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A Novel Mechanism for Persistence of Human Cytomegalovirus in Macrophages

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Human cytomegalovirus (HCMV) infection of monocyte-derived macrophages (MDM) results in delayed and nonlytic productive viral growth. During late stages of replication, infectious virus remains cell associated in cytoplasmic vacuoles. In order to understand HCMV survival and persistence in MDM, we examined mechanisms involved in the formation and trafficking of HCMV-containing vacuoles in these cells. Utilizing double-label immunofluorescence with antibodies to viral and cellular proteins, HCMV-containing vacuoles were associated with the Golgi apparatus marker mannosidase II but not with markers to early endosomes (transferrin receptor and rab5) or late endosomes and early lysosomes (LAMP-1 and -2). In addition, as late-stage viral infection progressed in MDM, the cells displayed increasing abnormalities in the Golgi apparatus. Analysis of structural features of infected cells revealed the disruption of the microtubule network. These observations suggest a novel mechanism by which HCMV is vacuolized in MDM, avoiding degradation and release from the cell.

Examination of cell types naturally infected by human cytomegalovirus (HCMV) in vivo has identified monocytes/macrophages as important cells in the biology of the virus (1, 10, 12, 20). In order for HCMV to persist in the host, the virus must avoid immune surveillance. A common mechanism that viruses use to evade immune detection is to restrict viral replication in cells until activation signals trigger the production of infectious virus. In the case of HCMV, virus infection of progenitor myeloid cells (23, 26, 27, 34, 35) as well as monocytes (13, 22, 33, 38, 39) is restricted to early events. However, differentiation of these cells into macrophages results in the production of infectious virus (19, 25). Therefore, one strategy that HCMV utilizes to evade immune detection is to remain quiescent in undifferentiated monocytes until the cells have differentiated into macrophages.

During HCMV productive infection of macrophages, the optimal situation for virus-host cell interactions is to maintain cellular survival during accumulation of virus. Recently, the viral replication cycle of HCMV in monocyte-derived macrophages (MDM) was shown to be significantly delayed relative to replication in human fibroblasts (HF) (14). In MDM, viral production peaked at 13 to 15 days postinfection (dpi) in contrast to 4 to 5 dpi in HF cells (14). HCMV, similar to other herpesviruses, exhibits a temporal order of gene expression during productive infection of cells. Immediate early genes are activated first, followed by early and late genes (37, 41). The altered growth of HCMV in MDM correlated with the delayed kinetics of viral immediate early and late gene expression in these cells. The retarded production of HCMV gene products may prevent the rapid increase of toxic viral factors in the cell. The delay in viral gene expression may therefore be a unique adaptation of HCMV to persist in MDM.

HCMV infection of MDM was also shown to be nonlytic and exclusively cell associated (14, 19). Infectious virus accumulated in discrete cytoplasmic vacuoles which were not associated with the plasma membrane. An important aspect of HCMV intracellular survival in the macrophage is evasion of host cell-mediated degradation induced by fusion of vacuoles with lysosomes. Pathogens utilize multiple strategies to ensure their intracellular survival within the endosomal-lysosomal system of MDM. One mechanism of microbial survival in macrophages is exemplified by *Mycobacterium tuberculosis* and *Mycobacterium microti* (15, 16). These macrophage-tropic pathogens avoid destruction by producing an amine which raises the pH of the vacuoles in which they reside, preventing the vacuoles from becoming acidic, active lysosomes (15, 16). Alternatively, organisms such as *Leishmania mexicana* and *Leishmania amazonensis* have adapted to resist degradation by lysosomal contents (2, 7). *Listeria monocytogenes* is an intracellular pathogen which survives in the macrophage by lysing the bacterial phagosome prior to phagosome-lysosome fusion (18, 29). Finally, *Salmonella typhimurium* enters a vacuole which is not acidified and does not appear to fuse with lysosomes (3). Therefore, intracellular pathogens have evolved a number of mechanisms to survive and persist in macrophages.

Although a number of viruses in addition to HCMV survive and persist in macrophages, the mechanisms involved in intracellular survival are unknown (31, 32). To address this issue, we examined the formation of HCMV-containing vacuoles in MDM. Our results suggest that HCMV uses a novel strategy for MDM intracellular survival through disruption of the microtubule network.

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MATERIALS AND METHODS

Isolation and culture of MDM. Peripheral blood mononuclear cells (PBMC) were isolated from blood samples of HCMV-seronegative donors selected from a pool of donors at Oregon Health Sciences University. Heparinized whole blood

