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Comparison of ribavirin and oseltamivir in reducing mortality and lung injury in mice infected with mouse adapted A/California/04/2009 (H1N1)

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Abstract

Aim—To compare the efficacy of ribavirin and oseltamivir in reducing mortality, lung injury and cytokine response profile in pandemic influenza H1N1 (2009) infection.

Main Methods—We assessed survival, weight loss, lung viral load (by RT-PCR), lung injury (by protein content in bronchoalveolar lavage), and inflammation (cell counts, differentials and cytokines in the bronchoalveolar lavage) in BALB/c mice after infection with mouse-adapted pandemic influenza strain A/California/04/2009.

Key Findings—Our results indicate that ribavirin (80 mg kg⁻¹) and oseltamivir (50 mg kg⁻¹) are equally effective in improving survival (100% vs. 0% in water treated controls), while ribavirin proved to be more effective in significantly preventing weight loss. Both drugs diminished the injury of the alveolar-capillary barrier by decreasing the protein detected in the BAL to baseline levels, and they were also equally effective in reduction lung viral loads by 100-fold.

Administration of either drug did not decrease the amount of inflammatory infiltrate in the lung, but ribavirin significantly reduced the percentage comprised of lymphocytes. This study shows that these antivirals differentially regulate inflammatory cytokines and chemokines with ribavirin significantly reducing most of the cytokines/chemokines measured. Ribavirin treatment leads to a

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Th1 cytokine response while oseltamivir leads to a Th2 cytokine response with significant increase in the levels of the anti-inflammatory cytokine IL-10.

Significance—This study reveals new mechanistic insights in the way that ribavirin and oseltamivir exert their antiviral activity and supports the theory that ribavirin could potentially serve as an efficacious therapeutic alternative for oseltamivir resistant pandemic H1N1 strains.

Keywords

Acute lung injury; H1N1 2009; Influenza A; Oseltamivir; Pandemic; Ribavirin

INTRODUCTION

The H1N1 influenza pandemic began in Mexico in April 2009 and resulted in nearly 274,000 hospitalizations and 12,470 deaths in the U.S. alone (Centers for Disease Control and Prevention 2010a; Noah et al. 2009). Worldwide, more than 214 countries were affected and at least 18,449 deaths were reported (World Health Organization 2010a). Although vaccination remains the best means of preventing influenza virus infection, antivirals are the most efficient therapy for severe cases of influenza requiring hospitalization. Existing antivirals target the M2 viral ion channel function (amantadine, rimantadine), viral replication (ribavirin) or viral neuraminidase (oseltamivir, zanamavir, peramivir) (Moss et al. 2010). Currently oseltamivir is the most widely used antiviral for the treatment of influenza infection.

Many previous and currently circulating influenza strains are resistant to adamantanes (Centers for Disease Control and Prevention 2010b; Deyde et al. 2007), and the 2007-2008 H1N1 seasonal strains were also resistant to oseltamivir (Duan et al. 2010). Although most of the current circulating H1N1 strains (H1N1 2009, pandemic lineage) are still sensitive to oseltamivir, there is a recent report of the emergence of a multidrug-resistant pandemic influenza A (H1N1) virus where neither oseltamivir, zanamavir, nor peramavir were effective, due to a novel mutation (I223R in the neuraminidase) and this stresses the need for new antiviral agents (van der Vries et al. 2010). Ribavirin is a broad-spectrum antiviral agent with strong *in vitro*, strong preclinical, and variable clinical efficacy against various RNA and DNA viruses (Crotty et al. 2002; Leyssen et al. 2005, 2006). Despite the lack of solid clinical data, the World Health Organization recommends the use of ribavirin in conjunction with other agents in all populations except pregnant women as the drug has potential teratogenicity (World Health Organization 2010b). Given the concern over the emergence of oseltamivir-resistant strains of influenza, there is increasing interest in other candidates effective against severe influenza infections such as ribavirin (Chan-Tack et al. 2009; Rowe et al. 2010).

