Human cytomegalovirus in a SCID–hu mouse: Thymic epithelial cells are prominent targets of viral replication

(herpesvirus/animal model/pathogenesis/replication/hematopoiesis)

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ABSTRACT Animal models of human cytomegalovirus (CMV) infections have not been available to study pathogenesis or to evaluate antiviral drugs. Severe combined immunodeficient mice implanted with human fetal tissues (SCID–hu) were found to support CMV replication and may provide a model for this species-specific virus. When conjoint implants of human fetal thymus and liver were inoculated with a low-passage-number isolate of CMV, strain Toledo, consistent high-level viral replication was detected 5, 12, 15, 28, and 35 days after inoculation and virus replication continued for up to 9 months. Other human tissue implants, including lung and colon, were also found to support viral growth but with greater variability in levels and for a shorter duration. As expected, the species specificity of human CMV was preserved in this model such that virus was detected in the human conjoint thymus/liver implant but not in surrounding mouse tissues. The majority of virus-infected cells were localized in the thymic medulla rather than cortical region of the implant and immunofluorescence analysis identified epithelial cells rather than any hematopoietic cell population as the principal hosts for viral replication. Finally, treatment of infected animals with ganciclovir reduced viral replication, thereby demonstrating the value of this system for evaluating antiviral therapies. This animal model opens the way for a range of investigations not previously possible with human CMV.

Human cytomegalovirus (CMV), a ubiquitous human virus, is as an important pathogen of immunocompromised individuals and neonates (1, 2). Disease in immunocompromised individuals follows either from primary infection or from reactivation of latent infection. Due to the strict species specificity of this virus and to the absence of any animal model that employs human CMV, it has been difficult to systematically investigate viral pathogenesis, tissue tropism, or latency or to undertake preclinical evaluation of antiviral drugs. Instead, investigators have relied upon murine CMV and other nonhuman animal viruses as models of infection or have developed cell culture models to mimic viral persistence, latency, or tissue tropism.

The persistence of CMV after primary infection is associated with a leukocyte population and it has long been known that virus may be transmitted with blood. Monocytes have been implicated as sites of viral persistence in the normal seropositive adult (3–5); however, other blood cell types, including T cells and various bone marrow cell types (6–8), may also be involved. Epithelial cells have long been recognized as targets for the growth and dissemination of CMV (2, 9, 10), particularly in immunocompromised individuals. In the normal healthy individual, epithelial cells in the ducts of

the salivary glands and renal tubules are the predominant cell population supporting persistent viral production. Thus, many studies of CMV tissue tropism have been driven by the medical impact of virus transmission by leukocytes; however, epithelial and other primary cell types are permissive and may play important roles in viral pathogenesis.

The SCID–hu mouse [a severe combined immunodeficient (SCID) mouse carrying a human tissue implant] was initially developed to provide a homologous animal model for the analysis of the human hematopoietic and lymphoid systems by using implants of fetal liver along with thymus (Thy/Liv) (11, 12) or bone marrow (13). Human fetal lymph node, lung, skin, and colon have also been introduced into this mouse, although variability in the amount of growth, survival time, and extent of differentiation of each tissue type has been observed. The SCID–hu mouse has proved useful in studies on tissue tropism differences of cell-culture-propagated virus vs. clinical specimens (14–16) on antiviral drugs (16–18). The variety and differentiation of human tissues that may be implanted into the SCID–hu mouse make this an attractive model to evaluate susceptibility to human CMV.

We initiated studies to determine whether the SCID–hu mouse could provide a model for human CMV infections and concentrated our efforts on hematopoietic tissues. We have sought to identify the principal cell types that are targets for viral infection. Our results establish the validity of the system and show that productive CMV replication localizes to epithelial cells in the human Thy/Liv implant. Furthermore, we demonstrate the efficacy of ganciclovir in suppressing CMV replication in this tissue implant.

MATERIALS AND METHODS

Virus and Cell Culture. The Toledo strain of CMV (passage 8, 9, or 10) (19), from Stanley Plotkin (Pasteur Mérieux, Marnes), and a *lacZ*⁺ recombinant (RC256) derived from the Towne strain of CMV (20) were grown in human foreskin fibroblasts (HFFs), and stocks were prepared by sonicating infected cells in a 50:50 (vol/vol) mixture of tissue culture medium and autoclaved nonfat milk (milk/medium).

