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Low-dose ribavirin potentiates the antiviral activity of favipiravir against hemorrhagic fever viruses

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Abstract

Favipiravir is approved in Japan to treat novel or re-emerging influenza viruses, and is active against a broad spectrum of RNA viruses, including Ebola. Ribavirin is the only other licensed drug with activity against multiple RNA viruses. Recent studies show that ribavirin and favipiravir act synergistically to inhibit bunyavirus infections in cultured cells and laboratory mice, likely due to their different mechanisms of action. Convalescent immune globulin is the only approved treatment for Argentine hemorrhagic fever caused by the rodent-borne Junin arenavirus. We previously reported that favipiravir is highly effective in a number of small animal models of Argentine hemorrhagic fever. We now report that addition of low dose of ribavirin synergistically potentiates the activity of favipiravir against Junin virus infection of guinea pigs and another arenavirus, Pichinde virus infection of hamsters. This suggests that the efficacy of favipiravir against hemorrhagic fever viruses can be further enhanced through the addition of low-dose ribavirin.

Keywords

Favipiravir; Ribavirin; Junin Virus; Arenavirus; Viral Hemorrhagic Fever

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Potential conflict of interest

YF is an employee of the Toyama Chemical Co., Ltd., the manufacturer of favipiravir. All other authors declare that no competing interests exist.

1. Introduction

Favipiravir (T-705) is a nucleoside analog that was recently approved as an anti-influenza drug in Japan, and is in Phase 3 clinical trials in the USA for the same indication. It is active against a growing list of virulent RNA viruses including Ebola virus, and our own studies show that it is highly protective in rodent models of Argentine hemorrhagic fever (AHF), caused by Junin virus (JUNV) (Delang et al., 2014; Furuta et al., 2013; Gowen et al., 2013; Oestereich et al., 2014; Safronetz et al., 2013; Scharton et al., 2014; Smither et al., 2014). Favipiravir appears to act by directly inhibiting the viral RNA-dependent RNA polymerase (RdRP), with minimal effect on cellular nucleoside metabolism or intracellular nucleic acid pools (Furuta et al., 2005). There is little evidence that resistant viruses are selected during the course of therapy (Delang et al., 2014), indicating that the drug's target site is essential for efficient virus replication.

At present, the nucleoside analog ribavirin is the only FDA-approved drug with broad-spectrum activity against RNA viruses. However, even though many studies since the early 1980s show that ribavirin blocks the replication of many different viral agents *in vitro*, and that it is protective in numerous animal models of RNA viral infection, it is in regular clinical use only in combination with interferon for the therapy of chronic hepatitis C. Although small-scale trials have found ribavirin beneficial for the treatment of two arenaviral infections, Lassa fever and AHF, it can only be used off-label for those indications. The restricted clinical use of a drug with known broad-spectrum antiviral activity *in vitro* and in animal models reflects both ribavirin's somewhat limited efficacy and its side-effect of hemolytic anemia, caused by a dose-related reduction in intracellular guanosine triphosphate (GTP) levels (Graci and Cameron, 2006; Hitomi et al., 2011).

Taken together, the above considerations suggest that favipiravir might become the drug of choice for the treatment of severe RNA virus infections, eliminating any further need for ribavirin. However, because the two compounds have somewhat different mechanisms of action, the possibility exists that they could produce a synergistic effect if administered together, providing a continuing role for ribavirin. Such combination therapy is in fact beneficial in rodent models of two Old World viral diseases, Crimean-Congo hemorrhagic fever and Rift Valley fever (Oestereich et al., 2014; Scharton et al., 2014). In the present study, we extended the evaluation of favipiravir and ribavirin combination therapy to two small-animal models of the severe hemorrhagic fever caused by rodent-borne arenaviruses in South America. We found that the addition of a small dose of ribavirin markedly potentiated the protective efficacy of favipiravir in guinea pigs infected with JUNV, the causative agent of AHF, and in hamsters infected with a related arenavirus, Pichinde virus (PICV). Since the treatment of JUNV infection is currently limited to convalescent immune globulin, which exists only in dwindling stocks in Argentina, our data suggest that favipiravir, supplemented as needed by low doses of ribavirin, could become an effective and readily available therapy for AHF.

