Novel Method for Evaluating Antiviral Drugs against Human Cytomegalovirus in Mice

LOIS B. ALLEN,* SHIXIONG LI, GUSSIE ARNETT, BARBARA TOYER, WILLIAM M. SHANNON, AND MELINDA G. HOLLINGSHEAD

Southern Research Institute, 2000 Ninth Avenue South, Birmingham, Alabama 35255

Received 16 July 1991/Accepted 14 October 1991

A virus-host cell system in which human cytomegalovirus-infected human cells are entrapped in agarose plugs has been developed. This model provides an inexpensive method for the in vivo evaluation (with outbred, immunocompetent mice) of antiviral drugs against human viruses such as cytomegalovirus that replicate primarily or only in human cells.

Human cytomegalovirus (HCMV) is of minimal consequence in immunocompetent persons but can cause serious malformation in infants infected in utero and can be life threatening in immunocompromised patients (6, 10, 13, 14). It is desirable to expand the quest for new drugs and animal models for HCMV that will predict the clinical efficacy of new anti-CMV therapies. Previously, in vivo evaluations of potential CMV drugs have been performed with mice (2, 4, 7, 11) and guinea pigs (1, 8) by using the CMVs natural to those species. The murine model of CMV (MCMV) has frequently been used since it requires the least drug expenditure of the available animal models and can be used for analysis of drug toxicity and pharmacology studies. Unfortunately, MCMV may differ significantly from HCMV in drug susceptibility for example, MCMV is quite susceptible and HCMV is relatively resistant (4) to acyclovir. In addition, compounds which inhibit HCMV but not MCMV have been identified. These differences between CMVs from rodent species and HCMV have led us to pursue a method for using human cells and HCMV in a mouse model for the evaluation of potential anti-CMV drugs. In vivo systems with encapsulated or entrapped cells allow the use of inexpensive, outbred, immunocompetent mice since the entrapped, implanted cells are protected from the hosts' immune cells (5). For this purpose, we have developed an encapsulation system in which HCMV-infected human cells are trapped in agarose plugs and cultivated in vitro or implanted into conventional mice.

HCMV strain AD 169 which was cultivated in MRC-5 (human embryonic lung) cells was used in these studies. The growth medium for MRC-5 cells was Eagle's minimal essential medium plus 10% fetal bovine serum. Two percent serum was used for cell maintenance and for virus titrations. For HCMV titration, the MRC-5 cells were diluted to 10^5 cells per ml and seeded into 96-well microtiter plates.

For plug preparation, subconfluent cultures of MRC-5 cells were incubated for 3 h at 37°C in the presence of medium with or without HCMV. For in vitro chemotherapy experiments, we selected a multiplicity of infection of 0.016, while a multiplicity of infection of 0.16 was used for in vivo experiments. Following incubation, the virus inoculum and medium were removed and the cells were washed twice with a balanced salt solution. The cultures were treated briefly with EDTA (2 μg/ml) in 0.25% trypsin to release the cells, which were diluted to 10^5 cells per ml in medium containing 1.5% agarose (SeaPlaque; FMC Bioproducts). Plugs were formed by drawing 0.3-ml volumes of the cell-agarose mixture into 1-ml plastic syringes and allowing the agarose to solidify.

Prior to HCMV infection experiments, uninfected MRC-5 cells were suspended in agarose and cultivated in vitro. Maintenance of cell viability during cultivation was confirmed by the use of the neutral red uptake method. Cell viability exceeded 80% through day 5 and decreased thereafter. Cells were exposed to virus, and plugs were prepared and incubated in vitro. Plug samples were collected at time 0 and days 1, 3, 5, and 7, and titers of HCMV were determined. Plug samples were processed (homogenized, sonicated, and centrifuged), and supernatants were titrated in MRC-5 cells. Cytopathic effect was scored on day 9, and mean titers were calculated (12). High titers were observed by day 3, with peak titers on days 5 and 7.

We performed experiments in which HCMV-infected MRC-5 cell plugs were incubated for 7 days in 12-well plates in the presence of drug-free medium (2 ml) or medium containing one of three concentrations (50, 5, and 0.5 μg/ml) of 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG) (ganciclovir). Samples were collected, frozen, and processed for titration. In the event that the plug samples contained drug which might inhibit virus replication during the titration, the samples were diluted with medium and adsorbed onto the MRC-5 cells for 2 h. Adsorption was followed by sample removal and replacement with fresh maintenance medium. There was no evidence of virus replication in virus-infected cells from plugs treated with DHPG at 50 or 5 μg/ml, and there was a >99% reduction in virus titer (794) from plugs treated with DHPG at 0.5 μg/ml compared with those from drug-free virus control plugs (100,000).