SCID–hu Mice. Male homozygous C.B-17 *scid/scid* mice were bred and maintained as described (15). At 8 weeks of age, coimplants of human fetal thymus and liver tissue (from 18- to 23-week fetuses) were introduced under the kidney capsule as a conjoint implant using an 18-gauge trocar (11). Human fetal tissues were obtained with informed consent according to federal and state regulations. The grafts were used starting 1 month after implantation and were visually

Abbreviations: CMV, cytomegalovirus; FITC, fluorescein isothiocyanate; SCID, severe combined immunodeficient; Thy/Liv, thymus plus liver sandwich implant; pfu, plaque-forming unit(s); HFF, human foreskin fibroblast; mAb, murine monoclonal antibody.

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inspected (usually $>3 \text{ mm}^3$) just prior to inoculation. Lung tissue implants were made under the kidney capsule, intestine was implanted subcutaneously, and skin was introduced as full-thickness dermal grafts. All fetal tissue samples were screened for human immunodeficiency virus (17). All surgical procedures were carried out under anesthesia [5% (wt/vol) ketamine/2.5% (wt/vol) xylazine in phosphate-buffered saline (PBS)] and approved by animal use committees at SyStemix and Stanford University.

Virus Inoculation and Titration. One to 6 months after implant, SCID-hu mice were anesthetized and implants under the kidney capsule were exposed with an 8-mm incision over the left costovertebral angle. A titrated frozen virus stock was quickly thawed and $\approx 0.02 \text{ ml}$ was inoculated under the surface of the exposed implant using a 30-gauge needle. Animals were observed daily for abnormal behavior or illness. At various times after inoculation, animals were sacrificed by cervical dislocation and the implants were removed, minced, placed into 2 ml of milk/medium, and sonicated. Virus titers were determined by plaque assay on monolayers of HFFs with a limit of detection of 10 plaque-forming units (pfu) per ml of the sonicate.

In Situ Detection of β -Galactosidase and Antigens. A portion of the implants was immersed in PBS with 4% (wt/vol) paraformaldehyde at 4°C followed by PBS with 10% (wt/vol) sucrose and then 20% sucrose and embedded in OTC compound (Miles) in an acetone/dry ice bath. Frozen sections ($10 \mu\text{m}$) were collected on glass slides and fixed with hematoxylin and/or 5-bromo-4-chloro-3-indolyl β -D-galactoside ($150 \mu\text{g/ml}$) as described (21). Frozen sections ($6 \mu\text{m}$) for immunofluorescence analysis were made from tissues embedded in OTC compound without prior fixation and were subsequently air-dried and fixed with acetone for 1 min at room temperature. For double-label fluorescence analysis, tissue sections were incubated with murine monoclonal antibodies (mAbs) directed against human keratin (AE1 and AE3, Boehringer Mannheim) at a 1:50 dilution, followed with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Caltag, South San Francisco, CA) at a 1:100 dilution. Tissue sections were extensively washed in PBS, exposed to normal mouse serum, and then incubated with mAb to CMV nuclear antigen ICP36 (DAKO-CMV, Dakopatts, Glostrup, Denmark) at a 1:50 dilution. After extensive washing with PBS, a second-stage Texas red-conjugated goat anti-mouse IgG (Caltag) was used at a 1:100 dilution. Under these conditions the keratin-positive cells appeared yellow when photographed as double exposures first under FITC and then under rhodamine filters. FITC-conjugated anti-human CD45 mAb (HLE, Becton Dickinson), FITC-conjugated anti-human CD15 mAb (LeuM1, Becton Dickinson), and phycoerythrin-conjugated anti-human CD1 mAb (T6, Coulter) were used at 1:50 dilutions. Photomicrographs were taken using an epifluorescent microscope (Nikon).

Ganciclovir Treatment. Ganciclovir (from Julian Verheyden, Syntex) was dissolved in sterile water at 1.5 mg/ml. Animals received ganciclovir at 1.5, 0.5, or 0.125 mg/ml in drinking water from 6 h until 12 days after inoculation. For parenteral treatment, mice were injected interperitoneally (i.p.) with ganciclovir at 8 or 40 mg per kg per day, starting 4 h after inoculation and continuing daily for 12–13 days.

