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Ozone exposure in the culture medium inhibits enterovirus 71 virus replication and modulates cytokine production in rhabdomyosarcoma cells

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Abstract

In the present study, the effects of ozone exposure on enterovirus 71 (EV71) replication and related cytokine production were investigated. Rhabdomyosarcoma cells (RD) were exposed to 0.5, 1, 1.5 and 2 ppm ozone or filtered air under different exposure regimens before or after infection for 1 or 2 h. The results revealed that at a proper concentration of ozone, e.g., 1.5 or 2 ppm, ozone exposure restricted virus production, prolonged survival time of cells and modulated cytokine production related to EV71 infection. Upon exposure of non-infected cells to ozone at 1.5 or 2 ppm for 1 h, the production of IL-1β, IL-6 and TNF-α was primed and boosted by the subsequent EV71 infection, generating an inhibitory effect on EV71 replication during the post-infection period of 48 h. While infected cells were exposed to ozone for 2 h at 1.5 or 2 ppm, ozone did not affect cytokine production by RD cells in response to EV71 infection. The data showed that ozone effect on induction of cytokine was only found in uninfected cells. The ozone-induced cytokines produced prior to the onset of EV71 infection generated antiviral effects, which proved beneficial in suppressing the subsequent EV71 infection.

Keywords: Enterovirus 71; Ozone; Inhibition of virus replication; Cytokine production

1. Introduction

Enterovirus 71 (EV71), a positive single-stranded RNA virus, is a member of the enterovirus genus of the family of *Picornaviruses*. EV71 infections have been known to be associated with hand, foot and mouth disease (HFMD), acute flaccid paralysis, myoclonic jerk and severe neurological disease, including brain stem encephalitis and aseptic meningitis (Wang et al., 1999; Chan et al., 2000; Yan et al., 2000). EV71 infection usually occurs through the fecal–oral route, leading to viremia and invasion of the skin and mucosa. However, fatal cases may occur when EV71 attacks the central nervous system as well as other organs and lead to extensive neuronal degeneration, severe CNS inflammation and pulmonary edema (Wang et al., 1999; Liu et al., 2000; Lin et al., 2002a; Wu et al., 2002). Large outbreaks of EV71 infection with severe and fatal cases of brain stem encephalitis have occurred in Malaysia in 1997 (AbuBakar et al., 1999) and Taiwan in 1998 (Chang et al., 1999; Ho et al., 1999) and caused the death of more than 100 children. To date, no approved antiviral agent is available for the treatment of enterovirus 71 infections. Pathogenesis of EV71 with variations in the clinical symptoms may be related to tissue tropism (Ishimaru et al., 1980; Melnick, 1984). EV71 is disseminated from the initial replication sites to the central nervous system and establishes secondary infection (Liang et al., 2004). Thus, restriction of virus production and viral spread, as well as modulation in the production of cytokines may affect the severity of infection.

Ozone is a very strongly reactive oxidant, but its toxicity can be controlled. When properly used, ozone treatment can become an alternative medical approach. Experimental treatment has demonstrated that ozone is effective against a number of enveloped viruses and non-enveloped viruses (Akey and Walton, 1985; Herbold et al., 1989; Carpendale and Freeberg, 1991; Wells et al., 1991; Arimoto et al., 1996; Kim et al., 2003; Shin and Sobsey, 2003) and has profound effects on cellular function. The study on HIV inactivation using ozone indicated that ozone could exert several antiviral effects, including

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disruption of viral particles, reverse transcriptase inactivation and/or a perturbation of the ability of the virus to bind to its receptor on target cells (Wells et al., 1991). Ozone exposure caused damage to the viral RNA of the free poliovirus 1 particles leading to poliovirus inactivation (Roy et al., 1981).

Upon different exposure concentrations/durations, ozone can directly and indirectly affect the cellular functions of a number of cell types. These effects include the formation of free radicals, lipid peroxidation, loss of expression of cell-surface molecules, changes in membrane permeability, activation or inactivation of both surface and non-membrane bound proteins and enzymes (Leikauf et al., 1995; Pearson and Bhalla, 1997; Górnicki and Gutsze, 2000; Koike et al., 2001; Fu et al., 2002), as well as modulation of the production of mediators related to the immune response (Jakab et al., 1995; Jaspers et al., 1997; Koike et al., 2001; Nichols et al., 2001). A study on bronchoalveolar lavage (BAL) cells, which presents the antigen to helper T-cells in the lung, indicated that the number of BAL cells expressing cell-surface molecules associated with antigen presentation in rats could be increased by ozone exposure (Koike et al., 2001). Exposure of the primary human nasal epithelial cells or bronchial epithelial cells to ozone significantly increased the expression of surface ICAM-1 and elevated the amounts of IL-8, GM-CSF, IL-1, IL-6 and TNF-α (Beck et al., 1994; Rusznak et al., 1996; Nichols et al., 2001). Larini and Bocci (2005) reported a significant relationship between cytokine production by peripheral blood mononuclear cells and ozone concentration.

These ozone-induced effects, however, are partly due to a cascade of lipid ozonation products (LOP) (Pryor et al., 1995; Kafoury et al., 1999). The formation of LOP within cell membrane could activate lipid signaling elements phospolipase A_2 (PLA_2) and phospholipase C (PLC) leading to the production and release of pro-inflammatory cytokines. Additionally, ozone can directly react with proteins, with nucleic acids and with some amino acids (Berlett et al., 1996). Ozonation could cause modification of cytoskeletal proteins, resulting in structural changes of membranes and could also cause aberrations of chromosomes, mutational changes of *Escherichia coli* as well as degradation of yeast RNA (Hamelin and Chung, 1974; Shinriki et al., 1981; Uppu et al., 1995; Wróbel et al., 1997; Górnicki and Gutsze, 2000). The properties of ozone reacting with organisms and organics have been validated as a useful disinfectant of drinking water and waste water.

