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Antiviral prevention of sepsis induced cytomegalovirus reactivation in immunocompetent mice

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ABSTRACT

Introduction: Immunocompetent patients can reactivate latent cytomegalovirus (CMV) during critical illness and reactivation is associated with significantly worse outcomes. Prior to clinical trials in humans to prove causality, we sought to determine an optimal antiviral treatment strategy.
Methods: Mice latently infected with murine CMV (MCMV) received a septic reactivation trigger and were randomized to receive one of four ganciclovir regimens or saline. Lungs were evaluated for viral transcriptional reactivation and fibrosis after each regimen. Influences of ganciclovir on early sepsis-induced pulmonary inflammation and T-cell activation were studied after sepsis induction.
Results: All ganciclovir regimens reduced measurable MCMV transcriptional reactivation, and 10 mg/day for 7 or 21 days was most effective. Lower dose (5 mg/kg/day) or delayed therapy was associated with significant breakthrough reactivation. Higher doses of ganciclovir given early were associated with the lowest incidence of pulmonary fibrosis, and delay of therapy for 1 week was associated with significantly worse pulmonary fibrosis. Although bacterial sepsis induced activation of MCMV-specific pulmonary T-cells, this activation was not influenced by ganciclovir.
Conclusion: These results suggest that antiviral treatment trials in humans should use 10 mg/kg/day ganciclovir administered as early as possible in at-risk patients to minimize reactivation events and associated pulmonary injury.

1. Introduction

Cytomegaloviruses (CMVs) for all species are endemic and display classic characteristics of the *Betaherpesvirinae*. Following immune control of the primary lytic infection, CMV establishes life-long infection in its host. CMV becomes dormant in multiple end organs, a state also referred to as latency, and can later be reactivated by a variety of stimuli, including immunosuppression and inflammation (reviewed in Hummel and Abecassis, 2002). We first became interested in cytomegalovirus (CMV) reactivation in critically ill patients in the late-nineties (Cook et al., 1998), and since then it has become increasingly clear that up to 30–35% of latently infected immunocompetent individuals experience CMV reactivation during critical illness. This finding has now been reproduced independently by 7 different groups (Chiche et al., 2009; Heininger et al., 2001; Jaber et al., 2005; Kutza et al., 1998; Limaye et al., 2008; von Muller et al., 2006; Ziemann et al., 2008). Roughly 60% of people older than age 6 in this country have been infected with human CMV (HCMV) (Staras et al., 2006), and this percentage increases with age (Musiani et al., 1988). Thus most patients harbor latent virus when they develop critical illness, making them "at-risk" for reactivation.

Although the occurrence of viral reactivation during critical illness is now indisputable, the real question remains: is HCMV a pathogen in immunocompetent patients during critical illness, or simply an innocent bystander identifying patients with severe disease? HCMV is a well described pathogen in those without fully functional immune systems, such as neonates, patients with HIV, and transplant recipients receiving concurrent immunosuppression (Gaytant et al., 2002; Gor et al., 1998; Simmons et al., 1977; Steininger, 2007). Interestingly, the preponderance of recent clinical data supports the hypothesis that HCMV is also a pathogen in immunocompetent patients that develop critical illness. Studies to date have demonstrated surprisingly consistent morbidity in these patients, including increased durations of mechanical ventilation, prolonged hospitalizations, and worsened survival (Chiche et al., 2009; Cook et al., 1998, 2003; Heininger et al., 2001; Jaber et

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al., 2005; Kutza et al., 1998; Limaye et al., 2008; von Muller et al., 2006; Ziemann et al., 2008). It is intriguing that HCMV reactivation is associated with increased durations of mechanical ventilation, particularly because lungs are a primary site of latent virus (Toorkey and Carrigan, 1989), and a consistent site of reactivation (Cook et al., 1998, 2003; Heininger et al., 2001; Jaber et al., 2005; Kutza et al., 1998; Limaye et al., 2008; von Muller et al., 2006; Ziemann et al., 2008). Even more importantly have been recent associations of CMV reactivation with worsened mortality (Limaye et al., 2008; Ziemann et al., 2008). Perhaps the strongest support for this association comes from a recent meta-analysis that suggests a doubled mortality risk in patients with reactivation during critical illness (Kalil and Florescu, 2009). Thus available clinical data are consistent with the hypothesis that pulmonary HCMV reactivation during critical illness is pathogenic.

Because of ethical limitations associated with CMV research in very sick humans, we have developed a murine model to study reactivation and pathogenesis. This model was modified from the first described murine CMV (MCMV) model (Gonczol et al., 1985), and utilizes a septic challenge as a trigger for viral reactivation (Cook et al., 2002). Fortunately, HCMV and MCMV share many similarities (Collins et al., 1993; Henson et al., 1966; Rawlinson et al., 1996): both establish clinical latency following acute primary infection (Ho, 1982), reactivation has been associated with sepsis in both (Cook et al., 2002, 2003; Cook et al., 2006a; Heininger et al., 2001; Kutza et al., 1998; Limaye et al., 2008), and importantly MCMV has the same proclivity as HCMV for several organs, including lungs (Balthesen et al., 1993; Koffron et al., 1998; Kurz et al., 1997). These characteristics make MCMV an ideal model to study the pulmonary effects of reactivation, and using this model we have recently demonstrated that MCMV reactivation by sepsis in immunocompetent mice causes lung injury (Cook et al., 2006b). These studies also suggest that antiviral treatment with ganciclovir can prevent both sepsis-induced CMV reactivation and CMV-associated lung injury in immunocompetent mice.