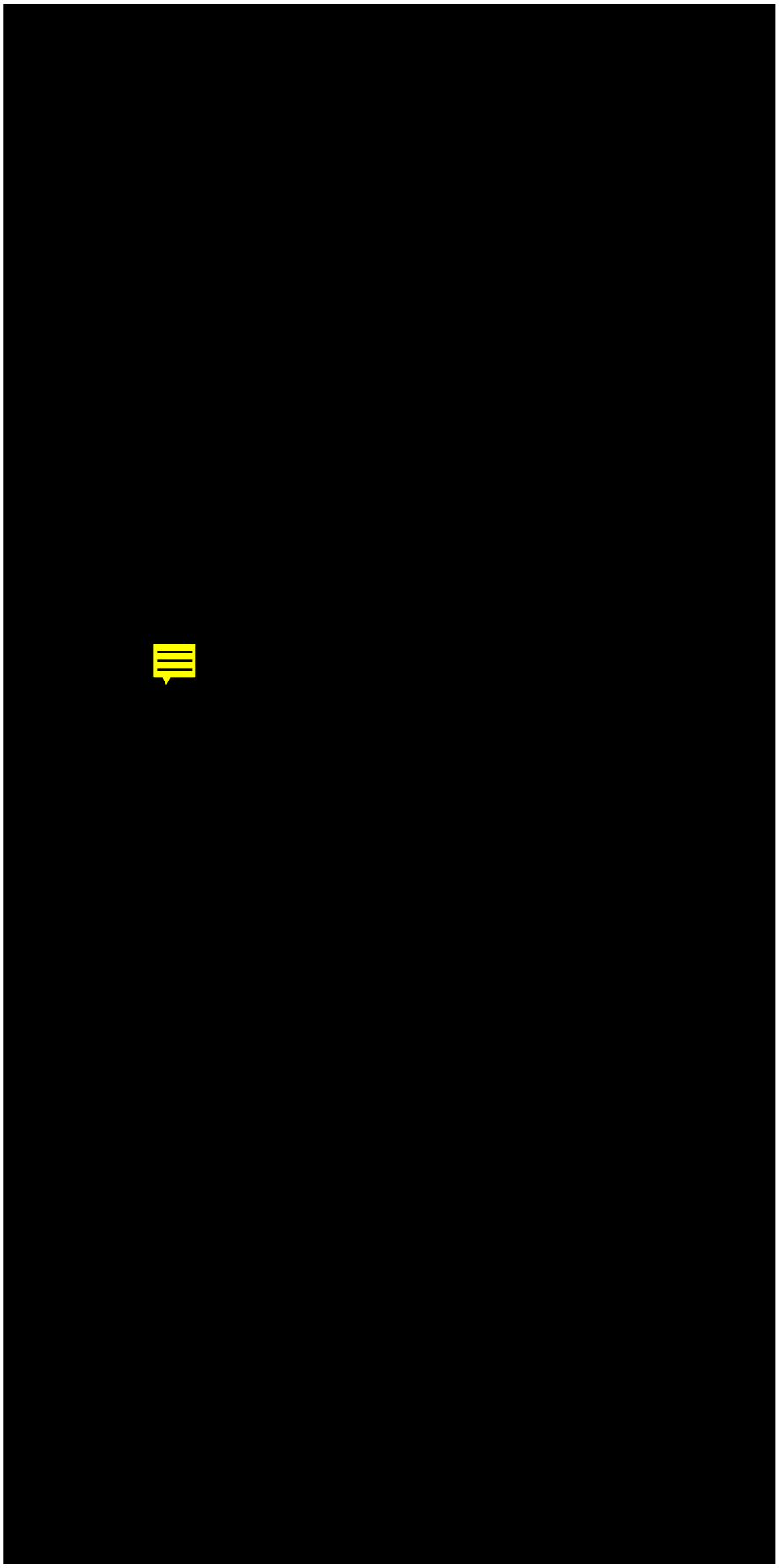


FIG. 1. Colocalization of HCMV and cellular organelle-specific proteins in MDM. MDM were infected with HCMV and examined 10 to 14 dpi by confocal microscopy by using the following antibodies: IE72 (green) and pp65 (red) (A), pp65 antigen (red) and membrane-associated mannosidase II antigen (green) (B), gB antigen (red) and membrane-associated mannosidase II antigen (green) (C), gB (red) and transferrin receptor (green) (D), gB (red), rab5 (green), and microtubulin (blue) (E), and gB (red) and LAMP-1 (green) (F). Uninfected (G) and HCMV-infected (H) MDM were examined for the presence of pp71 (red) and tubulin (green) antigen by double-label immunofluorescence. N, nucleus; V, vacuole; M, mannosidase II; T, transferrin receptor; R, rab5; Tu, tubulin; L, LAMP-1. Bars, 10 μm.

was underlaid with Histopaque (Sigma Chemical Co., St. Louis, Mo.) and centrifuged at 1,500 rpm in a Sorvall RT6000B centrifuge for 25 min at room temperature. The PBMC band was collected, washed twice with sterile saline and once with serum-free media, and resuspended at 4×10^7 cells per ml in Iscove's medium (GIBCO Laboratories, Grand Island, N.Y.) with 1.0% penicillin-streptomycin solution (GIBCO) and 10% pooled human serum (prepared from the Oregon Health Sciences University donor pool). Approximately 1.5×10^7 PBMC were plated per chamber onto Lab-Tek 2 chamber slides (Nunc, Inc., Naperville, Ill.) or approximately 5×10^7 PBMC were plated onto 60-mm-diameter Primaria culture dishes (Becton Dickinson, Lincoln Park, N.J.) and incubated at 37°C with 7% CO_2 for infections. Adherent cells were induced to differentiate by cocultivation with concanavalin A (5.0 μg/ml; Sigma)-treated nonadherent cells for 24 h. Subsequently, all nonadherent cells were removed and adherent MDM were cultured in complete 60/30 medium (60% AIM-V medium and 30% Iscove's medium [GIBCO] supplemented with 10% human serum and 1% penicillin-streptomycin). The adherent cells were greater than 99% esterase positive at 72 h (19). MDM cultures were fed every 3 days with 50% fresh medium and 50% spent medium clarified by centrifugation. Replacement of this conditioned medium at each feeding was necessary for optimal MDM differentiation. Day 1 of differentiation is defined as the day after the initial PBMC isolation and stimulation with concanavalin A.