2. Materials and methods

2.1. Ethics statement

All animal procedures complied with USDA guidelines and were conducted at the Laboratory Animal Research Center or the Vivarium in the BioInnovations Center, both at Utah State University, an AAALAC-accredited institution, and approved by the Utah State University Institutional Animal Care and Use Committee.

2.2. Animals

Female Syrian golden hamsters (81-90 g) and male Hartley guinea pigs (300-350 g) were purchased from Charles River Laboratories (Wilmington, MA) and quarantined for at least 72 hours prior to virus challenge.

2.3. Viruses

PICV, strain An 4763, was provided by David Gangemi (Clemson University, Clemson, SC). The virus was passaged 4 times in Vero 76 cells and once in hamsters to generate the stock (4 Vero 76, 1 hamster; 3.9×10^8 plaque-forming units (PFU)/ml) prepared from pooled and clarified liver homogenates. The Candid 1 vaccine strain of JUNV was provided by Robert Tesh (World Reference Center for Emerging Viruses and Arboviruses, The University of Texas Medical Branch, Galveston, TX). The Candid 1 virus stock (1 BSC-1, 1 Vero; 1×10^7 PFU/ml) was generated from a clarified Vero cell infection lysate following single passages in BSC-1 and Vero cells. The molecular clone of the Romero strain of JUNV was provided by Slobodan Paessler (University of Texas Medical Branch, Galveston, TX). The virus was rescued in BHK-21 cells as previously described (Emonet et al., 2011), and the stock (1.1×10^8 PFU/ml) used was prepared from a single passage in Vero cells. Viruses were diluted with minimal essential media (MEM) just prior to in vitro assays and animal challenges. Studies with PICV and the Candid 1 strain of JUNV were conducted in biosafety level 2 (BSL-2) laboratories. Work with the pathogenic Romero strain of JUNV was conducted in BSL-3+ laboratories by vaccinated personnel.

2.4. Compounds

Favipiravir was obtained from the Toyama Chemical Co., Ltd. (Toyama, Japan). Ribavirin was from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). Both compounds were suspended in 0.4% carboxymethylcellulose (CMC; Sigma-Aldrich, St. Louis, MO) prior to administration by oral gavage (p.o.) in the hamster PICV infection drug efficacy study. For the JUNV drug efficacy studies in the guinea pig infection model of AHF, both compounds were dissolved in 2.9% sodium bicarbonate solution (7.5% sodium bicarbonate solution diluted with sterile water; Sigma-Aldrich) for intraperitoneal (i.p.) administration as a single injection. For the hamster PICV coadministration study, favipiravir and ribavirin doses were administered as separate treatments. Thus, the placebo-treated hamsters received 2 separate doses of 0.4% CMC and is represented as "Placebo + Placebo" treatment.

2.5. Cell Culture Antiviral Assays

Vero cells (ATCC, Manassas, VA) were maintained in MEM supplemented with 0.18 % NaHCO_3 and 10 % fetal bovine serum (FBS). Varying concentrations of favipiravir (starting at 12 $\mu\text{g/ml}$ with 6 serial 2-fold dilutions selected based on an EC_{50} of 1.6 $\mu\text{g/ml}$) and ribavirin (starting at 100 $\mu\text{g/ml}$ with 6 serial 2-fold dilutions selected based on an EC_{50} of 16 $\mu\text{g/ml}$) were added to test wells containing 70-80% confluent Vero cells (in MEM containing 2% FBS) at the time of JUNV (Candid 1 strain) infection at multiplicity of infection of approximately 0.001. For toxicity determinations done in parallel, the same favipiravir and ribavirin combinations were added to uninfected Vero cells. Plates were incubated at 37°C, 5% CO_2 for 7 days, at which time culture supernatants were collected for endpoint titration of infectious virus and the plates processed to assess cell viability by neutral red vital dye uptake as previously described (Gowen et al., 2007).

