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Dose and duration-dependence of ganciclovir treatment against murine cytomegalovirus infection in severe combined immunodeficient mice

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Abstract

The present study investigates the full dose–response curve and treatment duration dependence of ganciclovir (GCV) against murine cytomegalovirus (MCMV) infection in severe combined immunodeficiency (SCID) mice. Animals inoculated intraperitoneally with 6.3×10^3 pfu of MCMV per mouse developed typical wasting syndrome rapidly and died around day 12 post-inoculation. Once-daily treatment with subcutaneous GCV for 5 days dose dependently delayed MCMV-induced wasting syndrome and mortality at a dose range of 1–80 mg/kg per day, whereas a dose of 160 mg/kg per day induced reversible side-effects. The effect of GCV treatment on mean death day (MDD) was significantly correlated to reductions of viral titers in the lung ($r = 0.969$, $P < 0.05$). Treatment duration dependence was examined at the dose of GCV at 80 mg/kg per day for 1, 5, 8 and 12 days. The protective duration, over vehicle-treated mice, was constantly 3–4 days plus the duration of GCV treatment, as evidenced by the delay of viral replication, wasting syndrome and death. At a sub-optimally effective dose of 10 mg/kg per day of GCV, maximum protection was achieved with a 8-day treatment regimen. Prolongation of this treatment to 12 days failed to further delay mean death day and wasting syndrome that started on day 10, indicative of insufficient suppression of viral replication. Treatment with a single dose of GCV failed to show a complete dose–response curve since only minimal protective effects were observed at the dose of 80 mg/kg while side-effects were associated with the dose of 160 mg/kg. The treatment duration dependence and requirement for sufficient dosage of GCV against CMV infection observed in the current model are consistent with clinical observations. It also suggests that 5–8 days treatment duration may be a good balance considering the opportunity for identifying active compounds and speeding up the turnaround time in drug evaluations.

Keywords: Ganciclovir; Cytomegalovirus; Dose–response; SCID mice

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1. Introduction

Human cytomegalovirus (HCMV) causes severe disease and mortality in immunocompromised hosts. Current available treatments include ganciclovir (GCV), foscarnet, and cidofovir. All of them are associated with limited efficacy, high frequency of relapse, and toxic side effects (Meyers, 1991; Collier and Corey, 1992; Jabs, 1992; Paul and Dummer, 1992; Bowden, 1993; Jacobson, 1994; Gerard and Salmon-Ceron, 1995; Naesens et al., 1996). A clinical need therefore exists for more effective, less toxic, and more convenient treatments for HCMV infections (DeAmond, 1991; Snoeck et al., 1993; Polis and Masur, 1995). Several animal models with utility for screening of anti-CMV drugs have been described. These include experimental infections with murine cytomegalovirus (MCMV), rat and guinea pig cytomegalovirus (Hsiung and Chan, 1989; Kern, 1991). MCMV infection in mice has been used extensively for several reasons (Kern, 1991). Firstly, mice are relatively inexpensive small rodents that can be easily obtained and maintained in statistically sufficient numbers for rapid in vivo screening. Secondly, MCMV shares many similarities to HCMV in terms of genetic organization, gene products, pathogenesis, tropism, latency and reactivation. Moreover, the relative in-vivo efficacy of the anti-CMV drugs foscarnet, GCV and cidofovir against MCMV is similar to that in the clinic (Kern, 1991; Naesens et al., 1996). Nevertheless, the enhanced efficacy and lack of relapse after a short course of these antivirals in MCMV infection models using normal mice do not mimic HCMV infections in immunocompromised patients (Smee et al., 1991; Neyts et al., 1992, 1993a,b). In addition, acyclovir has a rather pronounced anti-MCMV activity (Hsiung and Chan, 1989), but its activity against HCMV was very limited (only useful in certain immunocompromised patients (Bowden, 1993; Sasadeusz and Sacks, 1993)).

