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Protection from H1N1 Influenza Virus Infections in Mice by Supplementation with Selenium: A Comparison with Selenium-Deficient Mice

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Abstract The present paper describes protective effects of supplemental selenium in mice infected with influenza virus. The effects of supplemental selenium on serum selenium levels, mortality, lung virus titers, and cytokine titers were investigated in mice inoculated intranasally with suspensions of influenza virus. Whereas the mortality of the virus-infected Se-deficient mice was 75%, along with a marked reduction in body weight, lower levels of TNF-α and IFN-γ and lower serum selenium concentrations, the mortality of mice maintained on feed containing 0.5 mg Se/kg in the form of sodium selenite was 25%.There were no significantly differences, however, in viral titer between the Se-adequate and the selenium-supplemented groups. The data indicate that selenium supplementation may provide a feasible approach to improving the immune response to viral infections, such as lethal influenza infection.

Keywords Influenza virus · Selenium · Mortality · Viral titer · TNF-α · IFN-γ

Introduction

Several studies have shown that selenium deficiency enhances the susceptibility to infections including respiratory virus infections [1–3]. This increase in susceptibility is believed to result from an impaired host immune response due to a deficient diet.

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Furthermore, selenium deficiencies of the host can also affect viral pathogens themselves resulting in viral species that are more virulent than the parent species [4, 5]. Barnard DL reported that the prophylactic use of ZnAL42 is effective against avian influenza H5N1 or H1N1 virus infection in mice [6].

The pandemic 2009 (H1N1) is spreading to numerous countries and causing many human deaths. By August 13, 2009 the World Health Organization (WHO) has announced as many as 182,166 confirmed cases and a death toll of 1,799 with a mortality rate of 0.99%. In Mainland China alone, the Centers for Disease Control reported 2,861 cases by August 19, 2009. The influenza virus shows high genetic variability, resulting in the rapid emergence of pathogens resistant to antiviral drugs pathogens. It is suggested that supplementation with selenium might provide prophylactic protection against influenza infection. Therefore, we developed in vivo models of Se-deficient, Se-adequate, and Selenium-supplemented mice and determined the effects of supplementation with selenium on serum selenium levels, mortality, lung virus titers, and influenza-induced cytokine production.

Materials and Methods

Animals

Kunming strain mice weighing 20–22 g were maintained at room temperature under alternating natural light/dark photoperiods, and had access to standard laboratory food and fresh water ad libitum. This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals. Care was taken to minimize discomfort, distress, and pain to the animals.

All the chows had the recommended nutrients differing only in the selenium levels (Table 1).

The mice (n=120) were randomly assigned to continue receiving Se-adequate chow (0.2 mg Se/kg in the form of sodium selenite pentahydrate), Se-deficient chow (0 mg Se/kg in the form of sodium selenite pentahydrate), and Selenium-supplemented chow (0.3, 0.4, and 0.5 mg Se/kg in the form of sodium selenite pentahydrate) for 8 weeks, respectively.

Influenza Virus Infection

Influenza A/NWS/33 (H1N1) was obtained from the Chinese Academy of Sciences. Virus pools were prepared by infecting confluent monolayers of Madin-Darby canine kidney epithelial cells (MDCK) cells, incubating them at 37°C in 5% CO_2, and harvesting the cells at 3-5 days when viral cytopathic effect was 90-100%. The medium for the virus-infected cells contained MEM, 0.18% $NaHCO_3$, 20 μg of trypsin per mL, 2 μg of EDTA per mL,

Table 1 Composition of the Diets Used in the Experiments (g/kg)

Ingredients	Se-adequate chow	Se-deficient chow	Se-supplemented chow
Casein	200	200	200
Corn starch	500	500	500
Maltodextrin	150	150	150
Corn oil	50	50	50
Se	0.0002	0	0.0003, 0.0004, 0.0005

and 50 μg of gentamicin per mL. All virus stocks were placed in ampoules and frozen at −80°C before use.

Mice (5 weeks old) were inoculated intranasally with 50 μL of viral suspension (2×10^5 PFU/mouse). A number of mice (n=12) were observed for 21 days to monitor body weight change and mortality. In other groups of mice, blood, and the lung were individually collected at 3 and 5 days after virus inoculation ($n = 4 - 6$ each).