2.6. Efficacy of favipiravir and ribavirin combination therapy in PICV-infected hamsters

Hamsters were weighed and block randomized on the morning of the infection so that the average weight per group across the entire experiment varied by less than 3 grams. Hamsters ($n = 15$ for treatment groups, $n = 26$ for placebo group) were challenged i.p. with a 0.2 ml inoculum containing 250 PFU of PICV. Sham-infected normal control animals ($n = 6$) were injected i.p. with 0.2 ml MEM. Twice daily p.o. treatments of favipiravir (200, 100, or 0 mg/kg/day) combined in a 3×3 factorial design with ribavirin (35, 17.5, or 0 mg/kg/day) were initiated 6 days post-infection (p.i.) and selected based on previously published experiments (Gowen et al., 2008). The animals were observed for morbidity and mortality for 34 days and weighed every third day.

2.7. Efficacy of favipiravir and ribavirin coadministration in JUNV-challenged guinea pigs

Animals were weighed 3 days prior to virus challenge, grouped to minimize variation of the average weight per group across the entire experiment, and implanted subcutaneously (s.c.) with IPTT-300 programmable temperature transponders (Biomedic Data Systems, Seaford, DE). Guinea pigs ($n = 6$ for treatment and placebo groups) were challenged i.p. with 0.1 ml inoculum containing 100 PFU of JUNV. Sham-infected normal control animals ($n = 2$) were injected i.p. with 0.1 ml MEM. In the first coadministration efficacy study, twice daily i.p. treatments of favipiravir (300 or 0 mg/kg/day) combined in a 2×2 factorial design with ribavirin (50 or 0 mg/kg/day) were initiated 3 days p.i. based on previously published data (Gowen et al., 2013). For the second study in which lower drug doses were administered, twice daily i.p. treatments of favipiravir (250 or 0 mg/kg/day) combined in a 2×2 factorial design with ribavirin (25 or 0 mg/kg/day) were initiated 3 days p.i. The animals were observed for 42 days for morbidity and mortality in both efficacy studies and, when possible, animals with body temperatures below 3 °C of their starting temperature or having lost 20% or more of their starting weight were humanely euthanized and blood and tissues were collected for virus titer and histopathology analysis. At the conclusion of the studies, the surviving animals were euthanized and blood and tissue samples were collected and analyzed for infectious virus and histopathology.

2.8. Tissue and serum virus titers

Virus titers were assayed using an infectious cell culture assay as previously described (Gowen et al., 2007). Briefly, liver, spleen and brain tissue samples were homogenized in a volume (ml) of MEM 3 times the tissue mass (g) and the homogenates and serum were serially diluted and added to triplicate wells of Vero cell monolayers in 96-well microplates. The viral cytopathic effect was determined 11 days p.i. and the 50% endpoints were calculated as described (Reed and Muench, 1938) and represented as the log₁₀ 50% cell culture infectious dose (CCID₅₀)/ml or g for serum and tissues, respectively. The assay detection limit for liver, spleen and brain tissues was 3.05 CCID₅₀/g and the limit of detection for serum virus was 1.49 CCID₅₀/ml.

2.9. Statistical analysis

The Mantel-Cox log-rank test was used for analysis of Kaplan-Meier survival curves using Prism 5 (GraphPad Software, La Jolla, CA). For the JUNV cell culture and the PICV hamster drug combination treatment studies, analyses to determine synergistic effects of the coadministered treatments on virus yield reductions and survival outcomes, respectively, were performed using the MacSynergy II program (Prichard and Shipman, 1990). The 3-dimensional peaks above and below the plane, with the plane defined as no synergy or antagonism, is a representation of the quantitated synergy or antagonism of the interaction of favipiravir and ribavirin. The dose response curves for both drugs individually were used to calculate the theoretical additive effect of the drugs, which were subtracted from the experimentally determined dose response to produce the 3-dimensional peaks. MacSynergy II calculates a volume of synergy or antagonism. Because the in vitro data were plotted in log₁₀ scale, the calculated values were multiplied by 10. Data plotted as percentages (animal survival) required no such adjustment to the calculated values. The resulting volumes of synergy and antagonism value were interpreted as follows: <25 as insignificant, 25-50 as weak, 50-100 as moderate, and >100 as strong synergy (Ilyushina et al., 2008).