Presently, there is no direct data about efficacy of antiviral agents in reducing Acute Lung Injury (ALI) in influenza-infected mice. Increased levels of protein in the BAL are a hallmark of acute respiratory distress syndrome (ARDS) and therefore lung injury (Ware and Matthay 2000). This is an important issue since there were numerous incidents of ALI and ARDS in patients with H1N1 infections (Hubmyr and Farmer 2010). Moreover, the “cytokine storm” seen in cases of fatal pandemic H1N1 infection has been implicated in the

dysfunction of vascular permeability that leads to edematous fluid built up in the lungs and subsequent lung failure (Cheng et al. 2011; Walsh et al. 2011; Wang et al. 2010).

In the present study we compared the efficacy of ribavirin with that of oseltamivir in terms of survival, lung viral load, injury to the alveolar-capillary barrier, and inflammation in BALB/c mice after infection with mouse-adapted pandemic influenza A/California/04/2009. Our results indicate that ribavirin is equally effective with oseltamivir in improving survival, limiting viral replication and egress of plasma protein in the alveolar spaces. Interestingly, ribavirin reduced the lymphocytic infiltration in the lung but did not alter total inflammatory cell counts, which was the case for oseltamivir as well. Lastly, ribavirin promoted a different cellular cytokine pattern than oseltamivir suggesting differential Th1 and Th2 inflammatory responses.

MATERIALS AND METHODS

Experimental Animals

Female BALB/c mice (Charles River Laboratories, Wilmington, MA) were acclimated in BSL2 containment at least 3 days prior to use. Mice were 7-11 weeks old and weighed 17-22 grams at the time of challenge. Each mouse was uniquely identified by ear punch and by a subcutaneous implantable micro-identification device (IPTT-300 Chip; Biomedic Data Systems Inc.; Seaford, DE). The Provantis application (Version 7; Instem Life Systems, Ltd.; Staffordshire, United Kingdom) was used for the direct online capture of body weights, clinical observations, body temperatures, husbandry and dosing. Experimental protocols and animal procedures were approved by the Southern Research Institute Institutional Animal Care and Use Committee (IACUC).

Viruses

A mouse-adapted A/California/04/2009 (H1N1) pandemic strain virus was obtained from St. Jude Children's Research Hospital (Memphis, Tennessee, USA) (SJ # 175190). The virus was amplified in embryonated hen's eggs according to standard procedures. The virus was diluted in Dulbecco's Phosphate Buffer Saline (DBPS) for challenge. The average Egg Infectious Dose (EID₅₀ ml⁻¹) of the virus was approximately 10^{9.32} EID₅₀ ml⁻¹. All manipulations, dilutions, and infections were performed in a class II biosafety cabinet and maintained on ice until the completion of the virus challenge.

Antiviral Compounds

Ribavirin was purchased from MP Biomedicals (Solon, OH, USA). Oseltamivir was acquired as Tamiflu capsules from a local pharmacy. Both compounds were suspended in sterile water for oral treatment of mice.

Experimental Design

Thirteen (13) mice were assigned to the respective treatment groups using a computer-generated randomization procedure. Mean group body weight measured one or two days prior to virus challenge was used to calculate drug dosing. Mice were anesthetized with intraperitoneal injections of Ketamine/Xylazine (50 mg kg⁻¹ and 5 mg kg⁻¹ respectively)

and ninety μl of H1N1/mouse ($10^{5.35}$ EID₅₀) were instilled intranasally. A Bair Hugger warm air unit was used to keep animals warm during recovery from the anesthesia. The mice received 100 μl of either ribavirin (40 mg kg⁻¹), or oseltamivir (25 mg kg⁻¹) by oral gavage starting at 4-5 h prior to infection and at 12 ± 1 hr intervals for the next five days. Control mice were infected with H1N1 and received equivalent amounts of sterile water. Additionally, 3 naïve mice were utilized for BAL protein, total and differential cell counts as well as cytokine profiles.

Mortality

Ten (10) animals from each group were followed for up to 14 days post infection. Body weights, temperatures, clinical signs and time of death were monitored and recorded daily.