RESULTS

CMV Replicates in the SCID-hu Mouse. The initial aim of these studies was to determine whether human CMV was capable of replicating in human fetal tissue implants. We investigated the time course of viral replication in human Thy/Liv implants carried by SCID-hu mice after an inoculum of 4×10^5 pfu of the low-passage Toledo strain of CMV was injected directly into the exposed implant. As expected

FIG. 1. Time course of infection in Thy/Liv SCID-hu mice. SCID-hu mice were inoculated directly into the exposed implant with 4×10^5 pfu of CMV (strain Toledo) and groups of two to five animals were sacrificed 2, 5, 12, 15, 28, and 35 days after inoculation. Implanted tissues were removed from sacrificed animals and sonically disrupted, and CMV was titrated by plaque assay on HFF monolayers. The lower limit of detection was 10 pfu per tissue sample and is indicated by a dotted line. Animals with no detectable virus are depicted with data points below this line. The graph is a composite of eight experiments using animals carrying tissues from 12 fetuses, and the total number of animals graphed for each time point is indicated by the number in brackets above each data set. The geometric means of each set of data points are indicated by horizontal bars.

based on the slow replication of CMV, titers of virus were largely not detected 2 days after inoculation but began to rise and were readily detected 5 days after inoculation (Fig. 1). At this dose of virus, consistent high titers between 10^4 and 10^7 pfu per implant were sustained until at least 35 days after inoculation. Although we did not study the time course of virus replication beyond 35 days in detail, virus was recovered (10^3 – 10^4 pfu per implant) from two of four animals held for 9 months (data not shown).

There was no difference in levels of viral growth in implants derived from 12 fetal tissue sources, suggesting that the susceptibility was reproducible. There were no outward signs of viral damage in infected tissues. Infected animals showed no obvious physical or behavioral changes when compared to uninoculated animals. On gross inspection, the excised infected Thy/Liv implants from infected animals showed no distinguishing morphological characteristics compared to control mice. In the course of studying several dozen CMV-inoculated mice, $<5\%$ of the inoculated implants yielded very low or no detectable titers of virus between 5 and

FIG. 2. Influence of inoculum size on infection. Groups of four to six SCID-hu mice bearing Thy/Liv implants were inoculated with 4×10^5 , 4×10^4 , or 4×10^3 pfu of CMV (Toledo), animals were sacrificed 12 days after inoculation, and the virus titers in the implants were determined as described in Fig. 1. Data were collected from two experiments.

FIG. 3. Localization of CMV-infected cells within Thy/Liv implant 12 days after inoculation. (A) Enlarged owl-eye cell (indicated by arrowhead) typical of CMV cytopathology 14 days after inoculation. ($\times 290$.) (B) 5-Bromo-4-chloro-3-indolyl β -D-galactoside-stained infected cells (indicated by arrowhead) with hematoxylin background staining, demonstrating individual infected cells in the thymic medulla tissue 7 days after inoculation with a 50:50 mixture of Toledo and RC256. ($\times 75$.) All frozen sections are 10 μ m thick. Medullary area (M), cortical area (C), and Hassall's body (H) are indicated.

35 days after inoculation. The reasons for such variability may include poor inoculation or some other variation between implants. Significantly less variability was observed in later experiments as the manipulations became more routine.

To determine the influence of the input dose on the levels of replication, we inoculated groups of animals with 4×10^5 pfu, 4×10^4 pfu, or 4×10^3 pfu of virus. All three inocula resulted in readily detectable growth 12 days after inoculation, and titers decreased in parallel with the dilution of the input virus (Fig. 2). These results established a dose-response relationship and showed the susceptibility of Thy/Liv SCID-hu mice to as little as 4000 pfu of CMV (in 0.02 ml). This level of sensitivity should facilitate the investigation of growth properties of freshly isolated strains that grow poorly in cultured cells.

CMV replication was restricted to the implant and was not detected at any time in peripheral blood leukocytes where a small population may be derived from the Thy/Liv implant (11, 22) (data not shown). Consistent with the well-established species specificity of this virus (23), Thy/Liv implants were found to be virus-positive, but the surrounding mouse kidney taken from four animals was found to be free of virus (data not shown). It should be noted that uninoculated human fetal tissues failed to yield CMV when assayed,

FIG. 4. Colocalization of keratin and CMV antigen to infected thymic cells 12 days after inoculation. (A) Photograph of keratin (FITC)-positive cells in the frozen sectioned tissue. (B) Photograph of the CMV antigen (Texas red)-positive cells in frozen sectioned tissue. (C) Double-exposure photograph showing yellow cytoplasm and red nucleus (arrowhead) of CMV-infected cell. All frozen sections are 10 μ m thick. ($\times 720$.)

suggesting that the fetal tissues did not act as a source of virus.