Ozone may damage the virus particles, change the cellular functions, thereby activating the host's immune system by inducing the production of cytokines and stimulating metabolism by improving oxygenation and reducing local inflammation (Carpendale and Freeberg, 1991; Friedman and Stromberg, 1993; Bocci, 1996, 1999). In the case of respiratory syncytial virus (RSV) infection, one study showed that exposure to ozone reduced the involvement of respiratory epithelium in the infectious process and resulted in a less widespread infection (Wolcott et al., 1982). Another set of studies showed that ozone mediated an alteration in the viral antigen distribution that, in turn, led to significantly reduced influenza disease mortality and prolonged the survival time of mice (Jakab and Hmieleski, 1988; Selgrade et al., 1988). These studies primarily indicated that biological effects, such as changes in cellular function and immune response of the host as mediated by ozone, greatly contributed to the observed effects on treatment. Bocci (1998) reported that ozone could inactivate HIV in saline *in vitro*, whereas very high ozone concentration may not inactivate HIV present in blood ex vivo. In viral diseases ozone treatment activates the immune system indirectly via a cascade of messengers (Bocci, 2006).

Our previous work (Lin and Wu, 2006) has demonstrated the potential of ozone for EV71 inactivation. In the present study, we investigated the effects of ozone exposure on EV71 replication in rhabdomyosarcoma cells (RD) and the mechanisms related to immune response, i.e., cytokine production. By applying different exposure regimens, including exposures before or after infection, varying the ozone concentrations and exposure times, the ozone effects on EV71 replication and production of related cytokines were examined.

2. Materials and methods

2.1. Virus

Enterovirus 71 (kindly provided by Dr. MS Ho, IBMS Academic Sinica) was propagated in Vero cells by infection with an MOI (multiplicity of infection) of 1 in medium M199 with 2% fetal bovine serum and incubated at 37 °C in 5% CO_2. When 80% of the cells showed the typical enteroviral cytopathic effect (CPE), the infected cells were subjected to two freeze–thaw cycles for the virus harvest. The cellular debris was removed by centrifugation at 2000 × *g* for 6 min and the resultant supernatant was stored at −70 °C until used.

2.1.1. Ozone exposure

RD cells (rhabdomyosarcoma cells) used in these experiments were routinely maintained in DMEM medium (GIBCO, Invitrogen Corporation) containing 10% fetal bovine serum (FBS). For ozone exposure, 1×10^6 RD cells were seeded in TC dishes (40 mm × 10 mm) and then incubated overnight until they were confluent. Confluent cells with medium were exposed either to ozone at 0.5, 1, 1.5 or 2 ppm (ozone pre-exposed cells), or filtered air (air pre-exposed cells) for 1 h in a previously described exposure chamber (Lin and Wu, 2006). Following the exposure, ozone was removed, and the supernatant was discarded, and the cells were then infected with EV71 at an MOI of 1. The virus was allowed to adsorb for 1 h at 37 °C. Then the cells were washed three times with phosphate-buffered saline (PBS) and incubated at 37 °C, 5% CO_2 in 1 ml of freshly grown medium until the time of sampling. Dishes taken immediately after infection served as the time-zero samples.

For the performance of ozone exposure after infection, confluent cells growing in TC dishes were infected with EV71 at an MOI of 1. When ∼45% of the cells showed CPE (16 h), the whole dish was exposed either to ozone at 0.5, 1, 1.5 or 2 ppm (ozone-exposed infected cells), or filtered air (air-exposed infected cells) for 2 h. Dishes taken immediately after exposure served as time-zero samples. Multiple identical dishes were set up for different exposure regimens. Samples were taken at

the appropriate time for the determination of the viral titer, cell viability and cytokine production.

2.1.2. Cell viability

Cell viability was determined by the MTT assay, which was used to measure the mitochondrial function of living cells with the ability to metabolically reduce 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) to a formazan dye. The MTT test was carried out according to the manufacturer's instructions with minor modifications. Briefly, a 50-μl volume of MTT (Roche) solution was added to each dish containing the test cells. Then the dishes were incubated at 37 °C for 4 h. At the end of the incubation, 500 μl of the solubilization solution (10% SDS in 0.01 M HCl, Roche) was added to each dish and the dishes were allowed to stand overnight at 37 °C. A 100-μl volume of the resulting solution was removed from each dish and the spectrophotometrical absorbance was measured at 570 nm using a microtiter plate reader. Results were expressed as %control.

2.1.3. Viral titration

Virus titration was performed on the controls and the ozone-treated samples. Monolayers of Vero cells in 96-well plates were inoculated with serial 10-fold dilutions of virus and incubated at 37 °C and 5% CO_2. On the fourth day, endpoint titers were determined by the Reed and Muench method (Reed and Muench, 1938) as the reciprocal of the lowest viral dilution, which gave CPE in more than 50% of the cells.

2.1.4. Nitric oxide measurement

The amount of NO produced in the medium was determined by measuring the accumulation of its stable degradation product, nitrite. A 100 μl of sample was mixed with an equal volume of Griess reagent (Sigma) in a 96-well plate. The formation of a chromophore was measured at 540 nm. A range of sodium nitrite dilutions served to generate a standard curve for each assay.

2.1.5. Cytokine measurement

Cytokine levels in the culture medium were measured by using commercial enzyme-linked immunosorbent assay (ELISA) kits (Endogen, Pierce Biotechnology), according to the manufacturer's instructions. The amount of cytokine in each sample was interpolated from duplicate standard curves performed for each assay.

2.1.6. Virus replication in anti-cytokine monoclonal antibody-treated RD cells

RD cells were seeded in TC dishes for overnight until they were confluent. Before ozone exposure, neutralizing anti-human TNF-α, IL-1 and IL-6 monoclonal antibodies (MAbs) (Diaclone, Besancon, France) were added individually or together to the culture dishes at a concentration of 10 μg/ml each. After 1 h incubation, the cultures were exposed to ozone at 2 ppm for 1 h and then were infected with 1 MOI of EV71. Samples were taken at the appropriate time for the determination of the viral titer.

2.1.7. Statistics

All experiments were performed at least three times and standard deviations of the means (S.D.) from all data were determined. Comparisons between ozone-exposed and air-exposed uninfected/infected cells were performed by Student's paired *t*-test. A value of p less than 0.05 (*) or 0.01 (**) was considered significant.