3. Results

3.1. Favipiravir and ribavirin synergistically inhibit JUNV replication *in vitro*

The effect of treating JUNV-infected cells with ribavirin, favipiravir or the two drugs together was investigated using an 8 × 8 combinatorial design. Combination treatment resulted in enhanced reduction of virus yield, as reflected by the volume of the area above the expected inhibitory effect for each drug independently (Figure 1). The net volume of synergy resulting from favipiravir + ribavirin interaction was >170, indicative of a strong synergistic effect (Ilyushina et al., 2008). Toxicity associated with the tested drug combinations was limited, with 4% cytotoxicity observed with 12 µg/ml of favipiravir, 11% with 100 µg/ml of ribavirin, and 20% for the two drugs combined.

3.2. Addition of low-dose ribavirin enhances favipiravir protection of PICV-infected hamsters

In the initial combination therapy study in PICV-infected hamsters with treatment beginning on day 6 p.i., we found that 200 mg/kg/day of favipiravir plus 35 mg/kg/day of ribavirin

resulted in 80% survival, as compared to 20% survival in placebo-treated controls (Figure 2A). However, the same dose of ribavirin alone also produced 80% protection, so no beneficial effect of combined therapy was evident. Similarly, a regimen of 100 mg/kg/day of favipiravir combined with 35 mg/kg/day of ribavirin did not improve upon ribavirin alone (Figure 2B). At a level of ribavirin that gave less protection, it was possible to detect a benefit of combined therapy. When ribavirin was decreased to 17.5 mg/kg/day, only 20% of infected hamsters survived (Figure 2C), and decreasing favipiravir from 200 to 100 mg/kg/day also reduced the level of protection (Figure 2D). When the low dose of 17.5 mg/kg/day ribavirin was added to either dose of favipiravir, there was a significant increase in survival. Further analysis using the MacSynergy II program indicated favorable interactions between favipiravir and low-dose ribavirin, with a volume of synergy of 53.1, consistent with a moderate synergistic effect (Figure 3).

3.3. Addition of low-dose ribavirin enhances favipiravir protection of JUNV-infected guinea pigs

In a previous study in guinea pigs challenged with a lethal dose of the Romero strain of JUNV, we found that treatment beginning on day 3 postexposure with 300 mg/kg/day of favipiravir achieved approximately 80% survival, while only 33% of animals given 50 mg/kg/day of ribavirin survived infection (Gowen et al., 2013). When we repeated the experiment, using the same drug doses, we obtained similar results for treatment with each drug alone (Figure 4). Unexpectedly, treatment with the two drugs together did not result in increased survival, suggesting that the doses were perhaps too high for combined therapy to produce a measurable synergistic effect.

In a second experiment, we reduced the dose of both drugs, giving 250 mg/kg/day of favipiravir and 25 mg/kg/day of ribavirin (Figure 5). As before, placebo-treated animals developed fever and weight loss beginning 5-6 days after virus challenge (Figure 5B, C). All animals that received either drug alone succumbed to infection, though their deaths were delayed by 6-7 days compared to the placebo group (Figure 5A). In contrast, five of six guinea pigs that received the combination therapy survived, a significant ($P < 0.01$) improvement over either drug alone. Animals in the combined treatment group stopped gaining weight on approximately day 7 p.i., then began to recover before the end of the treatment period. In contrast, those that only received ribavirin progressively lost weight, beginning on approximately day 11 p.i., while animals treated with favipiravir alone continued to gain weight through approximately day 13-14, then rapidly lost weight (Figure 5B). Interestingly, increased body temperatures were measured on days 6-12 in placebo-treated animals and on days 13-18 in those that received only favipiravir, but fever was not detected in animals that received both drugs or ribavirin alone (Figure 5C).

The marked survival benefit of favipiravir + ribavirin suggests that treatment suppressed viral replication in the tissues of infected guinea pigs. To detect such an effect, we performed necropsies on all animals that became moribund and were euthanized. We found that all placebo-treated animals had high levels of JUNV in the serum, liver, spleen and brain (Figure 6). In contrast, virus was detected in the brains of the 11 drug-treated guinea pigs and in the spleens of 4 that received either ribavirin or favipiravir alone, but no virus

was detected in the serum or liver of any drug-treated animal. The brain was the only location where virus was detected in the one guinea pig that required euthanasia despite receiving combination therapy. Histologic studies showed that the detection of infectious virus correlated with the presence of inflammatory lesions or necrosis in the same tissues (Supplementary Table 1). Taken together, these data suggest that untreated guinea pigs died from a systemic viral infection, while those that died after receiving ribavirin or favipiravir alone, or the two drugs together, succumbed to late-onset encephalitis.