HCMV infection of MDM. Two recent isolates of HCMV were used to infect primary cultures of MDM. These isolates (I-G and PH) were isolated from transplant patients with HCMV disease, passaged through HF, and frozen below passage 12 in liquid nitrogen (19, 33). Frozen samples from this stock were thawed and passaged for three additional rounds through HF cells prior to MDM infections. Supernatants from HCMV-infected HF cells were used as the source of MDM inoculum, which was diluted 1:1 with MDM-conditioned media. Briefly, MDM cultures containing approximately 5×10^6 cells per 60-mm-diameter culture dish or 3×10^6 cells per slide chamber were infected at 8 to 10 days postdifferentiation with infected HF supernatants containing approximately 10^8 PFU per dish. Virus inoculum was incubated with the MDM for 2 h, removed with thorough washing, and replaced by conditioned MDM medium.

Immunocytochemistry. HCMV-infected MDM grown on chamber slides were fixed for 20 min at room temperature in buffered picric acid-paraformaldehyde (2% paraformaldehyde–15% picric acid) and permeabilized with 0.3% Triton X-100 in phosphate-buffered saline (PBS). Monolayers were blocked with 20% normal goat serum in PBS and incubated for 1 h at 37°C with a 1:100 dilution of one of the following antibodies raised against HCMV gene products: a polyclonal rabbit antibody to pp71, a monoclonal antibody to pp65, or a monoclonal antibody to glycoprotein B (5). Polyclonal antibody to the pp71 protein was generated by immunizing New Zealand White rabbits with recombinant protein generated in an *Escherichia coli* expression system (21). Cellular organelles were identified with the following primary antibodies: monoclonal antibodies H4A3 and H4B4 raised against the lysosome-associated membrane proteins LAMP-1 and LAMP-2, respectively (4, 6); a monoclonal antibody raised against alpha-tubulin (Amersham Life Sciences, Arlington Heights, Ill.); a monoclonal antibody, E7, raised against beta-tubulin (9); a polyclonal antibody specific for the Ras-like GTPase rab5 (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) which localizes to early endosomes (8); a polyclonal rabbit antibody to mannosidase II which is a Golgi apparatus resident enzyme (28); and a polyclonal goat anti-human antibody to transferrin receptor which is also an early endosome marker (42). Binding of primary antibody was detected with fluorescein isothiocyanate-, tetramethyl rhodamine isocyanate-, or Cy-5 (Biological Detection Systems, Inc., Pittsburgh, Pa.)-conjugated secondary antibodies raised in the appropriate species and visualized on an upright Leitz fluorescent microscope or a Leica Confocal Laser Scanning Microscope equipped with a Leitz Fluorovert-FU Microscope and argon-krypton laser.

The surface expression of major histocompatibility complex (MHC) class II on MDM was determined by measuring the fluorescence intensity of macrophages labeled with a fluorescent anti-HLA-DR antibody (L243; Becton Dickinson, Mountain View, Calif.). MDM were fixed in buffered picric acid-paraformaldehyde prior to staining. The Slowfade Antifade Kit (Molecular Probes, Inc., Eugene, Oreg.) was utilized to ensure minimal fluorescence fading. The Quantimet 500 fluorescence analysis program (Leica) was used to quantitate fluorescence. MHC class II expression was measured on days 8, 10, and 12 postinfection. The fluorescence intensity of uninfected macrophages was adjusted to 100% for each time point, and the fluorescence intensity of HCMV-infected macrophages was expressed as a percentage of that observed in uninfected cells. The measurements for nine cells (mean ± standard deviation) were recorded for each time point.

Drug-mediated modification of MDM organelles. Prior to fixation, MDM were pretreated with 10 μM taxol (Molecular Probes, Inc.) for 30 min at 37°C (36) to ensure that depolymerization of microtubules was not due to experimental procedures (11). In the nocodazole experiments, macrophages were treated for various intervals up to 6 days with 2.5 μg of nocodazole {methyl-(5-[2-thienylcarbonyl]-1H-benzimidazol-2-yl)-carbamate; Sigma} per ml, which allows for the total depolymerization of the microtubulin network without resulting in cell death or lifting of MDM off the culture substrate.

BODIPY C_6-ceramide (3,3′-dihexyloxacarbocyanine iodide $DiOC_6$; Molecular Probes, Inc. [3]) was used to examine the integrity of the Golgi apparatus in MDM. Mock- and HCMV-infected MDM cultures were exposed to BODIPY C_6-ceramide (10 μg/ml) for 10 min at different dpi, rinsed, and maintained in normal culture medium for 1 h before visualization by fluorescence microscopy.