To mimic HCMV infection in the immunocompromised host more closely, a MCMV infection model in severe combined immunodeficiency (SCID) mice was developed by Neyts et al. (1992). These mice are deficient in functional T and B lymphocytes (Bosma et al., 1983), and are thus highly susceptible to infectious agents. Intraperitoneal infection of SCID mice with MCMV resulted in a wasting syndrome and mortality at a rate dependent on the titer of the inoculum (Smee et al., 1992; Reynolds et al., 1993). Cidofovir was very efficacious in delaying mortality (Neyts et al., 1992; Smee et al., 1992). GCV on the other hand had only limited effects at the doses tested. However, a full dose–response curve of GCV in this disease model has not been reported. Therefore, the consequences of prolonged treatment with an optimal dosage of GCV, similar to that applied clinically, were not known. In addition, although different treatment protocols have been applied in previous studies, the optimal choice of treatment duration balancing the opportunity of identifying active compounds and shortening the turnaround time for compound evaluation has not been established for in vivo screening. The present study was designed to address these questions in order to further validate the clinical relevance of this model, as well as to establish an optimal treatment duration for drug screening.

2. Materials and methods

2.1. Compounds

GCV was prepared in the Department of Chemistry at Bio-Méga. The purity was checked by HPLC to be more than 97%. The compound was dissolved in phosphate buffered saline at concentrations which can be used for delivering desired subcutaneous doses in a volume of 10 ml/kg.

2.2. Animals

Female SCID mice (C.B-17 scid/scid, 5 weeks old) were purchased from Charles River (North Carolina, USA or St. Constant, Quebec, Canada). Animals were maintained under pathogen free conditions in micro-isolator cages inside a semi-rigid isolator. All animal handling was carried out inside the class II bio-safety cabinet (Nuaire), according to protocols approved by Canadian Council on Animal Care.

2.3. Virus and animal infection

The Smith strain of MCMV was obtained from the American Type Culture Collection (Rockville MD). The virus was then serially passaged in 5 week old athymic nude mice and salivary glands collected to generate a stock of homogenate for animal infection. The titer, plaque forming units per ml (pfu/ml), of the salivary gland homogenate was determined by plaquing on primary mouse embryo fibroblasts (MEF). SCID mice were inoculated with designated inoculum in a total volume of 0.1 ml/mouse intraperitoneally. Sham-inoculation was achieved by inoculating animals with salivary gland homogenates from non-infected nude mice. All drug treatments were started 3 h post-inoculation, once daily for the duration as indicated in the Results section. Animals were observed daily for clinical signs of disease, body weight changes and mortality.

2.4. Organ collection and virus titration

Several organs including lung, spleen, salivary gland and ocular tissue (eye balls) were isolated on designated days after viral challenge or after termination of GCV treatment. Each sample was homogenized in Dulbecco's Minimal Essential Medium (DMEM, at a w/v ratio of 1 g:10 ml). Organ homogenates were then assayed for MCMV by serial two-fold dilution and observation of cytopathic effect (cpe) on primary mouse embryo fibroblast (MEF) cells. The last dilution producing cpe was considered to be the viral titer in that organ homogenate. The minimum sensitivity of these serial dilution assay is 100 pfu/ml. Viral titers in eye homogenates were determined by a plaque assay on MEF cells.

2.5. Experimental design and statistical analysis

All animals were randomized into different experimental groups. The initial experiments were designed to test the inoculum dependence of MCMV-infection under our experimental conditions. Based on these experiments, a virus titer of 6.3×10^3 pfu/mouse was selected for inoculation, and experimental end points for drug evaluation were selected. Dose-dependent effects of GCV were studied based on both 5-day and 1-day treatment regimens. Treatment duration dependence of GCV was tested using two different doses: a sub-toxic dose of 80 mg/kg and a minimal effective dose of 10 mg/kg.

Body weight changes at different time points post-inoculation were expressed as percentage over the initial body weight at the time of inoculation. All data were presented as mean $\pm$ standard error of the mean (SEM). Statistical differences were determined using ANOVA followed with Student–Newman–Keuls (SNK) multiple comparisons supplied from SAS software (SAS Institute, Cary, NC, USA). Correlation between mean death day and viral titer reduction was analyzed by linear correlation. $P < 0.05$ was regarded as statistically significant.