Assessment of lung injury

On day six post-infection, three mice from each group were euthanized by intraperitoneal injections of sodium pentobarbital. The thorax was opened and their lungs lavaged with one ml of normal saline that was inserted and withdrawn twice. The lavage fluid was collected and spun at approximately 200 x g for approximately 5 minutes to pellet cells. The supernatant was collected, transferred to a clean tube, and snap frozen in liquid nitrogen. Lungs were removed and snap frozen in liquid nitrogen and stored at -80°C .

Mice that were moribund (that were not used for tissue collection on day 6), or had weight loss that equaled or exceeded 30% were euthanized via CO₂ inhalation. All remaining animals were euthanized on day 14 via CO₂ inhalation.

Lung viral load assessment by RT-PCR

Lung tissue samples (200-300 mg each) were minced and RNA extracted with 500 μl of phenol/Tris-Cl buffer. 100 μl of aqueous buffer was precipitated with ethanol and the pellet was dissolved in 100 μl of water. 2 μl of this was analyzed using the Invitrogen SuperScript® III One-Step RT-PCR System with Platinum®Taq (Cat. No. 12574-026), the primer set targeted to the M gene of the influenza virus (25 pmoles each 5'-AGATGAGTCTTCTAACCGAGGTCG-3' and 5'-TCGAGATCGGTGTTCTTTCC-3'), and probe (6FAM-TCAGGCCCTCAAAGCCGA-MGBNFQ, ABI), in a final volume of 25 μl with nuclease free water. Quantitative RT-PCR was performed on a Mastercycler EP Realplex real-time PCR system (Eppendorf). The number of viral genomic M segments was calculated using a quantitated RNA standard transcribed from the M gene sequence.

Protein levels in BALF

Protein concentrations in cell-free BALF samples were measured with the BCA Protein Assay Kit (Pierce, Rockford, IL) according to manufacturer's protocol. A standard curve was prepared with known concentrations of BSA in 0.9% NaCl.

Cell counts and differentials

After BALF centrifugation and storage of the supernatant, cell pellets were washed for a minute with red blood cell lysis solution and centrifuged at 300 x g for 10 minutes. Cells were re-suspended in 100 μl of 2% BSA saline solution and cells were counted using a

Neubauer hemocytometer. Cytoslides were prepared and stained with hematoxylin and eosin (Hema 3 System, Fisher HealthCare, Houston, TX). Differential counts of BALF monocytes, neutrophils, lymphocytes cells were determined via light microscopy.

Assessment of cytokines and chemokines in the BALF

The concentrations of a number of cytokines in the BAL were measured using the Bio-Plex Cytokine Mouse 23-Plex Panel (Bio-Rad Laboratories, Hercules, CA) according to manufacturer's instructions. The panel includes the following cytokines: interleukin (IL)-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17, Eotaxin, granulocyte-colony stimulation factor (G-CSF), granulocyte macrophage-colony stimulation factor (GM-CSF), interferon (IFN)- γ , keratinocyte derived cytokine (KC), monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1 α , MIP-1 β , regulated upon activation normal T-cell expressed RANTES and tumor necrosis factor (TNF)- α . The corresponding limits of detection provided by the manufacturer are given (in pg ml⁻¹): 2, 7, 3, 2, 3, 2, 2, 15, 2, 2, 4, 9, 1, 148, 1, 7, 6, 3, 14, 24, 2, 5, 6.

Statistical analysis

All values are presented as Mean $\pm$ SD for the indicated number of mice. Kaplan-Meier survival curves were analyzed by log rank test to assess statistical significance among the survivors of each treatment group. Data were analyzed by unpaired Student's t-test comparing to naïve vs. water controls as well as ribavirin or oseltamivir vs. water controls. Differences were deemed significant when $p < 0.05$.

RESULTS

Ribavirin and oseltamivir treatments reduced H1N1 mortality

H1N1 infection of water treated BALB/c mice (controls) resulted in 100% mortality by day 8 post-infection. Daily oral administration of ribavirin or oseltamivir resulted in 0 % mortality ($p < 0.001$ compared to water controls respectively, **Figure 1A**). These results indicate that both antivirals were effective in diminishing mortality.