HCMV-containing vacuole isolation from MDM. Isolation of HCMV-infected vacuoles was performed as follows. At 13 dpi a serum-coated coverslip was placed over the MDM culture and incubated for 24 h. Removal of the coverslip displaced the plasma membrane from the cell, exposing the intracellular organelles. Subsequently, the cells were rinsed lightly to recover the HCMV-containing vacuoles, which were separated from free virus by centrifugation in a Sorvall RT6000B at $500 \times g$ for 15 min. Plaque assays were performed with pelleted cellular organelles and supernatant on HF cells as previously described (14).

RESULTS

MDM vacuoles contain infectious HCMV. In previous studies of HCMV-infected MDM, numerous intracellular virus-containing vacuoles were observed at later stages of infection (Fig. 1A and B). Since infected MDM cultures contain cells at different stages of differentiation and virus replication, identification of HCMV in these vacuoles by electron microscopy is exceedingly difficult. Therefore, to demonstrate that vacuoles staining for late viral antigens contain infectious virus, we isolated HCMV-containing vacuoles from infected MDM (17). In these experiments, the plasma membrane of MDM was re-

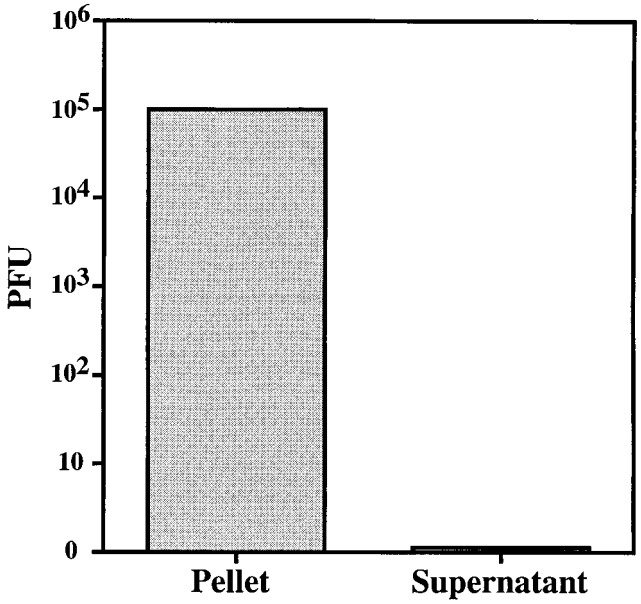


FIG. 2. Association of infectious HCMV with cytoplasmic organelles. MDM were infected with HCMV as described in Materials and Methods. The plasma membrane was removed at 14 dpi, and the cells were rinsed lightly to recover free organelles from the MDM. Following centrifugation, the cytoplasmic organelle pellet and supernatant were tested by plaque assay on HF cells.