We further investigated the ability of human CMV to replicate in other implanted human tissue types. After direct inoculation of CMV into implants of human fetal lung or colon, growth was observed although not as consistently or at the same high levels as observed in the Thy/Liv implant. Similarly, subcutaneous inoculation of implanted human skin resulted in low and variable levels of viral replication. In all of these instances, virus was only sporadically detected beyond a peak 5–9 days after inoculation (data not shown).

CMV Replicates in Epithelial Cells in Thy/Liv Implants.

Precise localization of productively infected target cells was carried out by histological observation and immunohistochemical staining of infected tissue sections 7–14 days after inoculation. Half of each implant was processed for titration of virus and the remainder was processed to make frozen sections. Cytopathic effects were more readily observed at later times after inoculation (12–14 days). Initial histological observation of hematoxylin-stained sections 14 days after inoculation revealed minimal CMV cytopathic effects; only occasional cells with an “owl-eye” appearance were observed (Fig. 3A).

To aid in localizing the foci of virus infection in the implants and to survey different tissue sections for evidence of virus infection, Thy/Liv implants were coinoculated with a 50:50 mixture of Toledo and RC256, each at 4×10^5 pfu. RC256 expresses β -galactosidase under control of strong early CMV promoter and acted as a marker of productively infected cells. As first shown with herpes simplex virus recombinant viruses (21), β -galactosidase is a simple method for detection and localization of herpesviruses in tissue sections. Initial experiments used to assess the replication potential of RC256 in tissue implants suggested that growth was more consistent and prolonged if the recombinant virus was introduced along with Toledo. Frozen sections from infected Thy/Liv implants 7 days after inoculation were stained first with 5-bromo-4-chloro-3-indolyl β -D-galactoside to reveal virus-infected cells and then with hematoxylin to reveal histology (Fig. 3B). At this time, infected cells were rarely clustered. The virus-infected cells appeared to be localized in thymic medulla, in an area rich in Hassall's bodies, rather than in cortical areas of the implant.

To identify the cell type(s) infected by CMV in the implants, immunofluorescence with a mAb directed at viral nuclear antigen was used to colocalize virus and various cellular markers. Animals were sacrificed and implants were examined for presence of viral antigens 9–14 days after inoculation with 4×10^5 pfu of the Toledo strain. The majority of cells that were found to express viral antigens appeared to be epithelial cells in the thymic medulla as best demonstrated by a double-label indirect immunofluorescence procedure using murine mAbs directed at a CMV nuclear antigen (Fig. 4A) and human keratins, as markers for epithelial cells (Fig. 4B). When viewed under separate FITC or rhodamine/Texas red filters, CMV antigen and keratin colocalized in the same cells. When photographed using a double exposure, cells with both antigens appeared to have yellow cytoplasmic fluorescence and red nuclear fluorescence (Fig. 4C). We further evaluated tissue sections by using mAbs directed at other cell populations. CMV-antigen-positive cells were negative for CD45 (the common leukocyte antigen found on thymocytes and monocytes), CD1 (a marker of thymocytes and thymic dendritic cells), and CD15 (a myelomonocytic cell marker). These results indicated that the CMV replication occurred in epithelial cells localizing to the region analogous to the thymic medulla.

Inhibition of CMV Growth by Ganciclovir. A preliminary experiment sought to evaluate the ability of ganciclovir to inhibit viral replication in Thy/Liv implants when added to the drinking water starting 6 h after inoculation. We found a dose-dependent reduction in titers 12 days after inoculation (Fig. 5A), similar to oral doses effective against murine CMV (24), with no obvious negative side effects of the drug. Parenterally administered ganciclovir (8 or 40 mg per kg per day) starting 4 h after inoculation reduced titers from two to four orders of magnitude (Fig. 5B), an effect remarkably similar to the prophylactic and therapeutic regimens currently in use on humans (25). These data predict that SCID-hu mice will aid in the evaluation of the antiviral activity of other drugs and biologics.