Ribavirin prevented significant weight loss caused by H1N1

As shown in **Figure 1B**, mice infected with H1N1 experienced progressive and significant weight loss during the next six days. Administration of ribavirin significantly prevented weight loss as compared to oseltamivir during days 7-10 as shown in **Figure 1B**. There was only a transient significant weight decrease in the ribavirin group by day 3 as compared to oseltamivir. Therefore, ribavirin and oseltamivir exert different effects on weight loss. However, all surviving mice had normal body weights at 14 days post exposure.

Ribavirin and oseltamivir reduced lung viral loads

Viral loads were quantified by means of quantitative RT-PCR in both lungs of each animal at day 6 (n=3 from each group). Treatment with either ribavirin or oseltamivir for five days (**Figure 2A**) decreased lung viral load a 10-fold compared to water controls.

Ribavirin and oseltamivir ameliorated alveolar-capillary barrier damage

BAL protein levels are an index of injury to the pulmonary microvascular and alveolar epithelium. As shown in **Figure 2B**, infection with H1N1 resulted in significantly higher BAL protein levels at day 6 as compared to naïve mice, (water controls: 296.7 ± 40.06 mg/100ml vs naïve: 55.6 ± 21 mg/100ml; $p < 0.001$). This value is consistent with increased alveolar permeability to plasma proteins resulting in non-cardiogenic pulmonary edema, which contributes to death from respiratory failure. Administration of ribavirin decreased the concentrations of protein in the alveolar spaces (86.87 ± 15.25 mg/100 ml vs. 296.7 ± 40.06 , respectively; $p < 0.01$). Similarly, administration of oseltamivir also decreased the concentrations of plasma proteins in the alveolar spaces at day 6 (114.7 ± 31.32 mg/100 ml vs. 296.7 ± 40.06 , respectively; $p < 0.01$). Thus, both antiviral drugs protected the lungs from injury.

Ribavirin reduces lymphocytic infiltration but does not affect the numbers of total cells in the BAL

Infection with H1N1 resulted in the influx of high number of inflammatory cells in the alveolar spaces at day 6 post-infection as documented by BAL total cell count analysis ($24 \pm 16 \times 10^5$ cells/ml vs. $4 \pm 1 \times 10^5$ cells/ml; $p < 0.05$). Infection led to alteration of the cellular differential with monocytes reduced and neutrophils and lymphocytes significantly increased in infected control mice as compared to uninfected naïve mice (see **Table 1**). At day 6, monocytes made up approximately 50%, neutrophils 25% and lymphocytes 25% of the total cell counts in both the control and oseltamivir groups, suggesting that the general infiltrate pattern in response to viral infection was not altered by oseltamivir administration. On the other hand, ribavirin administration exhibited significantly reduced infiltration of lymphocytes and slightly concomitantly increased monocyte and neutrophil counts (**Table 1**).

Ribavirin and oseltamivir induce different cytokine responses after H1N1 infection

As shown in **Table 2**, infection of BALB/c mice with influenza resulted in significant increases of most cytokines measured at day 6 post-infection as compared with naïve mice. Administration of antivirals resulted in different effects on the cytokine profile of infected mice. Ribavirin had significantly reduced levels of IL-9, IL-10, IL-12 (p40), G-CSF, MCP-1, MIP-1b in the BAL compared to controls. Moreover, there was a trend for lower levels of IL-5, IL-6, IFN- γ and RANTES. Finally, IL-1a was significantly higher compared to water controls. On the contrary, oseltamivir significantly decreased levels of IL-12 (p40) and G-CSF, and there was a trend for lower levels of MCP-1 compared to water controls. Conversely, the levels of IL-10, MIP-1a and RANTES were found to be significantly higher as compared to water controls and there was a trend for higher levels of IFN- γ and KC (IL-8).