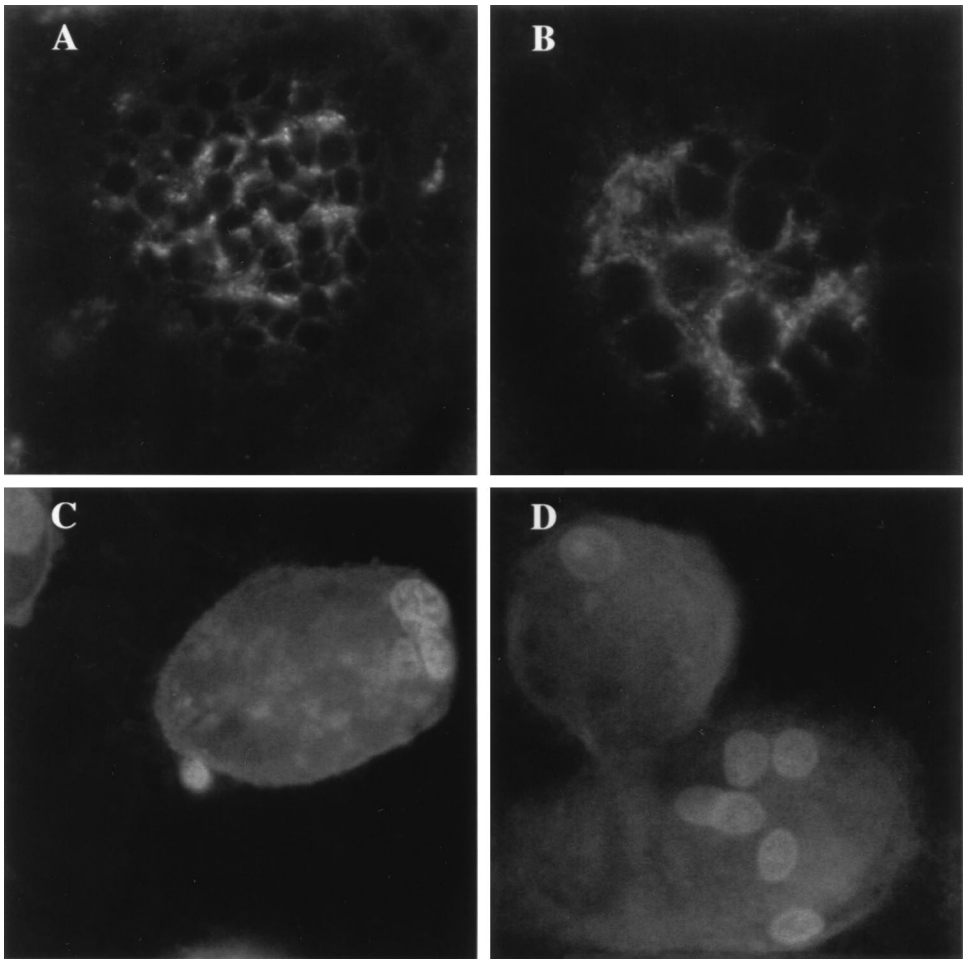


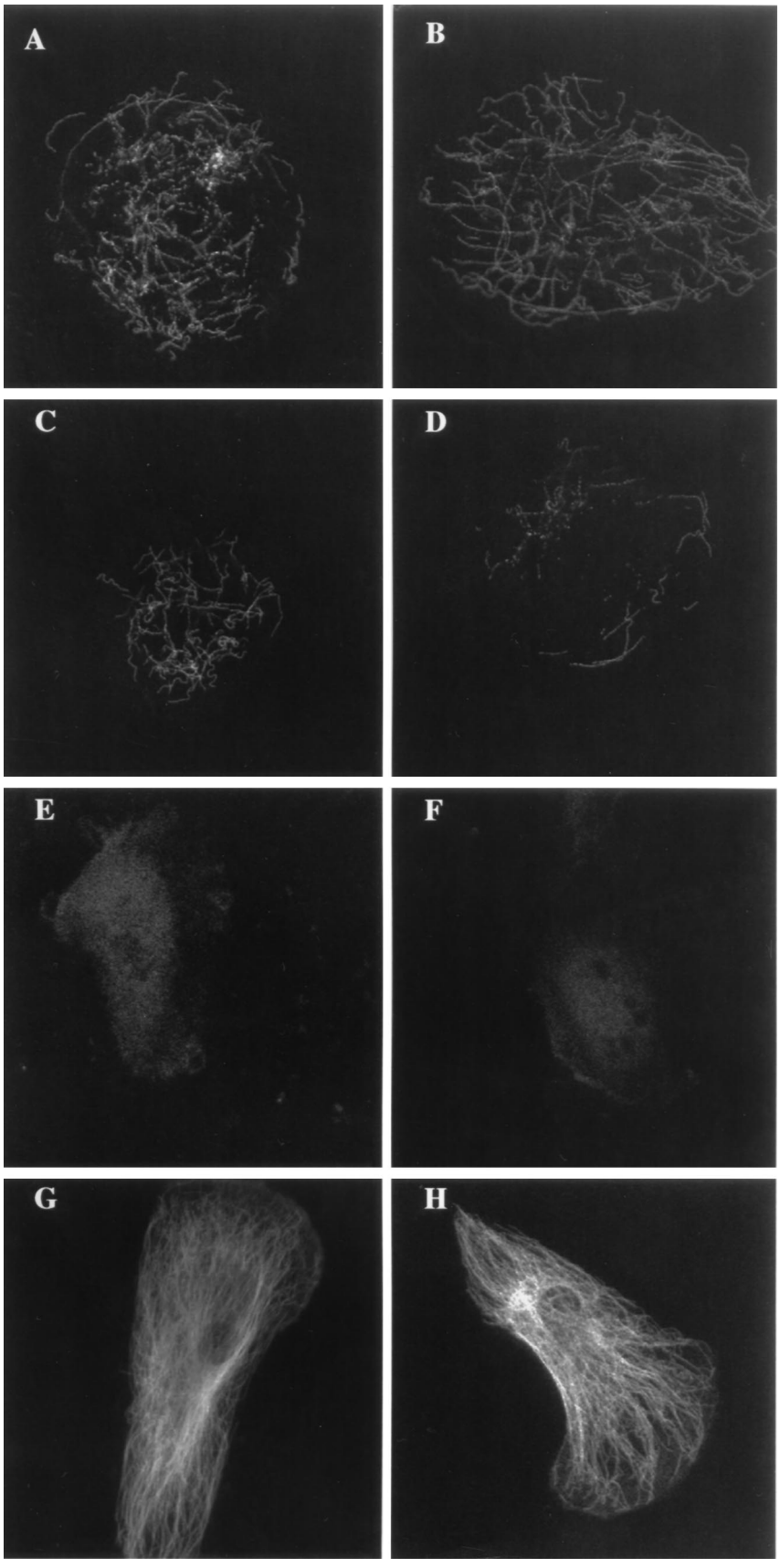
FIG. 3. HCMV disrupts the Golgi apparatus. The integrity of the Golgi apparatus was assessed by utilizing BODIPY C_6-ceramide, which specifically stains the Golgi apparatus in live cells. Uninfected (A and B) and HCMV-infected (C and D) MDM were exposed to the dye for 10 min, rinsed, and incubated for 1 h. Cells were fixed and examined by confocal microscopy. Magnification, ×400 (A, C, and D) and ×630 (B).