3. Results

3.1. Effect of ozone on RD cells

Healthy monolayers of RD cells (~99% viability) were exposed to various ozone concentrations (0.5, 1, 1.5 and 2 ppm) for 1 or 2 h to examine the susceptibility of RD cells to ozone exposure. The data in Fig. 1A indicate that cells exposed to 2 ppm and below for 1 h did not demonstrate significant cell injury. Viabilities of the cells were above 95% ($p > 0.2$) throughout

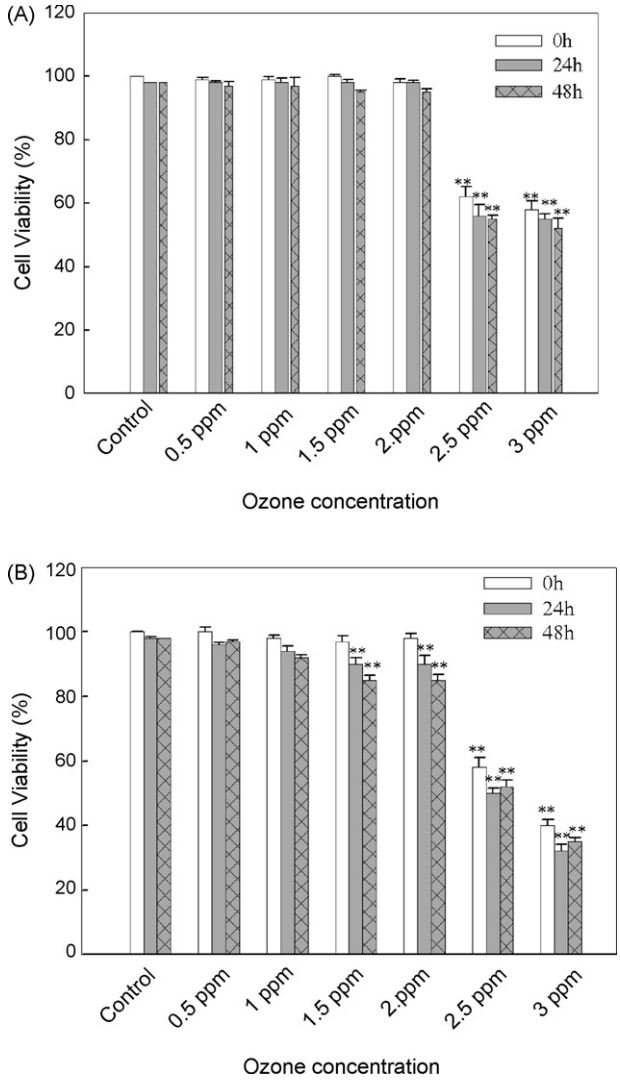

Fig. 1. Viabilities of normal control RD cells after exposure to different ozone concentrations for 1 h (A) or 2 h (B). Data represent the mean ± S.E.M., $n = 4$. **$p < 0.01$ indicates a statistically significant difference between air- and ozone-exposure cells.

the post-exposure period of 48 h. Exposure of cells to 1.5 ppm and above for 2 h caused a significant decrease in cell viability (Fig. 1B). There were ~90 and ~85% of viability ($p < 0.01$) for 1.5 and 2 ppm exposed cells at 24 and 48 h post-exposure, respectively. However, 2.5 and 3 ppm caused over 40 and 50% of cell mortality with 1 and 2 h exposure, respectively.

3.2. Effects of ozone on the susceptibility of RD cells to EV71 infection

To examine the susceptibility of ozone-exposed RD cells to EV71 infection, RD cells were pre-exposed to 0.5, 1, 1.5 and 2 ppm ozone or filtered air control for 1 h. After exposure, the cells were then infected with EV71 at an MOI of 1. Samples were collected immediately (0 h), 24 or 48 h after infection to estimate the cell viability and viral titer. Fig. 2A shows the patterns of virus production in ozone pre-exposed RD cells up to 48 h post-infection. Ozone exposure did not influence the adsorption of EV71 to exposed cells because the viral titers at 0 h of post-infection were comparable for air and all ozone pre-exposed cells. Throughout the 48 h of post-infection period, virus growth in the cells pre-exposed to 1.5 or 2 ppm ozone for 1 h was significantly inhibited; 3–4 log $CCID_{50}$ less infectious EV71 ($p < 0.01$) was produced as compared to air pre-exposed control at 24 and 48 h post-infection (Fig. 2A). The viabilities of these cells were retained at ~50% for 1.5 ppm and ~66% for 2 ppm exposure ($p < 0.01$) at 24 and 48 h post-infection (Fig. 2B). There was no additive effect of ozone on viability loss, which was mainly caused by virus infection, compared to uninfected controls exposed to the same levels of ozone. In contrast, pre-exposure of cells to 0.5 or 1 ppm ozone for 1 h did not significantly affect virus growth; cell viability was decreased with increased virus titer, as observed in air pre-exposed control.

