consistent and were observed in repeated experiments with MCMV, they are not universal for all herpesviruses. Herpes simplex virus type 1 replicates and

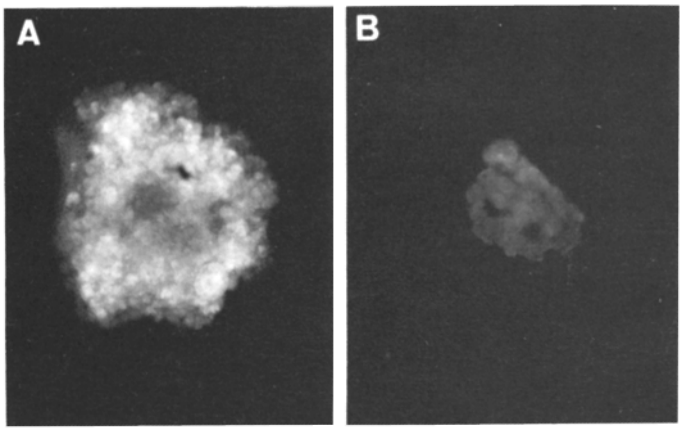

FIG. 5. Detection of MCMV antigens after induction of cell differentiation in MCMV-infected undifferentiated cells. Undifferentiated PCC4 cells were infected with MCMV and propagated as described in the legend to Table V. At 3 d postinfection and after either induction of cell differentiation with DMA (A) or maintenance of undifferentiation (B), the cells were allowed to attach to glass slides, fixed with ether-alcohol, and examined for MCMV antigens by indirect immunofluorescence. × 120.

makes infectious virus in undifferentiated cells as well as differentiated cells. In agreement with the observations concerning undifferentiated mouse cells and MCMV reported here, we have recently found that human CMV also fails to productively infect Tera-2 or PA-1 cells, undifferentiated human teratocarcinoma cell lines, but that it is permissive for differentiated lines.[2] The inability of MCMV to infect undifferentiated cells differs from most other viruses studied, inasmuch as Coxsackie B3 virus, encephalomyocarditis virus, murine hepatitis virus, Sindbis virus, Semliki Forest virus, LCMV, Pichinde virus, VSV, herpes simplex virus type 1, and vaccinia virus can replicate to a similar extent in undifferentiated and differentiated cells (36–38, Fig. 2).

It was of interest to determine the step at which MCMV replication in undifferentiated cells was blocked. Studies with radiolabeled MCMV indicated a similar number of binding sites on the undifferentiated PCC4 cells and the differentiated PCC4 cells (Fig. 3), as well as efficient transport of MCMV DNA to the nucleus (Table II). DNA hybridization kinetic experiments detected MCMV DNA in undifferentiated cells, but immunofluorescence results indicated that viral antigens were not made in these cells. Furthermore, infectious virus was not produced. MCMV DNA-cellular RNA hybridization experiments failed to show viral RNA in the cytoplasm from MCMV-infected undifferentiated cells. However, a low level of MCMV RNA could be detected in the nucleus by a pulse-labeling assay. Thus, the block in the MCMV infection of undifferentiated PCC4 cells appears to be at the level of RNA transcription. The finding of MCMV RNA in the nucleus of undifferentiated cells (Table IV) suggests that a portion of the viral genome may be transcribed. Experiments to determine which regions of the genome are transcribed in undifferentiated cells, the presence or absence of early MCMV antigens, and the reason(s) for failure of viral RNA transcription are currently underway.

The mechanism(s) involved in the block in viral RNA transcription in MCMV-

[2] Dutko, F. J., and M. B. A. Oldstone. Manuscript in preparation.

infected undifferentiated cells is unknown, but it may be due to a deficiency in RNA splicing. Indeed, our findings with MCMV in undifferentiated teratocarcinoma cells are reminiscent of the findings of others in similar cell lines with SV40. SV40 fails to replicate in F9 undifferentiated cells, but the block is not at the level of virus adsorption, penetration, uncoating, or transport to the nucleus (39). Such F9 cells infected with SV40 produce an unspliced early viral RNA transcript that is unstable (40). When F9 cells were induced to differentiate with retinoic acid (41), they produced stable spliced early SV40 mRNAs (42). Further, it is of interest to compare our findings with those obtained from herpes simplex virus latency in vivo in trigeminal ganglia. Using nucleic acid hybridization techniques, Puga and his colleagues (43) noted the presence of herpes simplex virus DNA but not viral RNA and postulated a molecular lesion in RNA transcription.