4. Discussion

Favipiravir's activity against a wide range of RNA viruses and its approval for the treatment of novel and re-emerging influenza viruses in Japan has inspired hopes that it may provide the type of broad-spectrum therapeutic activity that ribavirin was once hoped to have, but because of toxicity and limited potency, has failed to achieve in practice. The recent use of favipiravir to treat patients with Ebola virus disease in West Africa may represent the first step toward its development as a genuine broad-spectrum antiviral drug.

Our finding of synergistic antiviral activity of favipiravir and ribavirin against arenaviruses is consistent with previous work reporting a beneficial combined effect of the two drugs against two bunyaviruses, Crimean-Congo hemorrhagic fever virus and Rift Valley fever virus (Oestereich et al., 2014; Scharton et al., 2014). Favipiravir and ribavirin both exert direct antiviral effects on the RNA viral polymerase, but the mechanism of synergism is not clear. We suspect that the indirect antiviral effects of ribavirin, such as its proposed immunomodulatory activities and inhibition of IMPDH, may enhance the activity of favipiravir (Graci and Cameron, 2006). For example, reduced GTP pools resulting from IMPDH inhibition may enhance the binding of favipiravir-RTP to the viral RdRP, inhibiting further elongation or increasing misincorporation frequency. Favipiravir may also have unrecognized host effects that potentiate ribavirin's activity.

The toxicity of ribavirin has been known since the late 1970s (Bodenheimer et al., 1997; Chapman et al., 1999; Kochhar et al., 1980), but neither we nor others have formally assessed the toxicity of combinations of favipiravir and ribavirin. In our earlier study comparing the efficacy of ribavirin and favipiravir in JUNV-infected guinea pigs, we observed some toxic effects in animals treated with 50 mg/kg/day of ribavirin (Gowen et al., 2013), and in the present study, some toxic effects were noted when we reproduced those doses in combination with favipiravir. However, because our goal was to detect a synergistic effect of low-dose ribavirin, our next step was to reduce the dose by half, which appeared to eliminate toxicity. A formal evaluation of any toxic effects of the combined drugs in uninfected animals should clearly be performed.

The doses of favipiravir and ribavirin administered to rodents in the present study appear to reasonably reflect tolerated doses in humans. For favipiravir, a dose of 250 mg/kg in a rodent is equivalent to approximately 54 mg/kg, based on a 60 kg person. In the recent Ebola clinical trial, patients received 100 mg/kg for day 1, with a 40 mg/kg maintenance dose administered on days 2-10 (Schibler et al., 2015). Similarly, a rodent dose of 25 mg/kg of ribavirin is approximately equivalent to 5.5 mg/kg in humans, far below the amount given

to patients with AHF in a small clinical trial (85 mg/kg on day 1, 68 mg/kg on days 2-5, and 24 mg/kg for the last 6 days of treatment) (Enria and Maiztegui, 1994). Because favipiravir is well tolerated at a dose of 500 mg/kg/day (Mendenhall et al., 2011), the best strategy for future animal studies may be to increase the dose of favipiravir and further reduce the amount of ribavirin.

As noted above, convalescent immune globulin was proven to be efficacious for the treatment of AHF in a controlled trial in Argentina in the late 1970s, and it remains the only approved therapy in that country. However, because widespread vaccination in endemic areas has markedly reduced the incidence of the disease, the number of convalescent donors and the stock of immune globulin have correspondingly declined. The product is also not available outside Argentina, therefore, were JUNV used as a biological weapon in the United States, an expert panel has recommended the use of ribavirin for postexposure prophylaxis or therapy (Borio et al., 2002). Our findings suggest that favipiravir would be more efficacious for the treatment of JUNV infection, and that clinical recovery could be enhanced and survival rates improved through the addition of low-dose ribavirin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- The new antiviral favipiravir has shown efficacy against Ebola virus in patients and wide range of RNA viruses in animals.
- It appears to be more potent and less toxic than ribavirin, raising the question of a future role for that drug.
- We tested the ability of low-dose ribavirin to enhance the protective activity of favipiravir against arenavirus infection.
- Adding low-dose ribavirin to favipiravir significantly increased survival of arenavirus-infected hamsters and guinea pigs.
- Favipiravir plus low-dose ribavirin may be a highly effective therapeutic countermeasure against hemorrhagic fever viruses.