3. Results

SCID mice inoculated with MCMV developed a typical wasting syndrome with ruffled fur and progressed to mortality at a rate dependent on the titer of MCMV in the inoculum as described in previous publications (Smee et al., 1992). Consistent with previous publications, MCMV replication was identified in all the organs studied, including the lung, salivary gland, spleen and liver, with some differences in terms of time course (Neyts et al., 1992; Smee et al., 1992). Comparatively, viral replication in the lung tissue showed the best inoculum dependence on day 4 post inoculation (data not shown). The inoculum dependence at a later stage of infection (day 8) could not be determined since animals inoculated with the highest titer died before their organs could be collected. Based on the fact that a titer of 6.3×10^3 pfu/mouse induced highly reproducible disease in a reasonable time span for drug evaluation, this titer of inoculum was chosen for all subsequent experiments.

GCV dose dependently delayed the wasting syndrome in a dose range up to 80 mg/kg per day. The dose of 160 mg/kg per day is borderline toxic but more effective in terms of antiviral activity (one of 12 mice died of toxicity, Fig. 1). The

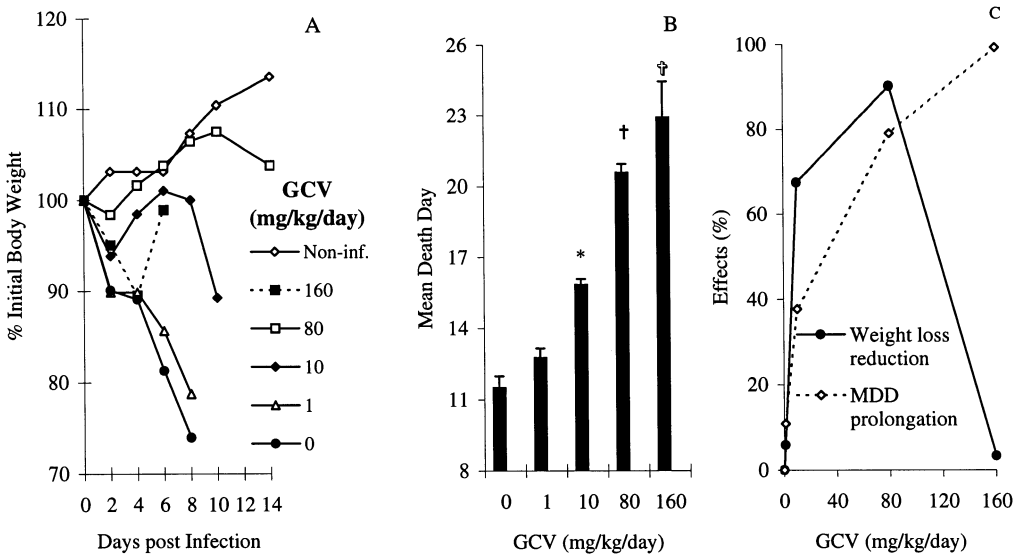

Fig. 1. Dose-dependent effects of GCV for 5 days on MCMV-induced wasting syndrome (panel A) and mean death day (panel B) in SCID mice. In panel C, % body weight loss reduction on day 4 and MDD prolongation by GCV were calculated using the values of vehicle treated animals as 100%. All animals, except the non-infected controls, were inoculated with MCMV at an inoculum of 6.3×10^3 pfu/mouse. GCV was administered s.c. once daily for 5 days at indicated doses. Statistical differences were determined using ANOVA followed by SNK multiple comparisons (*$P < 0.05$ from non-infected control; Closed cross, open cross $P < 0.05$ from previous groups).