Determination of Lung Virus Titers

Each mouse lung was homogenized and varying dilutions were assayed in triplicate for infectious virus in MDCK cells as described previously [7–9]. Briefly, after incubation at 37°C for 24 h, 0.002% L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma) was added, followed by a 72-h incubation. Chicken red blood cells were prepared at 1% in phosphate-buffered solution (PBS) and added to cultures. Virus titers were then calculated based on the hemagglutination pattern and reported as the 50% tissue culture infectious dose (TCID50).

Determination of Cytokines

Tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) in blood were assayed by the ELISA technique. Each sample was diluted tenfold with PBS, and then 50 μL of the dilution were incubated in a 96-well formatted flexible plate at 37°C for 1 h. After three washings with PBS, uncoupled binding sites in the wells were blocked with 10% skim milk in PBS. After three washings with PBS containing 0.05% Tween 20, the plate was incubated at 37°C for 1 h in the presence of rabbit antimouse/rat TNF-α or rabbit antimouse IFN-γ antibodies (Shanghai yueyan Biological Technology Co., Ltd, China). The plate was then developed using peroxidase-labeled goat antirabbit IgG (Asbio Technology, Inc., China) and peroxidase substrate [10].

Determination of Serum Selenium Levels

Plasma samples were obtained after centrifugation of blood in a microhematocrit centrifuge and were stored at −22°C until analysis. The plasma levels of selenium were measured by means of an atomic absorption spectrometer AAS-3200 (Shanghai, China) with a limit of sensitivity of 64 nM (5 ng/mL) according to the method introduced by Andrea P [4]. Undetectable concentrations were assigned a value of 5 ng/mL [5].

Statistical Analyses

Results are expressed as means±standard error of the mean. Increases in survivor numbers were evaluated using x^2 analysis with Yates' correction. Differences in others were compared with control values using the t test. The significant level of 5% (p<0.05) was used as the minimum acceptable probability for the difference between the means.

Results and Discussion

The ongoing spread of the human swine-origin H1N1 influenza virus meets the WHO criteria for a pandemic [11]. Although vaccines are an effective method of preventing

infection, their efficacy in protecting against a possible new pandemic influenza virus strain depends very much on the quality of vaccine [12]. It is suggested that nutritional supplementation in vitamins or trace elements might provide prophylactic protection against influenza infection including respiratory virus infections [13–16]. Selenium is an essential micronutrient in the diet of humans and other mammals. This trace element seems to be important for mounting immune responses to viral infections, whereas deficiencies in selenium have been demonstrated to result in more severe viral infections, including HIV and Coxsackie virus [17–21].

Our data in this study indicate that selenium supplementation would protect mice from lethal influenza infection. Se-supplemented mice demonstrated a dose-dependent increase in survival (Table 2) and a reduction in loss of body weight, an important indicator of the severity of infection (Fig. 1). Se-deficient mice showed high mortality (75%) with a marked reduction in body weight. In Se-supplemented (0.4 and 0.5 mg/kg) mice, although the loss in body weight was also found, the mortality (25%) was significantly lower than that of the Se-deficient group.

To examine whether or not selenium supplementation could suppress the virus loads in mice, virus titers in the lung were determined at 3 and 5 days of virus inoculation. The lung influenza viral titer was higher in Se-deficient animals. However, there were no differences in viral titer between the Se-adequate and the Se-supplemented groups. These results suggested that selenium did not show direct interaction with virus particles. Therefore, the protective effect of selenium observed in the mouse model was suggested to be involved in host mediated mechanism(s) such as the stimulating effect of host immune function. Thus, in the next experiments, the effects of selenium supplementation were determined on the levels of TNF-α and IFN-γ (Fig. 2).