DISCUSSION

Widespread use of a single antiviral during infection puts tremendous selective pressure on the influenza virus to decrease drug efficacy by mutating drug targets (Boltz et al. 2010). Although the side-effects of ribavirin include risk of hemolytic anemia and teratogenicity, it

remains a useful antiviral with preclinical influenza relevance, including *in vivo* efficacy against the pandemic influenza A/Mexico/4108/2009 strain, improving survival and reducing lung pathology in mice treated immediately post-infection (Rowe et al. 2010). For this reason, we compared the efficacy of ribavirin and oseltamivir, as determined by survival and lung injury reduction, against the mouse-adapted A/California/04/2009 H1N1 pandemic influenza strain. Our report supports and extends those of Rowe et al (Rowe et al. 2010). We have shown that infection with A/California/04/2009 H1N1 pandemic influenza is lethal, accompanied by high lung viral loads extensive perturbation of the alveolar-capillary barrier and high increases in inflammatory cytokines and chemokines.

We also have shown that both antivirals reduced mortality and viral load, and that ribavirin prevented the disease related weight-loss. From our data it is concluded that ribavirin exhibits a sustained effect on weight status even after stopping the administration of the drug while in the case of oseltamivir there was an acute, but recoverable, reduction in the weight immediately after stopping the drug treatment.

Protein levels in the BAL were decreased compared to infected controls, which suggests protection from alveolar-capillary barrier injury and improved tissue oxygenation. Conversely, both ribavirin and oseltamivir had no effect in reducing the infiltration of leucocytes in response to infection although ribavirin resulted in reduced lymphocytic infiltration as shown by differential cell counts in the BAL. Interestingly, ribavirin was more potent in reducing the levels of cytokines and chemokines in the BAL of infected mice than oseltamivir, suggestive of distinct ways of differential immunomodulation of the infection given that there was no mortality observed in the two groups. This effects on the levels of cytokines released by both the lung and inflammatory cells, may ultimately govern weight loss.

Increased levels of protein in the BAL are a hallmark of acute respiratory distress syndrome (ARDS) (Ware and Matthay 2000). In patients with the pandemic H1N1 infection there were numerous incidents of Acute Lung Injury and Adult Respiratory Distress Syndrome (Hubmayr and Farmer 2010). Increased levels of proteins in the BAL inactivate surfactant function and compromise gas exchange (Matalon et al. 1987). In previous studies, decreased levels of protein in the BAL have been associated with decreased mortality in various models of acute lung injury (Matalon et al. 1987; Yadav et al. 2010; Zarogiannis et al. 2011). Our data show for the first time that administration of ribavirin or oseltamivir mitigate injury to the permeability of the lung blood gas barrier to plasma proteins, even in the presence of significant viral loads. Additional studies are needed to elucidate the molecular mechanisms involved.

Although the pivotal marker of antiviral efficacy in animal models is post-challenge survival, significant and measurable cellular and humoral events during infection and viral replication contribute heavily to H1N1 pandemic influenza virus pathology. These events include edema and interstitial and intra-alveolar inflammatory cell infiltrate compared with seasonal H1N1 virus (Itoh et al. 2009). Our data show that antiviral treatment had little effect on the number of inflammatory cell infiltration in infected animals. However, in the ribavirin group we observed a significant reduction in the lymphocytic infiltration and we observed significant

changes in cytokine levels within the lungs. This finding is in good agreement with previous studies that reported ribavirin as a potent inhibitor of lymphocyte proliferation in vitro (Heagy et al. 1991). In contrast to our BALF cell counts data in the study of Rowe and coworkers (Rowe et al. 2010), lung histology after ribavirin treatment suggested that at day 6 post-infection low levels of perivascular inflammation and cell exudation were clearly observed compared with mock-treated infected groups. However, these findings are not directly comparable to ours since the drug administration scheme was different, using half our dose, for more days (7 vs. 5 in our case) and by intra-peritoneal route of administration. Therefore, the pharmacokinetic differences may account for their reduction in cell exudates. Recently, Sgarbanti and coworkers (Sgarbanti et al. 2011) showed that inflammatory cells are detected in the lungs of surviving mice not receiving treatment even 40 days after the infection and that the lung histology at 40 days is similar to the one at 6 days post-infection. Although we did not look at such time-point, it is possible that the reduced cytokine/chemokine production that we observed can lead to the accelerated resolution of the inflammation following flu infection.

