Vitamin E Supplementation Decreases Lung Virus Titeres in Mice Infected with Influenza

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Effects of vitamin E (E) supplementation on influenza infection were examined in young and old C57BL/6NIA mice fed 30 or 500 ppm of E for 6 weeks and subsequently infected with influenza A/Port Chalmers/1/73 (H3N2). Old mice fed 30 ppm of E had significantly higher lung virus titers on days 2 and 7 after infection than young mice fed 30 ppm of E. Titers on all 3 days were significantly lower in old mice fed 500 ppm of E than in those fed 30 ppm. Significant effects of E on lung virus titers in young mice were observed on only day 5, but E caused more reduction of virus titers in old than in young mice (25-fold vs. 15-fold). An age-associated decline in NK cell activity was restored by 500 ppm of E in old but not young mice. Pulmonary cytotoxic T lymphocyte activity on day 7 was not affected by age or E. These experiments demonstrate that high doses of E significantly enhance influenza viral clearance in aged mice but only modestly affect young mice.

Infections, particularly those affecting the respiratory system, rank among the leading causes of death in older adults [1]. Like humans, aged mice have more severe disease with an influenza infection [2], and most of our knowledge of the pathogenesis of and the host defenses against influenza was first established in mice and later confirmed in humans [2]. Many factors contribute to recovery from influenza infection. The most important is cellular immunity, in particular antinfluenza cytotoxic T lymphocyte (CTL) activity. In aged mice, lower CTL activity correlates with prolonged duration of disease [2]. Other immune functions that appear to aid in recovery from influenza include NK cell activity, high titers of neutralizing serum antibody, and, possibly, mucosal IgA [2].

Influenza infection in mice causes a decrease in lung and liver levels of the antioxidant nutrients [3]. One of the biologic changes associated with aging is a decrease in antioxidant defense status, which results in increased free radical formation and lipid peroxidation. Increased lipid peroxidation, including that of prostaglandin E2 (PGE2) production, has been implicated in the age-associated dysregulation of cytokine production and decrease in lymphocyte proliferation and CTL and NK cell activity [4]. Influenza infection is associated with the release of a variety of cytokines and eicosanoids in mice [5].

Vitamin E supplementation increases delayed-type hypersensitivity skin response, T cell proliferation, and interleukin (IL)-2 production [6, 7] and decreases PGE2 production and plasma lipid peroxide levels. Vitamin E supplementation also prevents antigen-induced decreased NK cell activity in old mice [8]. Therefore, in the present study we determined whether vitamin E supplementation would reduce lung virus titers in young and aged mice infected with influenza virus, and if so, whether this would be due to its effect on CTL, NK cell activity, or antibody titer.

Materials and Methods

Animals. Specific pathogen–free male young (4 months old) and old (22 months old) C57BL/6NIA mice were obtained from
NIA colonies at Charles River Laboratories (Kingston, NY). Mice were housed singly in microisolator cages at a constant temperature (23°C) with a 12-h light-dark cycle and fed ad libitum with a semipurified diet supplemented with either 30 (an adequate level of vitamin E) or 500 ppm of dl-α-tocopherol acetate (vitamin E) for 6 weeks [6]. We previously showed that there was no difference in food intake or weight gain between mice fed 30 or 500 ppm of vitamin E [6].

After the 6-week dietary period, mice were infected with Influenza A/Port Chalmers/1/73 (H3N2) virus according to the method of Bender et al. [9]. Mice were then sacrificed via CO₂ asphyxiation 0, 2, 5, or 7 days after infection.

**Tissue preparation.** Noses and lungs were removed and processed as previously described [10]. Livers were perfused with 3 mL of ice-cold sterile PBS, removed, immediately wrapped in foil, and placed in liquid nitrogen.