Figure 1. Ribavirin synergistically potentiates the antiviral effect of favipiravir in vitro
Vero cell cultures were treated with varying combinations of favipiravir and ribavirin and the log₁₀ reduction of infectious JUNV (Candid 1) was plotted and analyzed using MacSynergy II. A three-dimensional plot representative of the drug-drug interaction is shown. The calculated volumes above (193.4) and below (-19.7) the plane (regions of synergy and antagonism, respectively) yield a net volume of synergy of 173.7, indicative of a strong synergistic interaction.

Figure 2. Ribavirin/favipiravir dose-ranging studies in PICV-infected hamsters

Groups (drug-treated, $n=10$; placebo $n=21$) of PICV-infected hamsters were treated orally with favipiravir, ribavirin, or a combination of both compounds twice daily for 10 days starting 6 days p.i.. A, B) Treatment with 35 mg/kg/day of ribavirin alone was as effective as the same dose added to 200 mg/kg/day (A) or 100 mg/kg/day (B) of favipiravir. C, D) 17.5 mg/kg/day of ribavirin had no protective effect, but it significantly enhanced protection by 200 mg/kg/day (C) or 100 mg/kg/day of favipiravir (D). $*P < 0.05$, $**P < 0.01$, compared to placebo-treated animals by log-rank test. p.i., postinfection.

Figure 3. Synergistic interaction of ribavirin-favipiravir on survival data of PICV-infected hamsters

The survival data from the experiment described in Figure 2 was plotted and analyzed using MacSynergy II. The y-axis represents the percent of synergy, or antagonism, from the calculated theoretical additive drug interactions. The calculated volumes above (750) and below (-218.8) the plane (regions of synergy and antagonism, respectively) yield a net volume of synergy of 531.2, indicative of a strong synergistic interaction.

Figure 4. Initial dose-ranging study of ribavirin/favipiravir treatment of JUNV-infected guinea pigs

Animals in each group (n=6) were infected with 100 PFU of JUNV and treated i.p. with the indicated doses of favipiravir, ribavirin, a combination of both compounds, or placebo twice daily for 2 weeks starting 3 days p.i. Kaplan-Meier survival curves are shown. ** $P < 0.01$, *** $P < 0.001$, compared to placebo-treated animals. Tx, treatment; p.i., postinfection.

Figure 5. Low-dose ribavirin markedly enhances protection by favipiravir in JUNV-infected guinea pigs

Animals in each group (n=6) were treated i.p. with the indicated doses of favipiravir, ribavirin, a combination of both compounds, or placebo twice daily for 2 weeks starting 3 days p.i. Two sham-infected normal controls are included as baseline values for weight and temperature. A) Treatment with 25 mg/kg/day of ribavirin or 250 mg/kg/day of favipiravir alone did not prevent death, but the combination resulted in 83% survival. B) Shown are the group means and standard deviations of the percent weight change relative to starting weights 3 days prior to virus challenge. Animals that received both drugs gained weight nearly as well as normal controls. C) Body temperatures are represented as the group means and standard deviations relative to their starting temperatures 3 days prior to challenge. Fever was observed in infected animals receiving ribavirin or favipiravir alone, or placebo, but not in those treated with both drugs. *** $P < 0.001$ compared to placebo-treated animals; ^b $P < 0.01$ compared to animals receiving monotherapy. p.i., postinfection.

Figure 6. Combination therapy virtually eliminates infectious virus from tissues of JUNV-infected guinea pigs

JUNV titers were determined for serum, liver, spleen and brain samples collected following euthanasia on the days indicated. Samples were obtained from 5 moribund guinea pigs that had received either favipiravir or ribavirin alone, the single moribund animal that had received both drugs, and 5 animals from the placebo group. Where bars are not present, the virus concentration was below the limit of detection (1.49 log₁₀ 50% cell culture infectious dose (CCID₅₀)/ml of serum or 3.05 log₁₀ CCID₅₀/g of tissue).