dose–response curve of GCV against MCMV-induced body weight loss and mortality is summarized in Fig. 1 panel C, showing the optimal dose of GCV about 80 mg/kg per day, under the current experimental regimen. Several organs were isolated from 4 mice in each experimental group. Viral titers from the lungs on day 8 post-inoculation showed clear dose-dependent reduction by GCV (Fig. 2 panel A). In addition, the effects of GCV on mean death day was linearly correlated to its effects on viral titer reduction in the lung (Fig. 2 panel B). Due to the small sample size, the ocular tissue from all four mice were pooled for viral titration. As shown in Fig. 3 panel A, the effect of GCV on viral replication in the ocular tissue is dose dependent. In contrast, the effects of GCV on viral titers in the salivary glands and spleen at this time point seemed less dose dependent, and did not significantly correlate with mean death day (Fig. 3 panels B and C).

The treatment duration dependence of GCV at a dose of 80 mg/kg per day is summarized in Fig. 4. It is clear that the longer the GCV treatment duration, the longer the animals survived. However, when the data were analyzed by subtracting the GCV treatment duration from the mean death day, all GCV treated animals survived 3–4 days longer than the vehicle treated animals constantly (Fig. 4 panel A). The same 3–4 days of protection applies to body weight changes (data not shown). Consistently, when the lungs were isolated on day 8 post-inoculation, for viral titration, a treatment-duration-dependent reduction in viral titers was observed (Fig. 4 panel B). However, when lungs from GCV treated groups were isolated 11 days after termination of the GCV treatment, a time corresponding to 3–4 more days plus the vehicle treated control (day 8) and the GCV treatment duration, no significant reduction in viral titers from any GCV treated group was observed (Fig. 4 panel C).

At a dose close to ED_{50}, 10 mg/kg per day, GCV produced a treatment-duration dependent protection in terms of delaying wasting syndrome and mean death day up to a maximum of 8 days. Further prolongation of the treatment duration to

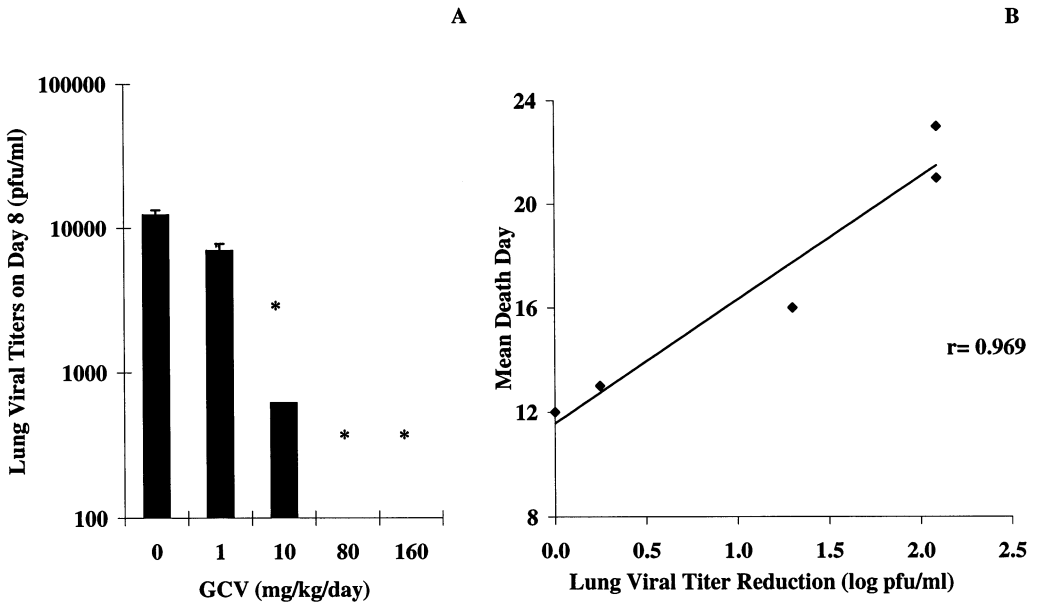

Fig. 2. Dose-dependent effects of GCV on MCMV replication in the lung. Panels A and B represent viral titer reduction by GCV, and its relationship to MDD, respectively. Animals were inoculated and treated as described in the legend of Fig. 1. Statistical differences in viral titer reduction were determined and indicated as in Fig. 1. Linear correlation coefficient is statistically significant ($P < 0.05$).