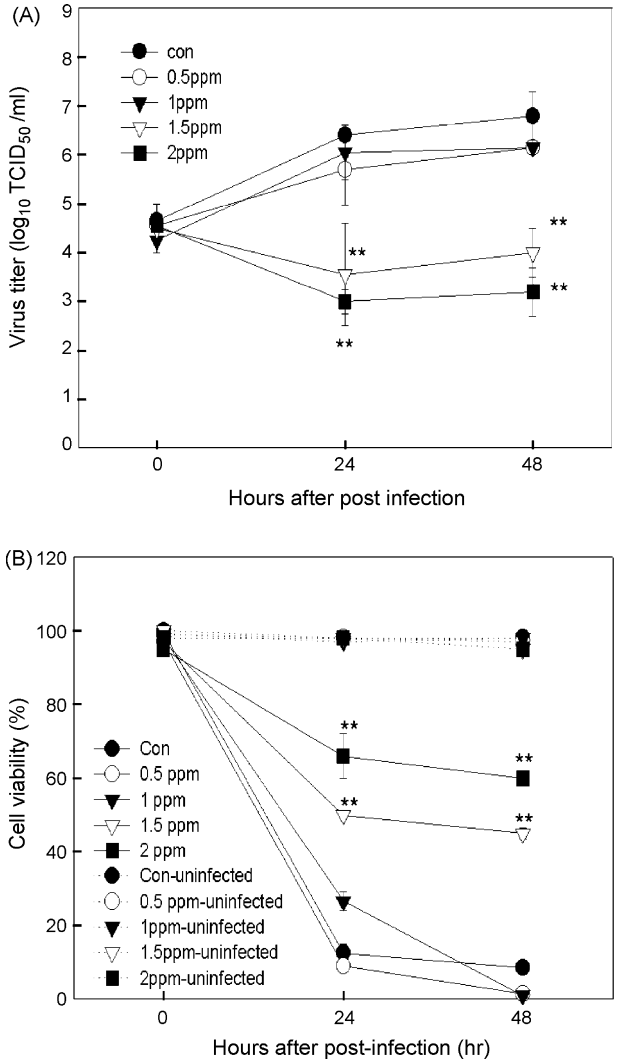

Fig. 2. Virus production (A) and cell viability (B) in normal RD cells exposed to various concentrations of ozone or filtered air for 1 h. After exposure, cells were immediately infected with EV71 (—) or left uninfected (. . .). Virus titer in cultures and viability were determined at 0, 24 and 48 h after infection. Data represent the mean ± S.E.M., $n = 4$. $^{**}p < 0.01$ indicates a statistically significant difference between air- and ozone-exposed cells with infection.

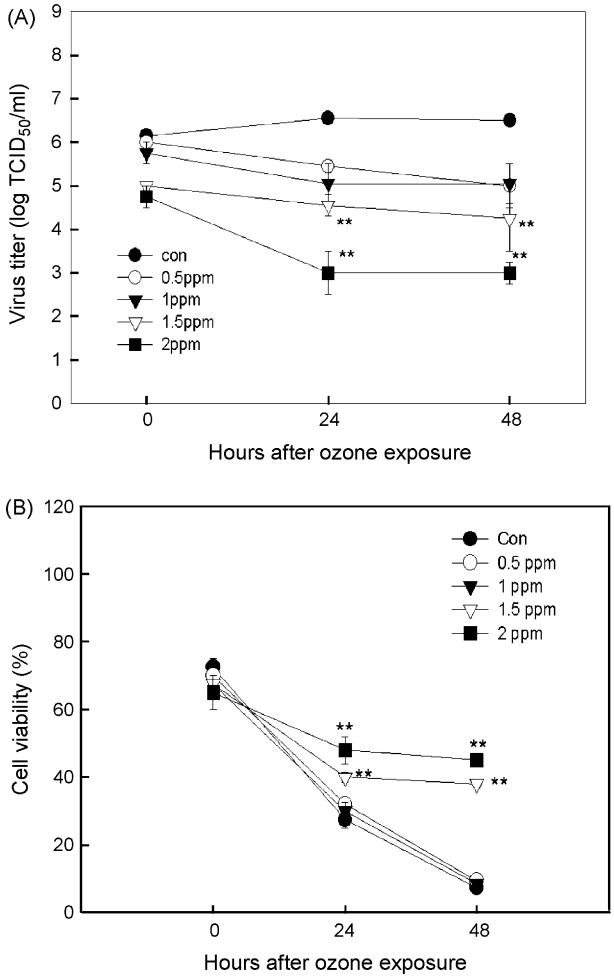

Fig. 3. Virus production (A) and cell viability (B) in infected RD cells exposed to various concentrations of ozone or filtered air for 2 h. Cells were infected with EV71 for 16 h before exposure to ozone. Virus titer in cultures and viability were determined 0, 24 and 48 h after exposure. Data represent the mean ± S.E.M., $n = 4$. $^{**}p < 0.01$ indicates a statistically significant difference between air- and ozone-exposed cells.

3.3. Effect of ozone on RD cells with an established EV71 infection

RD cells with established EV71 infection were exposed to ozone at 0.5, 1, 1.5 and 2 ppm or filtered air for 2 h. Cell viability and viral titer were assayed immediately (0 h), 24 or 48 h after ozone exposure. While the infected cells were exposed for 2 h, EV71 was inactivated in these infected cells (Fig. 3A). The viral titers in 0.5 and 1 ppm ozone-exposed infected cells were slightly reduced by 1 log $CCID_{50}$, while the 1.5 and 2 ppm ozone-exposed infected cells were significantly reduced by 3 and 4 log $CCID_{50}$ ($p < 0.01$), respectively, at 48 h post-exposure. No statistically significant differences in cell viability were noted among the air- and the 0.5 or 1 ppm ozone-exposed infected cells (Fig. 3B). However, 1.5 or 2 ppm exposed infected cells had a 45–40% viability throughout the 48 h post-exposure period.

3.4. NO production in response to ozone exposure

NO production in exposed culture supernatant was also examined. Ozone exposure at 1, 1.5 and 2 ppm, except for 0.5 ppm, induced a significant production of NO ($p < 0.01$) in the normal RD cells immediately (0 h) or 1 or 2 h after exposure (Fig. 4A and B). The trend of increasing amount of NO production was independent of ozone exposure concentration and duration. However, the NO concentrations declined during the subsequent post-exposure period to a level comparable with the air-exposed control. Similar results were obtained in the cases of ozone pre-exposed cells for 1 h followed by EV71 infection (Fig. 4C) and infected cells exposed to ozone for 2 h (Fig. 4D). Increased amounts of NO resulting from ozone exposure were not affected by EV71 infection. There was no ozone exposure concentration- or time-related effect on NO production.

3.5. Cytokine production

EV71 infection can produce cytokines such as interleukin (IL-1), tumor necrosis factor (TNF) and interleukin (IL-6), which are important in the regulation of inflammatory and immune events necessary for the elimination of the virus (Lin et al., 2002b, 2003; Liang et al., 2004). The presence of three cytokines was measured to understand the ozone-induced effect on cytokine production as related to the antiviral state in non-infected/infected cells.