Ribavirin and oseltamivir exert potent antiviral effects but at have distinct effects on the regulation of the cytokine/chemokine production in the lung. Namely, in the ribavirin group it was obvious that there are inflammatory cytokines and chemokines produced but in much lower level overall as compared to animals that did not receive treatment. The anti-inflammatory cytokine IL-10, a key anti-inflammatory cytokine, is 4-fold lower in these animals compared to water treated ones and therefore, the inflammatory response appeared reduced. Taken together survival, weight loss and cell counts, ribavirin has an anti-inflammatory effect in the lung when administered in influenza infection. This, probably, has to do with decreased activation of leucocytes in the site of infection. Indeed, there is evidence that ribavirin has an inhibitory effect on macrophage induced cytokine production as well as an inhibitory effect on Th2 cytokine production (Ning et al. 1998). Contrary to ribavirin, in the oseltamivir group we observed few differences in cytokines/chemokines profiles compared to control mice. There were increases mainly in the chemokines measured compared to control mice and notably there was a 4-fold increase in IL-10 levels, providing evidence for a Th2 response.

CONCLUSION

In conclusion we found that ribavirin is equally effective with oseltamivir in improving survival, reducing lung viral load and BAL protein content therefore decreasing lung injury resulting from H1N1 infection. Moreover, ribavirin was more effective in preventing weight loss that is an important clinical component of flu infection. Both ribavirin and oseltamivir had no effect in reducing the amount of total inflammatory cells infiltrating the lung but there was a significant reduction of lymphocytes in the lungs with ribavirin administration that could account for the differences in the pattern of cytokines in the BAL. From our data it is suggested that ribavirin administration can be an effective therapeutic alternative for oseltamivir resistant pandemic H1N1 infection with favorable immunosuppressing effects.

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Figure 0001

Figure 0002

Figure 1.

(A). Ribavirin and oseltamivir decrease mortality of BALB/c mice infected with mouse adapted A/California/04/09 H1N1 influenza virus. Mice were infected with mouse adapted A/California/04/2009 H1N1 influenza virus ($10^{5.35}$ EID₅₀) and 14-day survival was monitored. We note that in the ribavirin group, the single animal that died on day 1 was excluded from the analysis, as the death was caused by gavage error. Comparisons were made by Kaplan-Meier survival curve and log rank tests for each group (** $p < 0.001$ vs. water). (B). Ribavirin mitigates loss of body weight of BALB/c mice with mouse adapted A/California/04/2009 H1N1. 14-day time course of weight fluctuations of BALB/c mice receiving different treatments after infection with mouse adapted A/California/04/2009 H1N1 influenza virus is shown. Data was analyzed by two-way ANOVA. Symbols are as follows: Black Squares: Water ($n=10$); Open Circles: Ribavirin 80 mg kg⁻¹ ($n=9$); Black diamonds: Oseltamivir 50 mg kg⁻¹ ($n=10$). Values are means. (### $p < 0.001$ vs. Oseltamivir; @@ $p < 0.01$ vs. ribavirin). Comparisons vs. Water are omitted for clarity.

Figure 0003

Figure 0004

Figure 2.

(A). Ribavirin and oseltamivir reduce lung viral loads of mice infected with mouse adapted A/California/04/2009 H1N1. Mice were infected with mouse adapted A/California/04/2009 H1N1 influenza virus ($10^{5.35}$ EID₅₀) and 3 mice from each group were sacrificed in order to determine the lung viral loads at day 6. Values are the logs of viral loads (determined as described in Material and Methods) per microliter of homogenized lung tissues (means $\pm$ S.D). Data was analyzed by unpaired Student's t-test compared to water group. (** $p < 0.01$ vs. Water). Number of animals in all groups, $n=3$. Bars are as follows: 1st: Water; 2nd: Ribavirin 80 mg kg⁻¹; 3rd: Oseltamivir 50 mg kg⁻¹. (B). Ribavirin and oseltamivir reduce protein concentration in BALF of mouse adapted A/California/04/2009 H1N1 infected mice. After sacrifice at day 6, the lungs of mice were lavaged twice with 1 ml of normal saline. BAL samples were spun at 200 X g for 5 min at 4°C to pellet cells. The protein concentration in cell free BAL was measured by the BCA Protein Assay. Values are expressed as means $\pm$ S.D. Data was analyzed by unpaired Student's t-test compared to water group (** $p < 0.01$ vs. Water). Number of animals in all groups, $n=3$. Bars are as follows: 1st: Naïve 2nd: Water; 3rd: Ribavirin 80 mg kg⁻¹; 4th: Oseltamivir 50 mg kg⁻¹.