Ultimately, proof of pathologic causality will require antiviral treatment trials in critically ill patients at-risk for reactivation (Cook, 2007). These patients often tolerate drug side effects poorly, and therefore prior to embarking on such trials, we felt it critical to investigate the ideal dosing of antiviral medication. Although ganciclovir treatment was very effective in our previous studies, we chose several alternative strategies to reduce drug exposure. Using our murine model, we confirm that antiviral treatment with ganciclovir prevents sepsis-induced CMV reactivation and its attendant pulmonary injury. In addition, we report the influence of several antiviral dosing strategies on this reactivation induced injury mechanism. Finally, we present preliminary evidence that suggests that lung resident T-cells may contribute to CMV induced pulmonary injury during reactivation.

2. Methods

2.1. Animals, viral infection, and confirmation of latency

Female BALB/c mice (Harlan, Indianapolis, IN) 6–8 weeks of age were used in this study. Purified Smith strain (VR-194/1981) MCMV was obtained from ATCC (Rockville, MD). Primary CMV infection was achieved by intra-peritoneal (i.p.) injection of 2×10^5 PFU Smith-MCMV and latency was confirmed as previously described (Cook et al., 2006a,b). Mice were euthanized by cervical dislocation under inhalation anesthesia. Mouse tissues were dissected aseptically and snap frozen in liquid nitrogen, then stored at −80 °C. Primary infection and latency/reactivation were confirmed as previously published (Cook et al., 2002, 2006a). As previously published, we define latency as viral DNA present in host tissues, without transcription of viral genes (Cook et al., 2002, 2006a,b). All mice were housed adhering to the *Guide for the Care and Use of Laboratory Animals* prepared by the National Research Council (NIH Publication No. 86-23, revised 1985) following protocol approval by our Institutional Review Board.

2.2. Sepsis and CMV reactivation

We have previously shown that an LD_{50} model of polymicrobial sepsis induced by cecal ligation and puncture (CLP) will stimulate pulmonary transcriptional reactivation of latent MCMV in 100% of surviving mice (Cook et al., 2002). We defined transcriptional reactivation from latency as mRNA transcription of MCMV glycoprotein-B (GB) known to be expressed at early/late temporal phases (reviewed in Reddehase et al., 2002). In our model, transcriptional activity of MCMV-GB becomes detectable between 7 and 14 days following CLP, with peak transcription occurring 21 days after CLP (Cook et al., 2002).

Mice underwent CLP as previously described (Cook et al., 2002, 2006b) and were randomly divided into cohorts receiving saline (no treatment), ganciclovir 10 mg/kg/day × 3 weeks, ganciclovir 10 mg/kg × 1 week, ganciclovir 5 mg/kg/day × 3 weeks, or ganciclovir 10 mg/kg/day × 2 weeks, beginning 1 week after CLP. Three weeks after CLP, surviving mice were euthanized and lungs evaluated for viral reactivation and inflammatory mediator expression using PCR and RT-PCR. Tissue samples fixed in formalin and paraffin embedded underwent histologic analyses.

2.3. Antiviral therapy

Ganciclovir dosing of 10 mg/kg/day (subcutaneous in 0.2 cm^3 saline vehicle) was chosen because this has been previously shown to be efficacious in mice (Cook et al., 2006b; Duan et al., 1998; Lenzo, 2001) and is a standard dose in adults for CMV disease. Steady state plasma level comparisons were made between mice receiving subcutaneous and intravenous administration of ganciclovir and these were not significantly different after 5 days of treatment (data not shown). For reactivation experiments, we define 4 ganciclovir treatment groups: (a) 10 mg/kg/day for 21 days, (b) 5 mg/kg/day for 21 days, (c) 10 mg/kg/day for 7 days, or (d) delayed therapy, 10 mg/kg/day started 7 days after CLP (total of 2 weeks before evaluation). Groups a–c are considered prophylactic treatment, because therapy is being initiated on post-sepsis day 1, well before transcriptional activity of early/late genes can be detected. Group d could be considered pre-emptive therapy because it is started 1 week after sepsis onset, and mimics delayed treatment until viral activity is detected in humans. For T-cell experiments, mice received ganciclovir pretreatment (10 mg/kg/day) for 1 week prior to sepsis induction. This duration was chosen to allow development of steady state tissue concentrations (>5 doses) in an attempt to ensure treatment effect.

2.4. PCR and RT-PCR

PCR and RT-PCR were performed as previously described (Cook et al., 2006a). If the first reaction yielded no visible product, a second (nested) PCR or RT-PCR reaction was performed using 1 μl of this first PCR product. Primers for MCMV-GB and GAPDH were as previously published (Cook et al., 2009b). Each RT-PCR experiment was performed in triplicate, and if any one of the three replicates was "positive", the mouse was considered to have transcriptional reactivation. Concomitant "no-RT" reactions were performed for each sample for each run to confirm lack of DNA contamination. For inflammatory mediator mRNA quantitative PCR, RNA were extracted from tissues as previously described (Cook et al., 2009a). Relative mediator mRNA was calculated using the $2^{-\Delta\Delta CT}$ method