moved by placing a serum-coated coverslip over infected cultures at 13 dpi. The coverslip with plasma membrane was removed the following day, releasing free cellular organelles from MDM by a gentle rinse with media. HCMV-infected vacuoles were separated from other cytoplasmic contents by low-speed centrifugation, and both fractions were assessed for the presence of infectious virus by plaque assay (Fig. 2). The vast majority of infectious HCMV was observed in the MDM fraction which contains cellular organelles. These results indicate that the detection of HCMV late antigens in MDM vacuoles correlates with the presence of infectious virus and that very small amounts of infectious virus reside outside of these vacuoles.

Origin of HCMV-containing vacuoles in MDM. To determine how HCMV is compartmentalized in MDM, the origin of the vacuoles that contained virus was examined by double-label immunofluorescence utilizing antibodies directed against HCMV antigens and various cellular organelles. Utilizing antibodies to the Golgi apparatus-specific marker mannosidase II and either HCMV gB or pp65, the membranes surrounding HCMV-containing vacuoles stained with the Golgi apparatus marker (Fig. 1B and C). These results indicate the Golgi apparatus origin of these HCMV-containing vacuoles. Since Golgi apparatus-derived vacuoles enter the endosomal-lysosomal pathway, HCMV-containing vacuoles were examined with specific markers for early endosomes (transferrin receptor and rab5; Fig. 1D and E) and late endosomes-early lysosomes (LAMP-1; Fig. 1F). None of these markers were found to associate with the membranes of the HCMV-containing vacuoles in the cytoplasm of MDM. In addition, progression of HCMV infection also correlated with a reduction in lysosomal marker (data not shown). The results described above indicate that HCMV-containing vacuoles derived from the Golgi apparatus evade entry into the endosomal-lysosomal pathway.

HCMV disruption of the Golgi apparatus. Examination of HCMV-infected MDM with mannosidase II revealed abnor-

FIG. 4. Viability of nocodazole-treated MDM. To mimic the microtubular disruption observed in HCMV-infected MDM and assess cell viability, cell cultures were treated for various intervals with nocodazole. The integrity of the microtubule network was assessed by confocal microscopy by using antitubulin antibodies at the following times during treatment with nocodazole: 6 h (A), 12 h (B), 18 h (C), 24 h (D), 4 days (E), and 6 days (F). To determine MDM viability, re-formation of the microtubule network was analyzed in cells treated with nocodazole for 4 days (G) and 6 days (H); this was followed by removal of the drug. Cells were fixed at 24 h after removal of nocodazole for examination by confocal microscopy. Magnification, ×400.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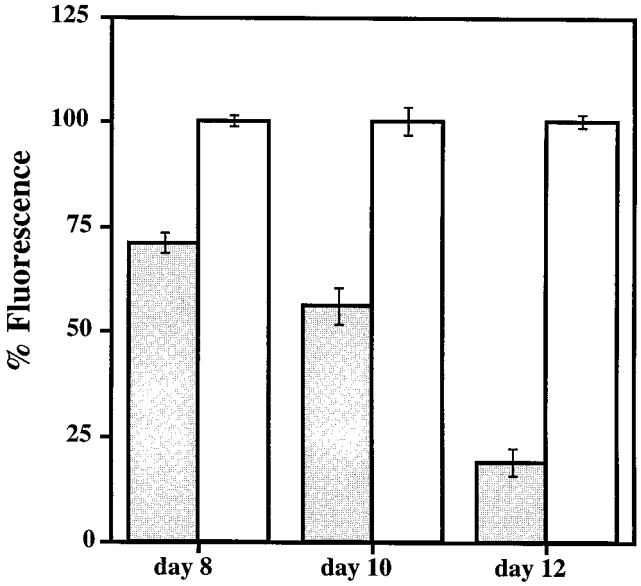

FIG. 5. Downregulation of MHC class II antigen in HCMV-infected MDM. The surface expression of MHC class II on macrophages was measured by recording the fluorescence intensity of macrophages labeled with a fluorescent anti-HLA-DR antibody. A Quantimet 500 fluorescence analysis program was used to quantitate fluorescence. MHC class II expression was measured on days 8, 10, and 12 postinfection. Fluorescence intensity of uninfected macrophages was adjusted to 100% for each time point, and fluorescence intensity of HCMV-infected macrophages was expressed as a percentage of that observed in uninfected cells. Each time point represents the measurements for nine cells (mean ± standard deviation).

mal Golgi apparatus structures associated with HCMV-containing vacuoles. To assess the integrity of the Golgi apparatus in infected cells, MDM were exposed to BODIPY C_6-ceramide, which can specifically stain the Golgi apparatus in live cells (30). Staining of uninfected MDM revealed the normal structure of multiple Golgi apparati interwoven between the nuclei of multinucleated cells (Fig. 3A and B). HCMV-infected MDM at 8 dpi also exhibited structurally normal Golgi apparati (data not shown). In contrast, late-stage (14 dpi) HCMV-infected MDM displayed a diffuse distribution of BODIPY C_6-ceramide (Fig. 3C and D). This observation indicates that HCMV disrupts the Golgi apparatus at late stages of infection.