12 days failed to provide better protection in both parameters (Fig. 5 panels A and B).

GCV treatment for 1 day only provided a transient protective effect on wasting syndrome at the dose of 80 mg/kg, while the side effect of 160 mg/kg remained. A further dosage increase to 320 mg/kg led to animal death within 48 h. In terms of mean death day, significant protection was only identified with 160 mg/kg, a dose also associated with side-effects (data not shown).

4. Discussion

Relapse of HCMV disease occurs frequently in the face of antiviral therapy with GCV and other available anti-HCMV agents (Orellana et al., 1987; Jabs, 1992; Paul and Dummer, 1992; Peters et al., 1992; Markham and Faulds, 1994; Gerard and Salmon-Ceron, 1995; Polis and Masur, 1995). It may occur as a result of inadequate suppression of viral replication and/or the emergence of viral resistance (Collier and Corey, 1992; Peters et al., 1992; Polis, 1992). Increased dosage of a drug, if tolerable, or use of more potent antiviral agents for an optimal treatment duration, might be expected to delay relapse.

However, relapse was not observed in MCMV-infection models using normal immunocompetent mice. In these models, the animal either died of an acute infection within the same week of inoculation, or recovered from the disease (Winkler et al., 1990; Braitman et al., 1991). Although latently infected animals can be reactivated by immunosuppression, no significant relapse occurs spontaneously (Jordan, 1983; Bruggeman, 1993). These observations place in question the clinical relevance of antiviral effects obtained from such models (Smee et al., 1991; Neyts et al., 1992; Smee et al., 1995).

Treatment duration dependence of GCV in the MCMV SCID model has not been reported, to our knowledge. Neyts et al. (1992) inoculated the mice with a severe challenge, 10^5 pfu/mouse that led to very rapid development of wasting syndrome and mortality (MDD = 8.5). GCV and HPMPC were applied subcutaneously, starting 2 h post-inoculation for five days, or starting day 4

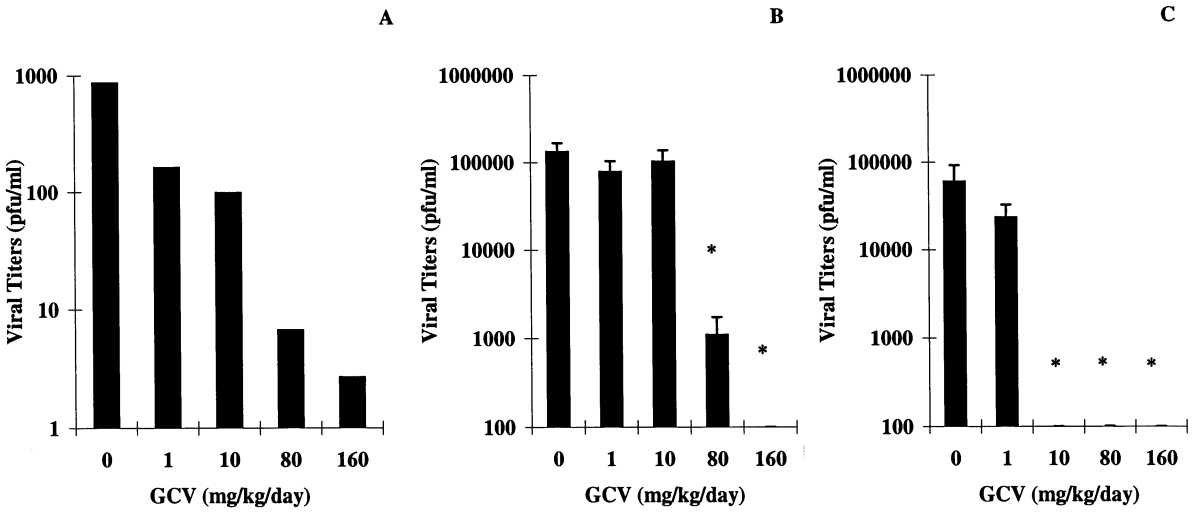

Fig. 3. Dose-dependent effects of GCV on MCMV replication in other organs. Panels A, B, C, represent viral titers in the ocular tissue, salivary glands, and the spleen respectively. Animals were inoculated and treated as described in the legend of Fig. 1. Statistical differences in viral titer reduction were determined and are indicated as in Fig. 1.