TNF-α and IFN-γ are well known to possess an antiviral effect against influenza virus infection. In particular, TNF-α has been reported to exert an anti-influenza virus effect [22]. Thus, the effect of selenium supplementation on the stimulation of TNF-α and IFN-γ production could contribute to their anti-influenza virus activity in animals. Indeed, the levels of cytokines including TNF-α and IFN-γ were increased in Se-supplemented mice (Fig. 3). At 3 days post-infection, there was no difference in cytokine levels between the Se-deficient and the Se-supplemented groups (Fig. 3a, b). At 5 days post-infection, however, both TNF-α and IFN-γ were increased in the blood samples of Se-supplemented mice, whereas less increase was observed in Se-deficient and Se-adequate mice (Fig. 3c, d). Therefore, the protective effect of selenium supplementation

Table 2 Analysis of Survival in Se-supplemented Mice after Influenza Infection

Different groups	No. of survivors/total no.	% Survivors
Se-deficient (0 mg/kg)	3/12	25
Se-adequate (0.2 mg/kg)	5/12*	41
Se-supplemented (0.3 mg/kg)	6/12*	50
Se-supplemented (0.4 mg/kg)	9/12**	75
Se-supplemented (0.5 mg/kg)	9/12**	75

n=12 mice per group at baseline

* $P<0.05$ compared to Se-deficient animals in the same experiment

** $P<0.01$ compared to Se-deficient animals in the same experiment

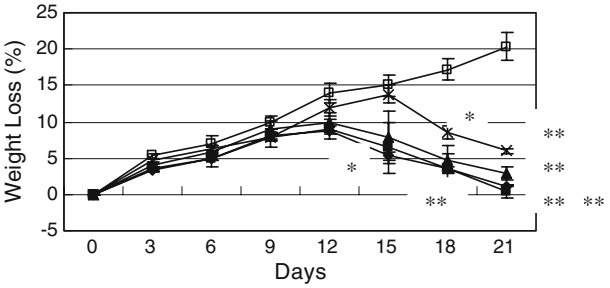

Fig. 1 Analysis of weight loss and recovery in Se-supplemented mice after influenza infection. *Blank square* Se-adequate group, *multiplication section* Se-deficient group, *filled triangle* Se-supplemented group 0.3 mg Se/kg, *filled square* Se-supplemented group 0.4 mg Se/kg, *filled diamond* Se-supplemented group 0.5 mg Se/kg. $n=12$ mice per group at baseline. Values are means±SEM, $^{*}P<0.05$, $^{**}P<0.01$ compared to Se-deficient animals

on lethal influenza virus infection might be due at least in part to the enhancement of TNF-α and IFN-γ production.

Several studies have shown that low serum selenium levels were associated with low percentage of NK cells [23]. In addition, deprivation of serum selenium in HIV infection is associated with increased levels of markers of disease progression and inflammatory response [24]. Table 3 shows the effects of selenium supplementation on serum selenium concentrations. Se-deficient mice had mean values for serum selenium (0.062 μg/mL). These values were significantly lower than those of Se-adequate and Se-supplemented mice (0.148 μg/mL, 0.159 μg/mL, 0.170 μg/mL, and 0.178 μg/mL, respectively). It is speculated that the effect of selenium supplementation on serum selenium concentrations may modulate the effect of viral or other infections and contribute to its anti-influenza virus activity in animals.

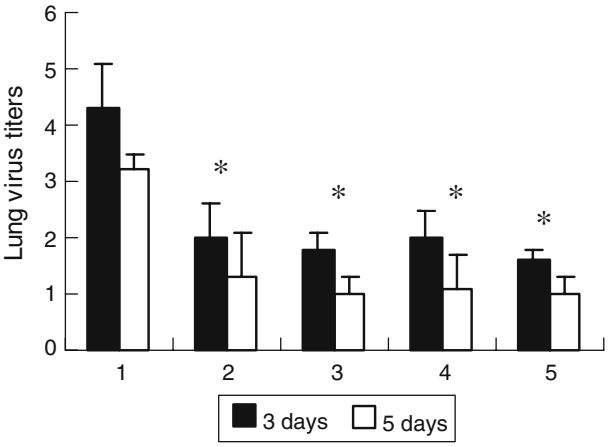

Fig. 2 Lung virus titers in mice after influenza infection. Values represent means±SEM expressed as TCID50, log10. *1* Se-adequate group, *2* Se-deficient group, *3* Se-supplemented group 0.3 mg Se/kg, *4* Se-supplemented group 0.4 mg Se/kg, *5* Se-supplemented group 0.5 mg Se/kg. $^{*}P<0.05$, compared to Se-deficient animals