Since the movement and structure of many cytoplasmic organelles are dependent on the integrity of the microtubule network (24, 40), we examined the organization of this structure in infected MDM. The microtubule network in uninfected MDM demonstrated normal structural features associated with multinucleated cells (Fig. 1G). However, as HCMV infection progresses in MDM cultures, cells showed increasing loss of the microtubulin network (Fig. 1E and H). Figure 1E demonstrates an HCMV-infected MDM at a late stage, and Fig. 1H shows two cells in the same field at different stages of viral infection. In the latter panel, the late-stage HCMV-infected cell at the top demonstrates the complete disruption of the microtubule network, while the beginning of microtubular disruption is observed in the earlier-stage HCMV-infected MDM at the bottom. Since the integrity of the Golgi apparatus is dependent on the microtubule network, the ability of HCMV to disrupt microtubules correlates with the disorganization of the Golgi apparatus that was observed at late stages of infection.

Since MDM are nondividing terminally differentiated cells, the absence of microtubule structure in viable infected cells was an unexpected result. To confirm that MDM remain viable without normal microtubule structure, cells were treated with nocodazole. Nocodazole treatment results in the depolymerization of microtubules, thus mimicking the disruption of this structure observed in HCMV-infected MDM. The viability of nocodazole-treated MDM was assessed by exposing uninfected MDM to the drug for various intervals for up to 6 days. After removal of nocodazole, the disruption and reformation of the microtubule network was determined by immunofluorescence staining with an antibody directed against tubulin (Fig. 4). MDM treated with nocodazole for up to 6 days retained the ability to reform a normal microtubule network, indicating that MDM retain viability in the absence of a cellular microtubule structure. Therefore, HCMV-mediated disruption of the microtubule network does not result in the imminent death of the cell.

HCMV disruption of the microtubule network is associated with alteration of MHC class II expression on the plasma membrane. HCMV disruption of the microtubule network would result not only in an alteration in protein processing events but also in intracellular membrane trafficking. MHC class II is assembled in the endoplasmic reticulum and transported from the Golgi apparatus to the cell surface through the endosome secretory pathway. HCMV-induced disruption of the microtubule network would likely interfere with these processes. Therefore, to address this issue, the presence of MHC class II in the plasma membrane was examined throughout the course of infection. For these experiments, double-label immunofluorescence was performed with antibodies directed against HCMV gB and MHC class II HLA-DR. Quantitation of MHC class II cell surface fluorescence was determined in HCMV-infected MDM over time by utilizing a Quantimet 500 fluorescence analysis program. As seen in Fig. 5, reduction of MHC class II cell surface expression correlated with the progression of HCMV infection. The greatest effect was detected at 12 dpi, which correlates with the HCMV-mediated disassembly of the microtubule network. These results suggest that HCMV interferes not only with viral protein trafficking to the cell surface but also with the trafficking of important cellular proteins which are involved in the antigen presentation pathways.

DISCUSSION

In this study, we examined mechanisms of HCMV survival in MDM. Analysis of HCMV storage vacuoles with known early endosome and lysosome markers indicated that the virus evades entry into the endosomal-lysosomal system. However, the presence of mannosidase II indicates that the virus-containing vacuoles are derived from the Golgi apparatus. How the virus-containing vacuoles evade lysosomal fusion is unknown, but disaggregation of the microtubule network may play a role in these processes.

Macrophages represent a key cell involved in the defense of the host against pathogens. The success of a pathogen that infects macrophages depends on the ability of the organism to evade lysosomal fusion or withstand lysosomal degradation. Macrophage pathogens have developed unique strategies to avoid cellular destruction, including the release of substances which allow for modification of acidity, resistance to a harsh environment, and disruption of vacuoles (2, 3, 7, 15, 16, 18, 29). HCMV uses a novel macrophage survival approach which involves evasion of virus-containing vacuole fusion with lysosomes. The mechanisms mediating this process may be the lack of vacuolar membrane proteins which promote lysosome fusion or the presence of viral proteins which prevent fusion.

Alternatively, lysosomal fusion may be inhibited by disruption of the microtubule network which mediates intracellular vacuole transport through the motor proteins kinesins and dyneins (40). One or all of these mechanisms may contribute to the inability of virus-containing vacuoles to fuse with lysosomes in MDM.

The ultrastructure of the cell is maintained in part by the microtubule network. This network functions to maintain placement and transport of intracellular organelles (24, 40). The major site of microtubule nucleation is the centrosome—the microtubule organization center. Polymerization and depolymerization of microtubules is a dynamic process in which the half-life of a microtubule is 10 min. HCMV may utilize multiple mechanisms to disrupt the microtubule network. First, HCMV may encode proteins which bind to the α and β tubulin monomers, thereby preventing polymerization, similar to the action of the drug colchicine. Second, the virus may block GTP and/or GDP binding to the microtubulin monomers, preventing tubulin assembly into microtubules. Lastly, HCMV may disrupt the microtubule organization center, inhibiting the nucleation event. Ultimately, destruction of the microtubule network would prevent transport of virus-containing vacuoles to the plasma membrane.