Very few viruses cannot productively infect undifferentiated cells while possessing the ability to infect differentiated cells. Polyoma virus can adsorb to undifferentiated cells and viral DNA is transported to the nucleus, but T or V antigens are not made (39, 44, 45). The nature of the block may be at viral RNA synthesis, since a low level of spliced viral RNA can be detected in polyoma virus-infected PCC4 cells (46, 47). Several murine leukemia viruses were also unable to productively infect undifferentiated cells, but could productively infect differentiated cells (37, 48, 49). Proviral DNA, but no infectious virus, was detected in undifferentiated cells (37). The minute virus of mice also fails to productively infect undifferentiated cells (50).

Using infected undifferentiated PCC4 cells, we were able to develop a model of CMV latency and subsequent activation. Whereas undifferentiated cells previously infected with MCMV failed to make infectious virus over several days in culture, the induction of cell differentiation resulted in the production of MCMV RNA, protein, and infectious virus.

This model of CMV in undifferentiated cells offers several unique opportunities for understanding both latency and MCMV infection. First, teratocarcinoma cells are similar to cells of the early mouse embryo in their potential to differentiate and in many of their surface antigens. Early mouse embryos are resistant to MCMV infection, whereas later-stage embryos are susceptible (51). Hence, study of teratocarcinoma cells in vitro and in vivo may provide leads for the understanding of CMV-associated birth defects. Second, several pluripotent teratocarcinoma cell lines can be induced to differentiate in vitro into cells of the embryonic ectoderm, endoderm, and mesoderm. The infection of such differentiated cells and precursor undifferentiated cells with MCMV may help lead to the control of viral and host gene expression in different cell types. Third, understanding the defect in MCMV replication in undifferentiated teratocarcinoma cells should allow the production and utilization of appropriate nucleic acid probes for the study of latent CMV infection in vivo. This information will be of value in understanding human CMV disease.

Summary

Murine cytomegalovirus (MCMV) does not productively infect OTT6050AF1 BrdU, F9, or PCC4 undifferentiated murine teratocarcinoma cell lines, as shown by immunofluorescence assays for viral antigens and by plaque assays for infectious virus. However, these cells were infected by a variety of other viruses. MCMV does

productively infect PYS2 and OTT F12 differentiated murine teratocarcinoma cell lines.

The replication of MCMV in the pluripotent PCC4 cell line was examined in detail. Undifferentiated PCC4 cells could be differentiated when propagated in the presence of dimethylacetamide, as judged by changes in the expression of H-2 antigens on the cell surface. Several viruses, including lymphocytic choriomeningitis virus, herpes simplex virus type 1, and vesicular stomatitis virus, replicated to a similar extent in differentiated and undifferentiated PCC4 cells. MCMV did productively infect differentiated PCC4 cells. In contrast, MCMV did not produce infectious virus, viral antigens, or substantial viral RNA in undifferentiated PCC4 cells.

The molecular block of MCMV replication occurred at the level of MCMV RNA transcription. Undifferentiated PCC4 cells have receptors for MCMV and bind similar amounts of radiolabeled virus as differentiated PCC4 cells. After MCMV binds to its receptors on undifferentiated cells, MCMV penetrates the plasma membrane and is transported to the cells' nuclei. MCMV DNA was present in the cytoplasm, and small amounts of MCMV RNA (<17% of that found in MCMV-infected differentiated PCC4 cells) were found in the nucleus. However, MCMV RNA was not detected in the cytoplasm of undifferentiated cells.

A latent infection was established by infecting undifferentiated PCC4 cells with MCMV, inactivating residual infectivity with antibodies to MCMV, and propagating cells under conditions that maintained the undifferentiated state. These MCMV-infected undifferentiated cells did not produce infectious virus, viral antigens, or viral RNA but did contain viral DNA detectable by DNA-DNA hybridization kinetics. Latency was terminated and infectious virus was made when such undifferentiated cells were induced to differentiate.

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