Table 1

BAL inflammatory cell counts and differentials.

	Naïve (n=3)	Water (n=3)	Ribavirin 80 mg/kg/day (n=3)	Oseltamivir 50 mg/kg/day (n=3)
# Cells $\times 10^5$	4 $\pm$ 1	24 $\pm$ 16 [#]	16 $\pm$ 13	20 $\pm$ 30
Monocytes (%)	87 $\pm$ 3	44 $\pm$ 6 [#]	56 $\pm$ 15	56 $\pm$ 9
Neutrophils (%)	8 $\pm$ 6	23 $\pm$ 10 [#]	32 $\pm$ 16	20 $\pm$ 5
Lymphocytes (%)	5 $\pm$ 4	33 $\pm$ 5 ^{##}	12 $\pm$ 5 [*]	24 $\pm$ 5

Ribavirin resulted in significantly lower percentage of lymphocytes in the BAL. No other differences were found compared with the water group. Number of animals in all groups, n=3. Values are expressed as means $\pm$ S.D. Data was analyzed by unpaired Student's t-test

[#]
 $p < 0.05$

^{##}
 $p < 0.01$; Water vs. Naïve

^{*}
 $p < 0.05$ Ribavirin vs. Water).

Table 2BAL fluid chemokine/cytokine levels (pg ml⁻¹).

	Naïve (n=3)	Water (n=3)	Ribavirin 80 mg/kg/day (n=3)	Oseltamivir 50 mg/kg/day (n=3)
IL 1a	3.55±1.2	<2	12.8±2.3^{***}	<2
IL 1b	8.16±6	45.4±8.2[#]	56.4±22.6	51.3±6.4
IL 5	<2	24.3±20.2[#]	2.3±0.8	26.6±13.9
IL 6	<2	209.2±166.2[#]	45.7±23.1	280.8±116.7
IL 9	23±6.3	36.3±7.2	22.1±1.3[*]	38.8±7.1
IL 10	<2	171.3±33.7[#]	39±47.4[*]	756.1±280.1[*]
IL 12 (p40)	12.7±4	529.1±49.3[#]	131.1±98.3[*]	139.6±50.1[*]
IL 13	72.8±20.2	105.1±29.5	73±8	87.6±19.2
IL 17	<1	<1	1.5±0.4	1.23±0.3
G-CSF	3.2±0.1	870.5±103.5[#]	141.1±21.9[*]	547.6±164.5[*]
GM-CSF	12.1±2.2	6±6.7	10.6±3.8	11.71±4.2
IFN-γ	<6	85.2±79.2[#]	8.7±11.3	650.6±447.8
KC (IL 8)	21.9±7	134.8±8.2[#]	182.1±43	234.4±84.3
MCP 1	<14	2620±1035[#]	383.5±135.1[*]	1013±310.5
MIP 1a	<24	101.8±7.4[#]	130±44.7	178.3±19.5[*]
MIP 1b	<2	93.8±5[#]	58.6±20.9[*]	158.4±19.1^{**}
RANTES	<5	73.8±10.3[#]	51.2±12.1	113.3±9.3[*]
TNF α	<6	<6	6.1±6.9	8.3±2.9

Chemokines and cytokines in cell free BALF measured by Bio-Plex Cytokine Mouse 23-Plex Panel (Bio-Rad, CA, USA). Number of animals in all groups, n=3. Values are expressed as means ± S.D. Data was analyzed by unpaired Student's t-test

^{##} $p < 0.01$; Water vs. Naïve

[#] $p < 0.05$

^{*} $p < 0.05$

^{**} $p < 0.01$

^{***} $p < 0.001$ Ribavirin or Oseltamir vs. Water).