Air pre-exposed uninfected RD cells did not produce detectable levels of cytokines. Immediately after infection with EV71, the cells gradually produced increasing levels of IL-6 (Fig. 5B) and TNF-α (Fig. 5C) during the infection process, but IL-1β was not detectable until 48 h of post-infection (Fig. 5A). Upon exposure of uninfected RD cells to ozone for 1 h, only 1.5 or 2 ppm ozone-induced a significant increase in the release of IL-1β, IL-6 and TNF-α immediately after the exposure (0 h), compared to air pre-exposed uninfected cells. When the unin-

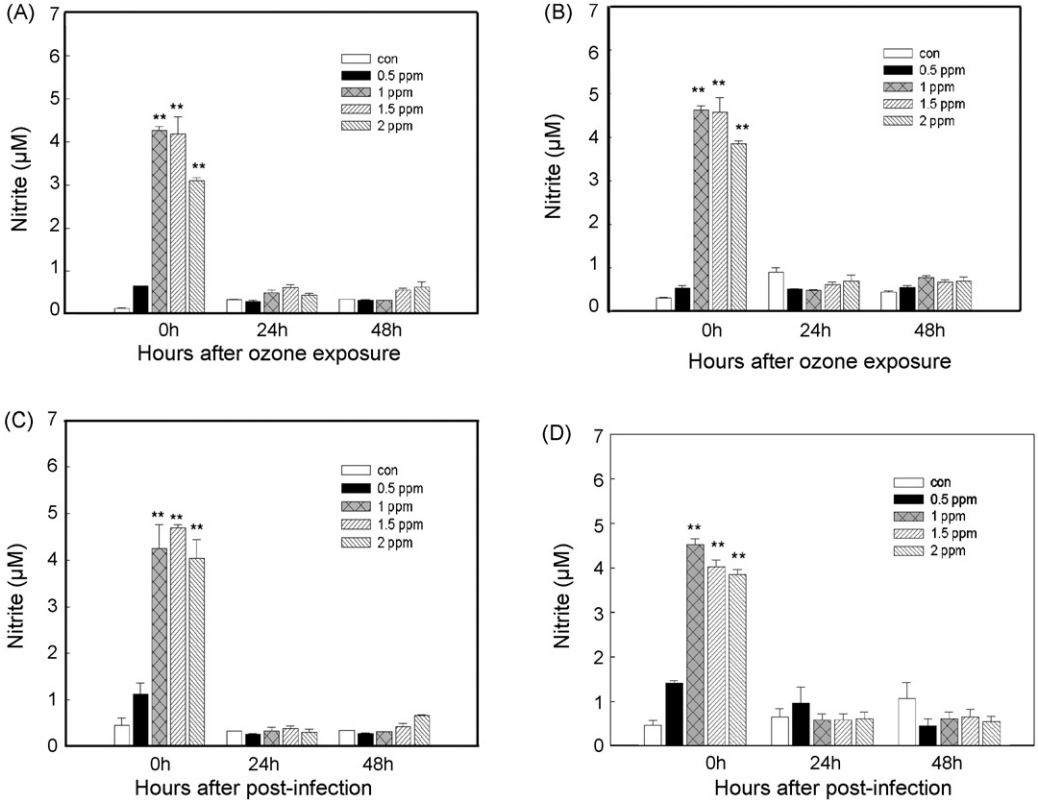

Fig. 4. Effects of ozone exposure on NO production in normal control RD cells exposed to ozone for 1 h (A) or 2 h (B), normal RD cells exposed to ozone before infection for 1 h (C), or infected RD cells exposed to ozone for 2 h (D). NO production was determined at 0, 24 and 48 h after exposure or infection. Data represent the mean ± S.E.M., $n = 4$. $^{**}p < 0.01$ indicates a statistically significant difference between air- and ozone-exposed cells.

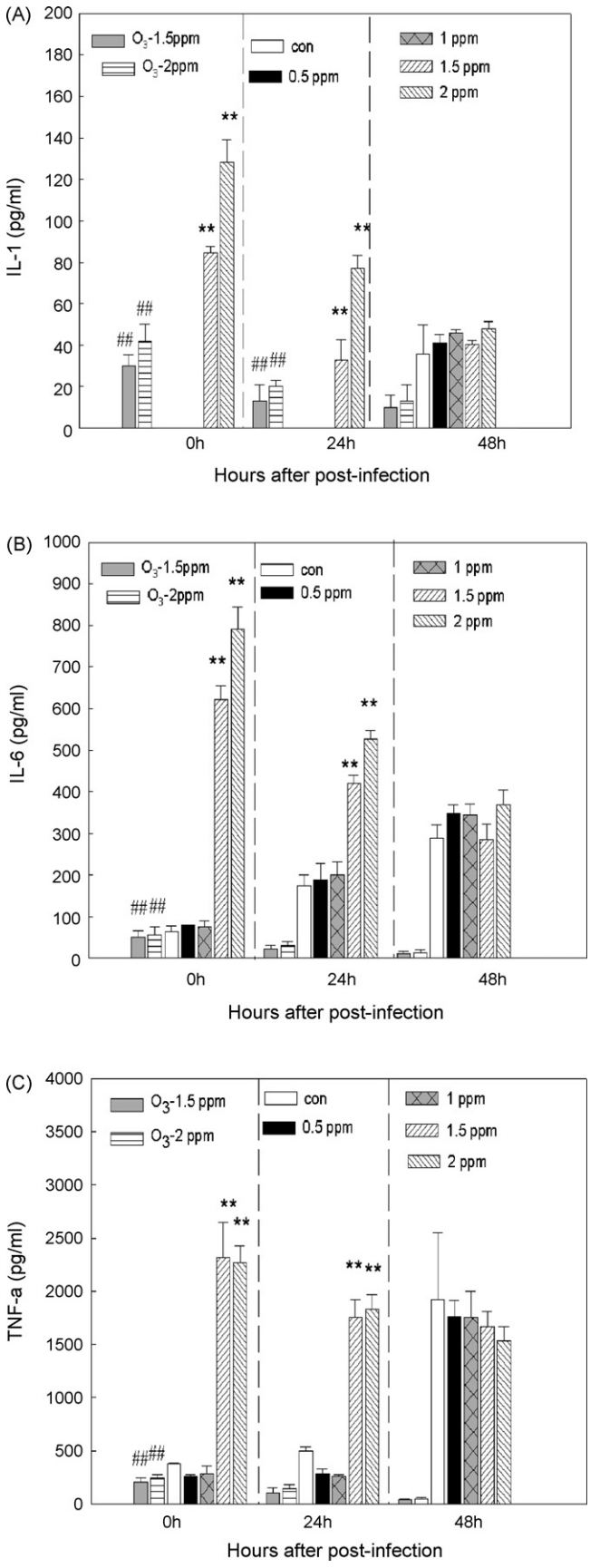

Fig. 5. Effects of ozone exposure on the production of IL-1β (A), IL-6 (B) and TNF-α (C) in normal RD cells exposed to ozone before infection for 1 h. Cytokine production was determined at 0, 24 and 48 h after infection. The cytokine production of ozone-exposed cells left uninfected was also determined (ozone: 1.5 and 2 ppm). Data represent the mean ± S.E.M., $n = 4$. $^{**}p < 0.01$ indicates a statistically significant difference between air pre-exposed and ozone pre-exposure cells infected with EV71 following the exposure. $^{\#\#}p < 0.01$ indicates a statistically significant difference between air pre-exposed and ozone pre-exposed cells left uninfected following the exposure.