post-inoculation for 5 days, or two periods of three consecutive days starting on day 0 and day 9 post-inoculation, or a single dose 3 days prior to inoculation. Smee et al. (1992) studied the effects of GCV and cidofovir in the SCID model with less severe challenge, infection with MCMV at $10^{3.5}$ or $10^{4.5}$ pfu/mouse that led to a MDD of 19 and 17 days respectively. They administered GCV and cidofovir intraperitoneally, starting 1 day post-inoculation for 10 days against MCMV infection at the inoculum of $10^{3.5}$. To test the hypothesis that a longer duration of treatment with cidofovir could be more effective, they administered cidofovir for 20 days starting 5 days post inoculation, against an inoculum of $10^{4.5}$ pfu/mouse. Since the prolonged treatment duration was only conducted in the experiment with an increased viral challenge and longer delay in treatment, this experiment did not prove or disprove the proposed hypothesis (Smee et al., 1992). Our current study compares the effects of differential treatment duration with either optimal or sub-optimal doses of GCV. The results clearly show an enhanced protection with an optimal dose of GCV and prolonged treatment. The constant period of protection after termination of treatment indicates that GCV at optimal dosage is sufficient to control viral replication and disease development only during the treatment period, but not to prevent relapse. Lack of viral titer reduction beyond a constant period of time following termination of GCV treatment further verified that viral replication was associated with relapsed disease. The fact that 12 days of therapy is not better than 8 days at the dose of 10 mg/kg per day revealed a clear insufficiency of GCV against disseminated viral replication or the possible development of resistant virus (Collier and Corey, 1992; Peters et al., 1992; Polis, 1992). These observations are consistent with clinical observations and support the hypothesis that proper adjustment of dosage is important for GCV treatment (Polis, 1992).

An empirical 4–8 days of treatment duration has been recommended for antiviral studies (Hsiung and Chan, 1989). Most of the previous publications using the SCID model applied a treatment regimen for 5–7 days (Neyts et al., 1992; 1993a; Smee et al., 1994; Yang et al., 1996). In addition, a complete dose–response curve for GCV has not been reported in this model, to our knowledge. Therefore, the effects of GCV, in terms of dose dependence and treatment duration dependence, were not established in this SCID model. Our results clearly demonstrate the time requirement