Similar to other persistent pathogens, HCMV has developed a variety of strategies to evade detection by the immune system. The most efficient mechanism is to remain quiescent in cells until activation by some external stimuli. We (19) and others (33, 38, 39) have found that HCMV expression is restricted in monocytes but not in differentiated MDM (14, 19). The quiescent state of the virus may represent the primary mechanism that HCMV utilizes to evade immune detection in these cells. As monocytes differentiate into permissive macrophages, the virus is sequestered in vacuoles which are unable to traffic to the plasma membrane. Therefore, infectious virus is retained in an environment protected from the immune response. Lastly, HCMV disruption of the microtubule network indirectly alters the trafficking of proteins involved in antigen-presenting pathways to the surface of the cell. The inability of the cells to present viral antigen would prevent detection by the immune system.

HCMV has developed a unique interaction with MDM which allows both viral and cellular survival as well as evasion of immune detection. Identification of viral gene products which mediate these events will be important in elucidating mechanisms involved in MDM persistence.

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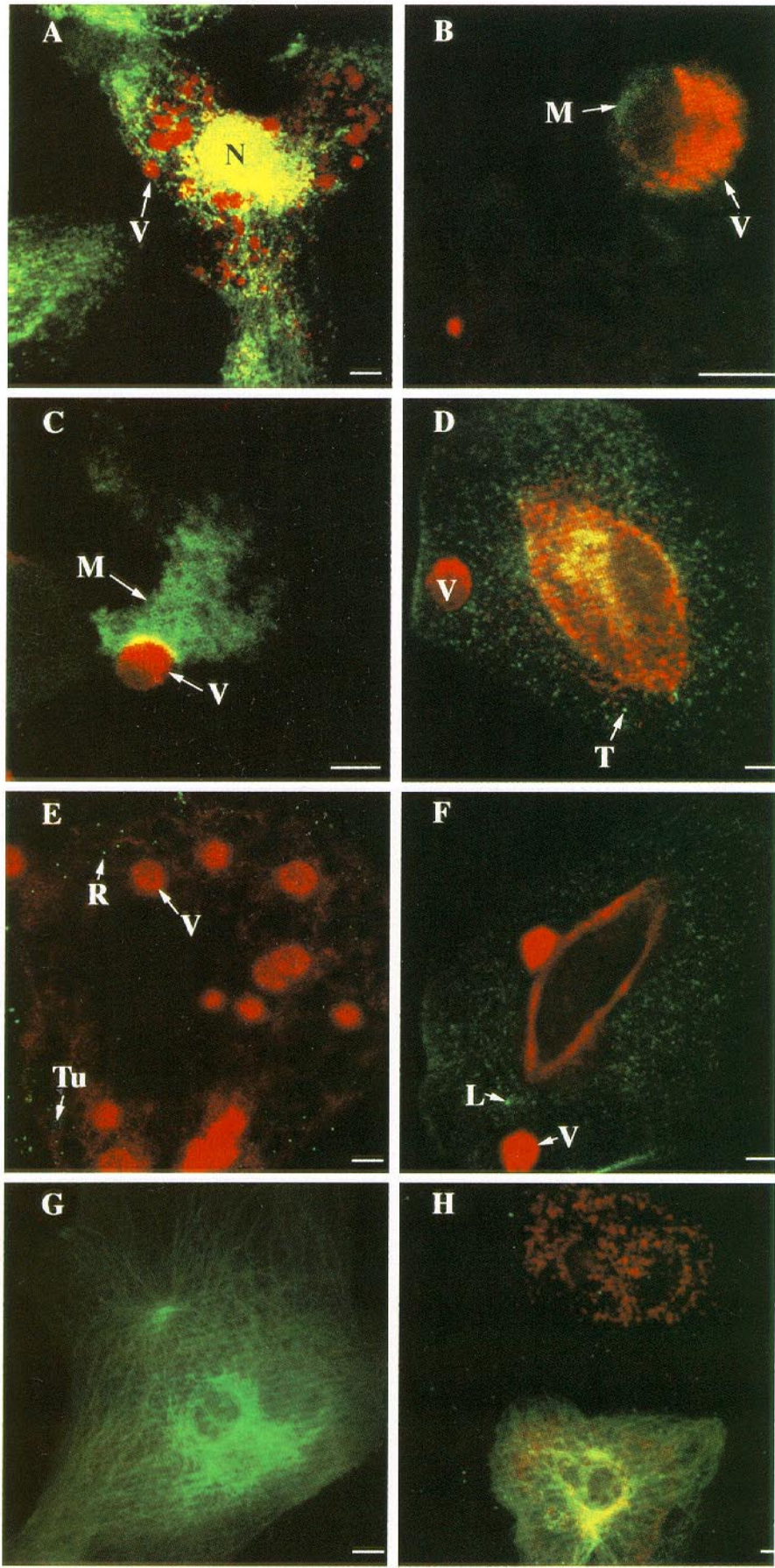
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