fected RD cells were immediately infected with EV71 following the exposure to ozone for 1 h at 1.5 or 2 ppm, the levels of IL-1β, IL-6 and TNF-α were elevated by threefold ($p < 0.01$), five to sixfold ($p < 0.01$) and sevenfold higher ($p < 0.01$) at 0 h post-infection, respectively, as compared to the air pre-exposed cells infected with EV71 following the exposure. In ozone pre-exposed cells, to 1.5 and 2 ppm followed by infection, production of the three cytokines were highest at 0 h, but declined to comparable levels with the air pre-exposed control followed by infection at the late phase of infection (48 h) (Fig. 5). In contrast, there were similar patterns with no statistical difference in the levels of IL-1β, IL-6 and TNF-α in the supernatants of air pre-exposed and ozone pre-exposed cells to 0.5 or 1 ppm after EV71 infection.

The ozone effect on cytokine production was also examined in cells with an established EV71 infection. Fig. 6 shows the production of IL-1β, IL-6 and TNF-α obtained from the cells infected with EV71 for 16 h before exposure to air or ozone. With an established infection, cytokine productions in all ozone-exposed infected cells displayed a similar trend of gradually increasing levels during 48 h of post-exposure period as the air-exposed infected control. Ozone exposure at 1.5 or 2 ppm slightly elevated (not statistically significant) the levels of IL-1β (Fig. 6A) immediately after ozone exposure, as well as IL-6 (Fig. 6B) and TNF-α (Fig. 6C) at 48 h of post-exposure, as compared with the air-exposed infected control. For all air/ozone-exposed infected cases the highest levels reached were at 24 h post-exposure for IL-1β and 48 h post-exposure for IL-6 and TNF-α.

3.6. *Effects of cytokine inhibitors on EV71 replication in ozone-exposed cells*

In order to understand the mechanism on how ozone-induced cytokine production in non-infected cells affects subsequent EV71 infection, we analyzed the effects of IL-1β, IL-6 and TNF-α inhibitors on EV71 replication in RD cells. Results revealed that non-infected RD cells pretreated with anti-IL-1β, anti-IL-6 or anti-TNF-α MAb alone followed by ozone exposure did not neutralize the inhibitory effects on subsequent viral replication at 24 and 48 h post-infection (Fig. 7). However, pretreatment of non-infected RD cells by anti-IL-1β, IL-6 and TNF-α MAbs mixed together blocked the inhibitory effects on viral replication. These results suggest that the ozone-induced inhibitory effect on EV71 replication is dependent on pro-inflammatory cytokines produced in uninfected RD cells prior to EV71 infection.

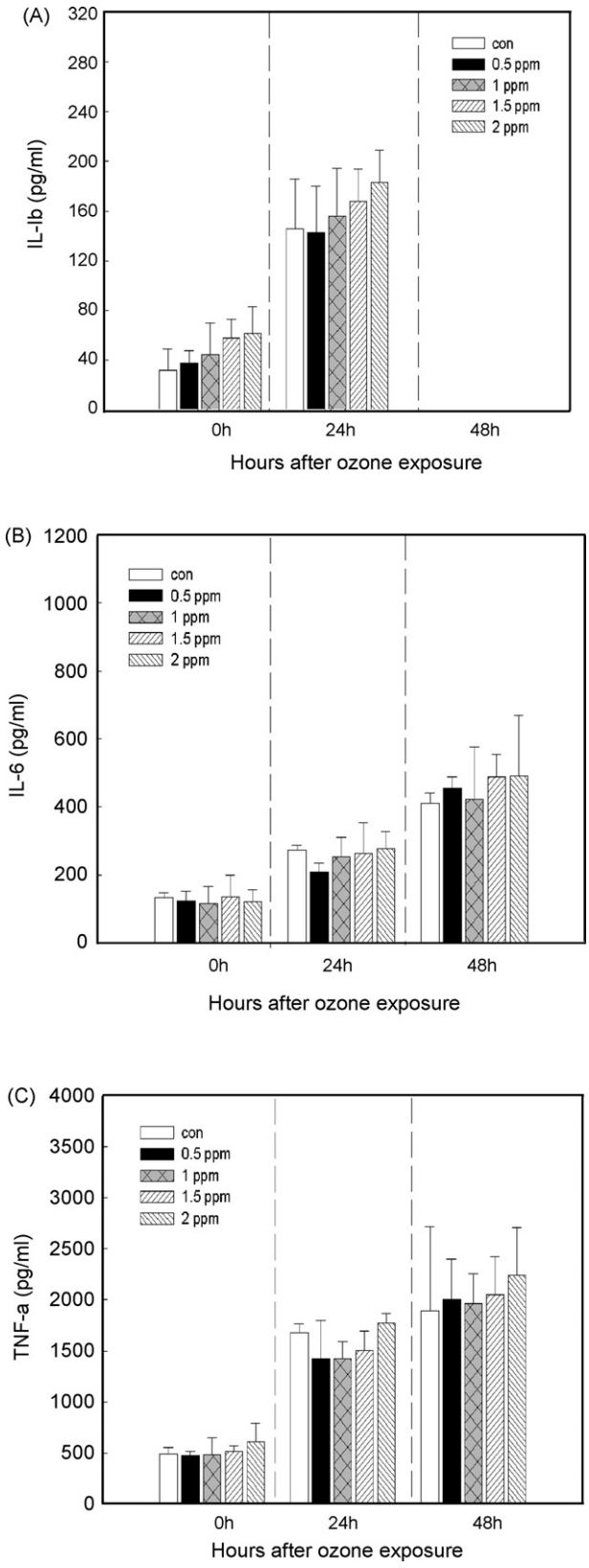

Fig. 6. Effects of ozone exposure on the production of IL-1β (A), IL-6 (B) and TNF-α (C) in infected RD cells exposed to ozone for 2 h. RD cells were infected with EV71 for 16 h before ozone exposure. Cytokine production was determined at 0, 24 and 48 h after exposure. Data represent the mean ± S.E.M., $n = 4$.