**NK cell and CTL activity.** Lung cells were enriched for T cells by adding the cell suspensions to nylon wool columns and incubating them at 37°C for 1 h, eluting nonadherent cells from the columns, and washing once in supplemented Iscove’s medium. Enriched T cells were resuspended in 10-mL of medium, counted using trypan blue dye exclusion, and adjusted to 10⁶/mL. These cells were subsequently used for either NK cell (2 days after infection) or CTL (7 days after infection) assays. Stein-Streilein et al. [11] reported optimal NK cell activity 2 days after infection. Our preliminary experiment using samples from days 5, 6, 7, 8, 9, and 11 after infection showed optimal CTL activity on day 7 after infection.

NK cell activity against Yac-1 target cells was assessed as previously described [8]. The CTL assays were performed as previously described [9]. Target cells (LB27) were sensitized with influenza A/Port Chalmers/1/73 (H3N2) virus (10⁷.⁵ EID₅₀) or influenza B virus and incubated at 37°C, 5% CO₂ for 1.25 h with occasional mixing. The final effector-to-target ratio for this assay was 20:1.

**Virustiters.** Virus titers for lung and nose samples were measured as previously described [9, 10].

**Serum antibody.** Sera were frozen, and a hemagglutination inhibition assay was performed [12].

**Statistical analysis.** Data were analyzed using the SYSTAT statistical package (SYSTAT, Evanston, IL) by a 2 × 2 factorial two-tailed analysis of variance with individual differences analyzed by single degree of freedom comparison using the Fisher’s least significant difference procedure and are reported as mean ± SE. Significance was set at P < .05.

### Results

**Virustiters.** Following influenza challenge of healthy young mice, the virus reached a peak pulmonary titer of ~10³–10⁴ TCID₅₀ on days 2–5 after inoculation and was cleared from the lungs by day 7 (figure 1). As previously reported [2, 9, 10], aging had a significant impact on viral clearance, with significantly higher virus titers in old mice than in young mice on days 2 and 7 after infection (P < .01). Vitamin E supplementation decreased virus titers of young mice only on day 5 (figure 1) but significantly decreased the virus titers of old mice on all days. Even on day 5, vitamin E had a more dramatic effect on viral clearance in aged than in young mice. Titers were 25-fold lower in old vs. 15-fold lower in young mice supplemented with 500 ppm of vitamin E than in old and young mice fed the control diet (30 ppm of vitamin E). Vitamin E had no impact on nasal virus titer (data not shown).

**Immune response.** Primary pulmonary CTL activity was measured against influenza H3N2-sensitized LB27 cells. In contrast to results of our previous studies with aged female mice [2], no age-associated decline in pulmonary CTL activity was observed in male C57BL/6NIA mice. In addition, vitamin E supplementation did not affect CTL activity in either young or old animals (50.1 ± 8.9, n = 7; 48.8 ± 9.7, n = 8; 47.1 ± 8.9, n = 4; 42.6 ± 8.9, n = 6 in young mice consuming 30 ppm of vitamin E, young mice consuming 500 ppm of vitamin E, old mice consuming 30 ppm of vitamin E, and old mice consuming 500 ppm of vitamin E, respectively). The fact that the CTLs exhibited very low lysis of influenza B–sensitized LB27 cells (11%–16%) demonstrated that we were measuring specific anti-influenza A CTL activity.
The NK cell activity was significantly lower ($P < .01$) in old animals fed 30 ppm of vitamin E (6.34 ± 6.03, $n = 7$). Vitamin E supplementation had no significant effect on NK activity in young animals (nonsignificant decrease; 14.51 ± 4.92, $n = 8$ in young mice fed 500 ppm of vitamin E vs. 28.25 ± 5.4, $n = 7$ in young mice fed 30 ppm of vitamin E); however, it tended to enhance NK cell activity in old mice, such that NK cell activity in the old animals fed 500 ppm of vitamin E (18.18 ± 5.3, $n = 6$) was not significantly different from that of young mice fed 30 ppm of vitamin E (28.25 ± 5.4, $n = 7$).

Serum antiinfluenza hemagglutinin (HA) inhibition titers were measured 2, 5, and 7 days after infection. Aged animals had significantly lower serum titers ($P < .001$, data not shown) than young mice. Vitamin E–supplemented animals tended to have higher serum titers than control mice (differences not statistically significant, data not shown).