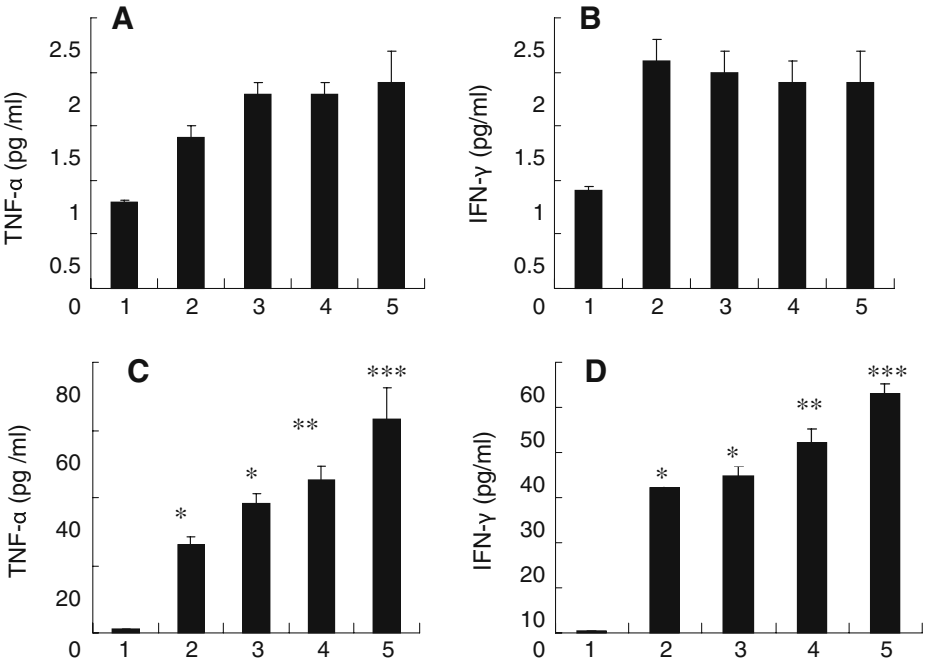

Fig. 3 Effects of Se-supplemented on TNF-α and IFN-γ levels in mice infected with influenza virus. *1* Se-adequate group, *2* Se-deficient group, *3* Se-supplemented group 0.3 mg Se/kg, *4* Se-supplemented group 0.4 mg Se/kg, *5* Se-supplemented group 0.5 mg Se/kg. Blood were collected from mice at 3 (**a**, **c**) and 5 days (**b, d**) after virus infection. Values represent means±SEM. *$P<0.05$, compared to Se-deficient animals. **$P<0.01$, compared to Se-deficient animals. ***$P<0.05$, compared to Se-deficient animals

Conclusions

The results presented here further our understanding of the importance of selenium supplementation in the response to viral disease. Se-deficient mice had an altered immune response to influenza virus infection. Lower serum selenium in Se-deficient mice

Table 3 Serum Se Concentrations (μg/mL) of Mice Fed Diets with Different Dietary Concentrations and Sources of Se

Different groups	*n*	Serum Se concentrations	SEM
Se-deficient (0 mg/kg)	3	0.062	0.001
Se-adequate (0.2 mg/kg)	5	0.148*	0.006
Se-supplemented (0.3 mg/kg)	6	0.159*	0.009
Se-supplemented (0.4 mg/kg)	9	0.170**	0.007
Se-supplemented (0.5 mg/kg)	9	0.178**	0.009

Blood were collected from mice at 21 days after virus infection

*$P<0.05$, compared to Se-deficient animals

**$P<0.01$, compared to Se-deficient animals

contributed to less production of TNF-α and IFN-γ and to lower survival rates. Our data in this study indicate that selenium supplementation may constitute a feasible approach to improving the immune response to viral infections, such as lethal influenza infection.

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