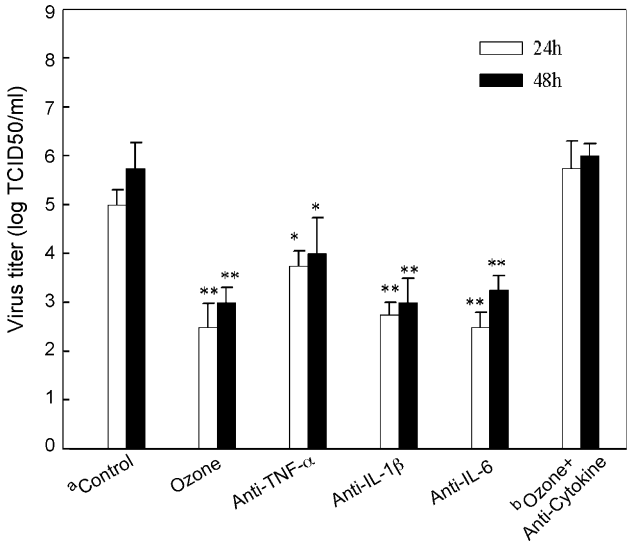

Fig. 7. Virus productions in uninfected RD cells pretreated with anti-IL-1β, anti-IL-6 or anti-TNF-α monoclonal antibodies individually or together, followed by ozone exposure at 2 ppm for 1 h. After exposure, cells were immediately infected with EV71. Virus titer in cultures was determined at 24 and 48 h after infection. Data represent the mean ± S.E.M., $n = 4$. *$p < 0.05$ or **$p < 0.01$ indicates a statistically significant difference between control and ozone-exposed cells. [a]Control: cells were exposed to air without pretreatment with any MAb and then infected with EV71. [b]Ozone + anti-cytokines: cells were pretreated with a mixture of anti-IL-1β, anti-IL-6 and anti-TNF-α MAb and then exposed to ozone, followed by infection.

4. Discussion

The present study demonstrated that ozone exposure affects EV71 growth in RD cells and modulates the production of cytokine in response to virus infection. The elicited effects and mechanisms varied with different exposure regimens, i.e., ozone exposure before or after infection.

The observations on cell viability revealed that ozone exposure at 2 ppm and below for 1 h did not result in cell injury, while exposure to 1.5 or 2 ppm for 2 h caused significant reductions in cell viability. Exposure of RD cells to 2.5 or 3 ppm either 1 or 2 h induced more pronounced mortality of cells. The results indicate that at proper exposure doses and duration, ozone was not cytotoxic to cells. Our study was therefore conducted using a range of ozone concentrations below 2.5 ppm. Exposure of uninfected RD cells to ozone for 1 h within the range eventually did not cause cell injury/mortality and, air and ozone pre-exposed cells initially took up a similar amount of virus as determined in the virus-exposed cell lysates at 1 h after virus inoculation (Fig. 2A), suggesting that the receptors related to EV71 binding were not modified.

The susceptibility of RD cells to EV71 binding was not altered after ozone exposure to 1.5 or 2 ppm. Ozone treatments for 1 h exposure before infection and 2 h exposure after infection at 1.5 and 2 ppm showed a decrease in virus production in these exposed cells. The results show that a proper concentration of ozone, e.g., 1.5 and 2 ppm which were not cytotoxic to cells, can be used to inhibit virus replication, although the mechanisms of inhibiting viral replication are different according to the exposure regimens used.

NO has inhibitory effects on a variety of viral infection (Nathan, 1995; Zaragoza et al., 1997; Lin et al., 1997; Akaike and Maeda, 2000). Exposure to ozone can induce NO in a variety of cells including neuronal, epithelial cells, macrophages and inflammatory cells (Pendino et al., 1995; Inoue et al., 2000; Fakhrzadeh et al., 2002). In our study, ozone effect on increasing NO production was seen on the non-infected and infected cells immediately after exposure at 1, 1.5 or 2 ppm for 1 or 2 h; however, the amount of NO production did not significantly differ among the ozone pre-exposed and ozone-exposed infected cells, indicating that there is no obviously direct relationship between restricted production of EV71 and NO production induced by ozone exposure. The cause may be due to a low level of NO (<10 μM) induced. Exposure of influenza and sendai viruses to biologically relevant concentrations of NO (<20 μM) produces no appreciable reduction of viral growth in cultured cells *in vitro* (Akaike and Maeda, 2000; Akaike et al., 2000). The low levels of endogenous NO may not exert direct antiviral activity but may offer some alterations in the cellular signal transduction pathway involved in host defence (Mannick, 1995).

Ozone can have significant effects on the production and the release of cytokines, such as IFN-γ, IL-8, TNF-α, IL-6 and IL-1β (Arsalane et al., 1995; Rusznak et al., 1996; Jaspers et al., 1997; Becker et al., 1998; Jörres et al., 2000; Park et al., 2004; Johnston et al., 2005), which play important roles in virus elimination (Ramshaw et al., 1997). EV71 infection can induce the production of cytokines of IL-1β, IL-6 and TNF-α (Lin et al., 2002b, 2003). Fibroblast cells in response to viral infection have been shown to secrete cytokines such as IP-10, GM-CSF, TNF-α, IL-6 and IL-1β (Miller and Krangel, 1992; Oppenheim and Feldmann, 2001).