FIG. 5. Ganciclovir inhibition of CMV growth. (A) Animals were left untreated or placed on drinking water containing ganciclovir at 125, 500, or 1500 μ g/ml starting 6 h after inoculation with 10^6 pfu of CMV (Toledo). (B) Thy/Liv SCID-hu mice were inoculated with 5 – 10×10^6 pfu, and 4 h after inoculation each received an i.p. injection of 1 mg or 0.2 mg of ganciclovir. Both experiments are composites of two experiments performed several months apart. Titers were determined 12–13 days after inoculation.

DISCUSSION

Studies on human CMV tissue tropism, latency, and pathogenesis have lagged behind work on other viruses where convenient small animal models exist. Here we have inoculated a human hematopoietic tissue implant with human CMV and found that thymic medullary epithelial cells support viral replication. Epithelial cells in salivary gland and kidney have long been recognized as important target cells and important sites of persistent replication (2, 9, 10). Although the replication of CMV in myeloid and lymphoid cells has been demonstrated after *in vitro* infection (6–8), we have not observed any evidence of widespread viral replication in these cell types. Indeed, CMV infections of leukocytes in normal individuals may be largely nonproductive (3, 4, 26). The other tissue implants that were tested, including lung, colon, and skin, supported growth less consistently or to lower levels than observed with Thy/Liv implants. Even though the SCID-hu model employs only a subset of human tissues, each implant that we studied was found to differentiate and function in a manner reminiscent of natural tissues and to provide an environment that cannot be mimicked by cells grown in culture. Thus, the system provides an opportunity to dissect aspects of the human CMV–host interaction related to tissue tropism and tissue-specific gene expression. Given the known specificity of CMV, this model should aid in dissecting the basis of interactions with a variety of differentiated human cell types and should enable investigation of nonproductive and productive infection.

We initially chose to use the Toledo strain of CMV because of its low-passage history, high-titered growth, and demonstrated virulence characteristics (19). Many of the parameters of the infection indicated a level of specificity not obvious from cell-culture studies on CMV. (i) The Toledo strain exhibited persistent growth that did not diminish over several weeks of observation. (ii) Consistently higher titer growth was exhibited by Toledo as compared to laboratory-passaged strains of CMV (unpublished observations). These characteristics may be related to substantially increased virulence exhibited by the Toledo strain relative to the Towne strain of CMV (19). These growth and virulence differences predict the existence of viral gene functions that may be missing from the laboratory strains. These determinants may be identified and mapped using the SCID-hu mouse model.

Although virus continued to be produced in Thy/Liv implants for long periods of time, viral replication was not as widespread as might have been expected based on the fetal origin of the tissues and the lack of any specific immune response to the virus in SCID mice. The few cells that were infected were distributed in the region of the implant believed to carry out functions analogous to the medulla of the mature thymus and thus to control the maturation of T cells (22, 27) and did not extend into the cortex. This pattern of infection suggested that epithelial cells in the cortex did not provide the requirements for productive CMV replication. Within the medulla, cell-to-cell spread of virus appeared to proceed slowly even in the absence of an immune response. The pattern of spread, level of replication, and cell-type specificity of CMV infection may be dependent on the virus strain, passage history, or other characteristics.

The most prominent cell type infected by CMV in the Thy/Liv implant was a thymic medullary epithelial cell. The developmental origin of epithelial cells in the thymic medulla is the pharyngeal pouch endoderm, which also gives rise to submandibular epithelial cells (28). Thus, the characteristics of the cells observed to replicate virus here may have many features in common with the target cells in the salivary gland. The cellular components required for CMV infection and replication may be maintained in these parallel lineages during development. Primary cultured epithelial cells, in general, are permissive for human CMV, although the level to which they support viral growth has been variable (2). In normal seropositive individuals, epithelial cells of the salivary gland ducts and kidney tubules have been shown to play central roles in virus infection and spread. Thus, the importance of epithelial cells in the biology of CMV infections was recapitulated in the model we have described here.

The availability of the SCID-hu mouse as a model for CMV may facilitate the direct isolation of viruses from human tissues and the investigation of low-passage clinical isolates of CMV that in general replicate poorly in HFF culture. The impact of viral replication hematopoietic cell function may now be addressed in a functionally intact organ. Here we have shown that this system provided an excellent venue to demonstrate the antiviral activity of ganciclovir. There is no reason that this model cannot provide a significant amount of information for the evaluation of other drugs and biologics directed against human CMV.

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