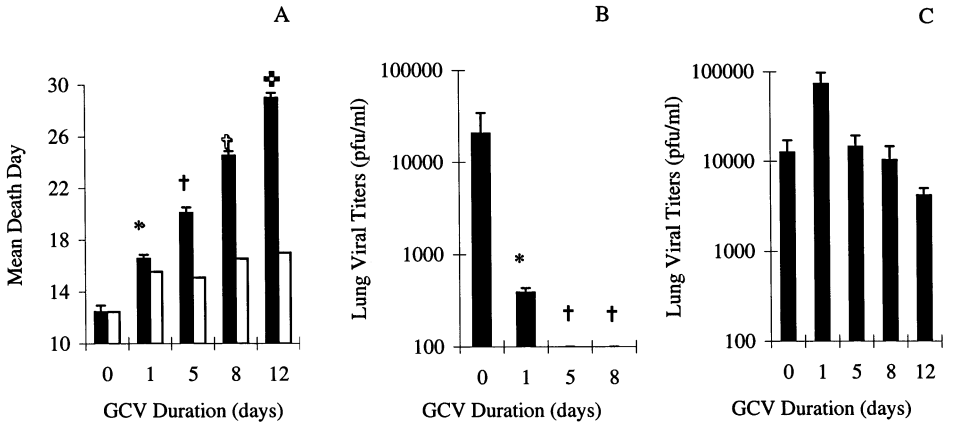

Fig. 4. Treatment duration-dependence of GCV on MCMV-induced mortality and viral replication in the lung. Panels A and B represent MDD and lung viral titers on day 8 respectively. In panel C, lung tissue was isolated on day 8 post-inoculation for the vehicle group, or 11 days after the termination of treatment for all GCV-treated groups. Animals were inoculated as described in the legend of Fig. 1. GCV treatment duration was indicated in the x-axis (0–12 days, 80 mg/kg per day). Statistical differences were determined and are indicated as in Fig. 1. In panel A, the solid bars present the MDD, the open bars represent MDD subtracting the GCV treatment duration.

to identify a clear dose–response curve for a drug such as GCV. Drug effects at lower doses may not be obvious when applied for a very limited duration, such as one day. The effective dose may be too close to the toxic dose, thus preventing the establishment of an effective dose–response curve. On the other hand, longer treatment duration may not make a sub-optimal dose more effective beyond certain increases in duration of treatment. Thus it seems that the empirical treatment duration of 4–8 days recommended for antiviral in vivo studies is applicable when using the described model for drug evaluation.

In summary, the current study has demon-

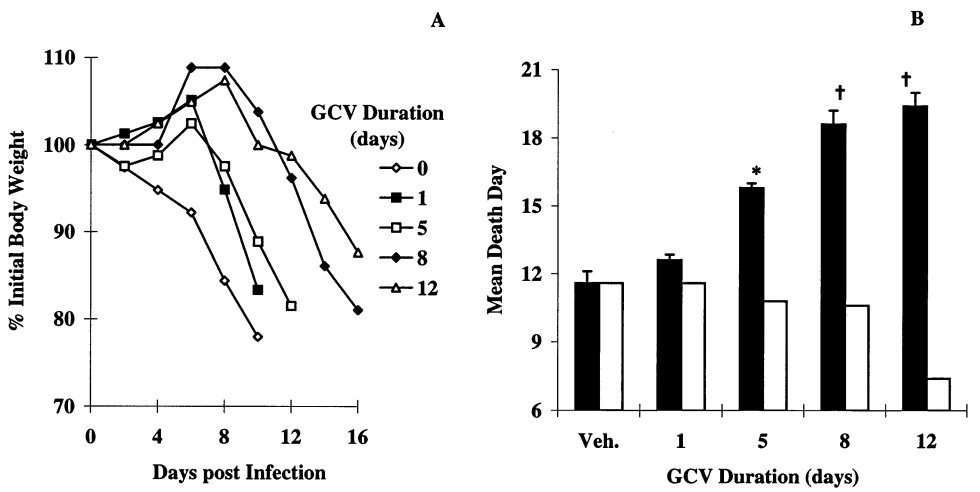

Fig. 5. Treatment duration-dependence of GCV on MCMV-induced wasting syndrome (panel A) and mean death day (panel B) in SCID mice. Animals were inoculated as described in the legend of Fig. 1. GCV treatment duration was indicated in the x-axis (0–12 days, 10 mg/kg per day). The mean death day subtracting the GCV treatment duration was plotted as open bars in panel B. Statistical differences were determined and are indicated as in Fig. 1.

strated that the described SCID MCMV-infection model provides an immunodeficient condition that allows the relapse of viral replication and CMV disease, upon termination of GCV treatment at an optimal dose, or during GCV treatment at an insufficient dose. The requirement for continuous therapy with optimally adjusted doses of GCV to maintain antiviral activity and the relapse upon termination of treatment are reminiscent of the situation in immunocompromised patients. The empirically recommended treatment duration for in vivo antiviral studies seems to apply here in the described model as a good balance considering both the opportunity to identify active compounds and shortening the turnaround time for drug evaluations.

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