Ozone exposure significantly induced cytokine production in normal RD cells. Exposure of uninfected RD cells to 1.5 or 2 ppm ozone for 1 h followed by EV71 infection could result in an additionally enhanced effect on the production of IL-1β, IL-6 and TNF-α at 0 h post-infection. Exposure of BEAS-2B cells to ozone followed by RSV infection also resulted in an additive effect on cytokine production (Becker et al., 1998). The increased production of cytokines was succeeded by a decrease in virus production (Fig. 5 and Fig. 2A) at 24 and 48 h post-infection. Pretreatment of uninfected RD cells with a mixture of anti-IL-1β, anti-IL-6 and anti-TNF-α MAbs neutralized the inhibitory effects of ozone exposure on viral replication. The reduction in virus replication was not due to direct reactivity of ozone with virus particles (Kafoury et al., 1999). It is therefore proposed that ozone-induced cytokine production, thus contributing to the inhibition of EV71 replication and viral spread. Interestingly, the inhibition of viral replication generated from the cytokine did not require the presence of three cytokines IL-1β, IL-6 and TNF-α together. In the absence of one of them, the antiviral effect could be still seen (Fig. 7).

Cytokines play central roles in the host response to viral infections and in the immunopathology associated with virus-induced disease. Enterovirus infection has been shown to reduce the secretion of cytokines such as IL-6 and to suppress cell sensitivity to TNF-α by decreasing the TNF receptor on the surface of the infected cells (Dodd et al., 2001; Neznanov et al., 2001). Induction of apoptosis to eliminate infected cells in early stage of infection process may be important to inhibit the propagation and spread of virus. However, EV71 infection induces high levels of pro-inflammatory cytokines and apoptosis at the late phases of infection resulting in virus-induced pathogenesis (Li et al., 2002; Lin et al., 2002b).

According to our findings, the ozone effect on induction of cytokine production was only demonstrated in uninfected cells. Thus, ozone exposure prior to infection appears to be important in the induction of cytokine effect on resistance to EV71 replication/infection. Ozone exposure at 1.5 or 2 ppm for 1 h before the onset of the infection process stimulated cytokine production in RD cells and triggered the antiviral activity of these cytokines, the subsequent EV71 infection then boosted the cytokine production and this amplified the antiviral activity. As a result, viral replication was inhibited. Antiviral activities of pro-inflammatory cytokines have been reported in many studies. In cell culture, TNF can inhibit the replication of various DNA or RNA viruses by interfering with the viral life cycle (Czarniecki, 1993; Ramshaw et al., 1997; Herbein and O'Brien, 2000). Macrophages pretreated with TNF-α resulted in a delayed and reduced production of HIV DNA long terminal repeat (LTR) reverse transcripts which involve the early events of the viral life cycle (Herbein et al., 1996). Seo and Webster (2002) reported that TNF-α-pretreated cell line produced significant endogenous TNF-α levels after infection with influenza virus and the endogenous TNF-α exerted strong antiviral activity against the virus. IL-6 has been reported to have both pro- and anti-inflammatory effects (Tilg et al., 1997; Jones et al., 1999).

Our finding that exposure of uninfected RD cells to ozone at 1.5 or 2 ppm for 1 h did not affect the ability of RD cells to absorb EV71, indicates that antiviral activity of cytokine induced from ozone probably did not involve virus–cell binding. Although there was a reduction on cell viability in the early period of post-infection (0–24 h, Fig. 2B), the inhibition of EV71 replication was not due to a cytotoxic effect of ozone on RD cells because at the exposure concentrations and duration used ozone was not cytotoxic to RD cells (Fig. 1). Antiviral activity of cytokines can be performed by induction of apoptosis/cytotoxicity, interfering with viral entry, and inhibition of synthesis of viral mRNA and viral structural proteins (Herbein and O'Brien, 2000; Khaiboullina et al., 2000; Seo and Webster, 2002). The use of a mixture of anti-IL-1β, anti-IL-6 and anti-TNF-α MAbs that block cytokine binding to its receptor and block cytokine-induced cytotoxicity, restored virus replication and spread, suggesting that antiviral activities of these pro-inflammatory cytokines probably involve the induction of apoptosis/cytotoxicity of infected cells in early phase of infection as a protective way against EV71 replication. The molecular mechanisms by which IL-1β, IL-6, or/and TNF-α inhibit EV71 replication in an early stage of infection, however, need further study.

On the contrary, ozone-exposed infected cells produced similar patterns of cytokine production to the ones in the air-exposed infected control (Fig. 6). Ozone exposure on the cells with an established infection did not affect cytokine production in response to infection during the infectious process. The increases

in the levels of cytokines during 48 h post-exposure were considered to be virus-dependent cytokine expression and were not related to the measured decrease in viral titer (Fig. 6 and Fig. 3A) because comparable levels of IL-1β, IL-6 and TNF-α were found in air-exposed infected control cells with higher virus production. It is therefore speculated that the significant reduction of the viral titer in the ozone-exposed infected cells at 1.5 or 2 ppm for 2 h was independent of cytokine production.

The reaction of ozone with membrane proteins can cause membrane injury to cultured cells, such as modification of proteins, loss of expression of cell-surface molecules and changes in membrane permeability (Leikauf et al., 1995; Górnicki and Gutsze, 2000; Fu et al., 2002). When infected cells were exposed to ozone at 1.5 or 2 ppm for 2 h, cell viability of 45–40% was retained with a non-elevated virus titer at 24 and 48 h post-exposure. It is proposed that the reduction in virus titer and cell viability in the early phase of post-exposure (0–24 h) was partly due to direct inactivation of extracellular virus by ozone and partly due to the disruption of cell membrane leading to cell death, followed by inactivation of the intracellular virus. By ozone contact, inactivation of virus could be caused by viral particle disruption and/or a perturbation of the ability of the virus to bind to its receptor on target cells (Roy et al., 1981; Wells et al., 1991). Our previous study has also demonstrated the effects of ozone on extracellular and intracellular EV71 inactivation (Lin and Wu, 2006). Cells surviving from ozone exposure in some way had a limitation to virus replication and spread that involve changes in membrane structure related to the release of EV71 by ozone.

In conclusion, our finding suggests that with an appropriate concentration, ozone reduced virus production and modulated cytokine responses in relation to EV71 infection. Importantly, ozone exposure to uninfected cells could stimulate cytokine production, which may then help suppressing virus replication upon EV71 infection. This information could potentially be useful in exploring agents for inhibition of viral replication and virus-induced immunopathology.

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