For in vivo studies, CD-1 mice (Charles River Laboratories) were anesthetized with methoxyflurane and an incision large enough to permit entry of the syringe barrel was made through the skin and abdominal musculature. For implantation, the plug was drawn back in the syringe, the syringe hub was cut away, and the syringe was inserted through the incisions. The plug was implanted, and the incisions were closed in two layers.

In initial in vivo experiments, plugs were implanted by intraperitoneal (i.p.) and subcutaneous routes. We found that plugs implanted i.p. were more easily recovered and produced higher virus titers; therefore, we selected the i.p. route for further study.

* Corresponding author.
To examine the in vivo virus replication curve, plugs were implanted i.p. and samples were collected daily on days 4 through 7. Titers of approximately 10,000 to 100,000 50% tissue culture infective doses were observed each day. Blood was collected daily from all mice on days 4 through 7. Sera were titrated in MRC-5 cells, and no virus was detected. In order to verify the virus production curve, we performed an additional experiment in which plug samples were collected at time 0 and days 1 through 7 (Fig. 1). Ten plugs were collected at each time point and processed, and titers in MRC-5 cells were determined. Low titers were observed at time 0 and on days 1 and 2. Appreciable increases occurred by day 3, with titers in the range of 100,000 50% tissue culture infective doses on days 4 through 7. The titers on days 4 through 6 in both experiments were essentially identical.

On the basis of the data from the virus production and cell viability studies, we felt that chemotherapy experiments should end on day 5. Therefore, we performed an experiment to evaluate DHPG. Mice were arranged into four groups of 10 animals each and implanted with HCMV-infected cells. Treatments (subcutaneous) included placebo (saline-treated) and three DHPG groups (100, 50, and 25 mg/kg of body weight per day). Treatments began 4 h before plug implantation. Virus infection of the MRC-5 cells began approximately 4 to 6 h prior to implantation. The animals were treated twice daily on days 0 through 4. In addition, groups of five mice received uninfected cell plugs and were treated with the same drug doses and schedules. Plugs were collected and frozen on day 5 approximately 18 h after the last treatment. The plugs were processed, and titers were determined as previously described. Differences in titers between the placebo (virus control)- and drug-treated groups were evaluated by the Mann-Whitney U test. Treatment with DHPG at 100, 50, and 25 mg/kg/day reduced titers by 92.5%, 73.0%, and 20.6%, respectively (Table 1). The activity seen with the 50-mg/kg/day dose of DHPG was verified in a second experiment. No adverse signs in the drug toxicity control animals were noted.

Previously, Freitas et al. (2) have shown that 9 mg of DHPG per kg per day produced significant increases in the numbers of survivors in the MCMV model. The doses found to be effective in the agarose plug model were 5 to 10 times higher than those needed in the MCMV model. The larger amounts of drug needed in our model may reflect a requirement for high levels in blood to achieve active concentrations in the peritoneal fluids. Also, adherent murine cells may decrease drug penetration into the plugs. A need for high levels in blood would be a problem only for drugs that are minimally selective and therefore somewhat toxic.

Recently McMahon et al. (9) described studies for developing an in vivo anti-human immunodeficiency virus model with calcium alginate-encapsulated cells. These workers found that murine peritoneal cells which adhered to the microcapsules interfered with the detection of cell viability. They postulate that the murine cells might produce cytotoxic products or physically or metabolically interfere with the influx of nutrients to the encapsulated cells. These potential problems cannot be ruled out with the agarose system described herein. However, the presence of significant virus titers in the agarose-entrapped MRC-5 cells does indicate that entrapped cells survive adequately to support virus replication and that dose-dependent antiviral effects were reproducibly achieved with a known positive control antiviral drug (DHPG).

Since HCMV replicates in MRC-5 cells suspended in agarose plugs and DHPG interferes with that replication, we believe that this agarose entrapment model provides a potential in vivo model for the evaluation of antiviral drugs against human viruses that do not infect mice or other rodents.

REFERENCES