**Discussion**

The most important finding of this study is that old mice supplemented with 500 ppm of vitamin E have significantly lower virus titers following influenza challenge than old animals fed normal dietary levels of vitamin E (30 ppm). This effect of vitamin E does not appear to be mediated through enhanced CTL activity, T cells that are important in viral clearance. On the other hand, preservation of NK cell activity and antioxidant status may contribute to the observed effect.

To our knowledge, this is the first time that a higher than adequate intake of a nutrient has demonstrated a beneficial effect on influenza in animals. The only other dietary intervention that has demonstrated any protective effect against influenza infection in old rodents is caloric restriction (40% reduction), an intervention that is unlikely to be practical for most humans [13]. The biologic effect of food restriction in the aged is due, at least in part, to its reduction of oxidative stress [14]. In this experiment, vitamin E was more effective in reducing virus titers in old mice that show higher oxidative stress than in young mice.

The mechanism for the effect of vitamin E on reducing influenza virus titers could not be determined from our experiments. We originally hypothesized that the effect of vitamin E would be mediated through enhanced CTL activity, but no such effect was observed. Nevertheless, this does not rule out the possibility that vitamin E might influence pulmonary CTL activity in vivo. We eliminated macrophages from the CTL preparation, and thus, the main source of prostaglandins, which have been shown to inhibit CTL activity. We previously showed that cyclooxygenase and 5-lipoxygenase products increase with age and that supplementation with vitamin E decreases their production [6, 7]. Therefore, it is feasible that elimination of macrophages in our CTL culture system eliminated vitamin E–induced differences in CTL activity. In this experiment, only one effector-to-target ratio was used in the CTL assay. It is possible that vitamin E could be effective in enhancing CTL activity at lower effector-to-target ratios.

Vitamin E supplementation prevented an age-associated decrease in NK cell activity of old mice, which suggests that the effect of vitamin E in old mice may be mediated through the preservation of NK cell activity. NK cell activity decreases in old mice, and NK cells may play a role in influenza infection [11].

Influenza virus infection has been shown to increase production of cytokines, including IL-1, IL-4, IL-6, interferon-γ, and tumor necrosis factor, which may contribute to the pathogenesis of the disease [3]. Treatment with antioxidant enzymes was shown to decrease pathogenicity of influenza virus [15]. Nuclear transcription factor κB (NF-κB) activation is needed for expression of mRNA for several of these cytokines. A recent report indicated that influenza virus HA activation of NF-κB might be involved in the influenza-induced increase in cytokine production. However, HA-induced activation of NF-κB was inhibited by the antioxidant dithiothreitol [16]. Other studies have shown that vitamin E supplementation of old subjects inhibited IL-6 production and prevented exercise-induced increases in IL-1 and tumor necrosis factor production by peripheral blood mononuclear cells [17]. Thus, the beneficial effect of vitamin E might be mediated through modulation of cytokines involved in the pathogenesis of influenza virus.

Clearly, antioxidant status is important for protection against influenza. This may explain the greater protective effect of vitamin E in old rather than young mice. Since one of the biologic changes associated with aging is increased accumulation of free radicals, leading to increased oxidative stress, old animals exposed to virally induced oxidative stress may require higher levels of antioxidant nutrients to control viral replication to the same level as in young animals. This is supported by our preliminary observation (Han et al., unpublished data) that lungs from influenza-infected mice have significantly higher H2O2 production and lower vitamin E levels than noninfected mice and that lungs from old animals have higher zymosan-stimulated H2O2 production. Furthermore, vitamin E supplementation decreased H2O2 production in old animals.

In conclusion, our results show that supplementation with 500 ppm of vitamin E decreases lung influenza virus titers on days 2, 5, and 7 after infection in old mice. This observation could be of great clinical relevance to the elderly, who have higher morbidity and mortality due to influenza. Further research to delineate the mechanism of the effect of vitamin E and determine the effectiveness of vitamin E supplementation in older humans is warranted.

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**References**