A Dietary Supplement Improves Outcome in an Experimental Influenza Model in Old Mice

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ABSTRACT: Twenty-month-old Swiss mice were allocated into three groups: (A) control; (B) infected group; and (C) infected but treated with 5 mg of the phytocompound MMT. Mice were infected intranasally with 30 μL of 75 HA viral units. MMT markedly blunted the nasal signs of virus infection and the febrile response. Formazan-positive cells, lung and plasma lipoperoxides, and TNF-α in lung tissue increased during viral infection, but improvement was seen in the MMT-treated group ($P < 0.05$). MMT also normalized SOD, catalase activities, and ascorbic acid and determined a significant decrease of lung but not nasal viral titer, although nasal inflammatory infiltrate dropped significantly. MMT has potential clinical applications with and has an excellent safety profile even in old animals.

KEYWORDS: influenza model; old mice; TNF-alpha; RANTES; phytocompound

INTRODUCTION

The available options for the prevention and treatment of influenza and flu syndromes still have several limitations, especially in the elderly. Although inflammatory and oxidative phenomena seem to cause and perpetuate tissue injury in this condition, there are only scanty reports in the literature on the efficacy of antioxidants as therapeutic agents during an influenza virus infection. Thus, in the present study we tested a natural compound, MMT (Kyotsu Jigyo Inc., Tokyo, Japan), containing a number of herbal ingredients with known antioxidant and anti-inflammatory properties.[1–5]

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MATERIALS AND METHODS

Experimental Design

Twenty-month-old Swiss mice (25–30 g) were housed and fed in a pathogen-free environment and were allocated into three groups: (A) healthy control; (B) infected group; and (C) MMT-treated infected group. Under ether anesthesia, mice were infected intranasally with 30 μL of 75 HA units of virus (A/Hong Kong/8/68 propagated in the allantoic fluid). The control group was given the same quantity of sterile allantoic fluid. The MMT group received 15 mg of a dietary supplement orally divided into three doses daily (MMT: ginger, *Strobilanthes cusia*, *Panax pseudoginseng*, *Eucommia ulmoides*, *Momordicae grosvenori*, licorice root, *Allium fistulosum*) from the day of inoculation till the day they were killed. Clinical signs of infection were observed throughout the study period, while total inflammatory cell counts in nasal washings, as well as virus titers in lung homogenates, were determined for 8 days.

Bronchoalveolar Lavage Fluid (BALF) Collection and Lung Tissue Storage

On the fourth day, BALF was collected and centrifuged to obtain a cell pellet. Quantitative analysis using real-time polymerase chain reaction (PCR) was also performed to determine regulated activation of normal T cell expressed and secreted (RANTES) and PCR products were quantified by densitometric analysis. Soon afterward, the rats were killed by cervical dislocation and the lungs were obtained after perfusion with cold PBS.

Assessment of Superoxide Radical Production

Cell suspensions were mixed with 50 μL of 0.2% nitroblue-tetrazolium (NBT) and after incubation were counterstained with Leishman's stain. Formazan-positive cells (F+) were blindly scored and all results in triplicate were expressed as the number of F+ cells per 200 cells.

Cellular and Biochemical Determination

1. Whole lungs were homogenized and supernatants were analyzed for ascorbate by high-performance liquid chromatography/electrochemical detection. TNF-α activity from tissue culture supernatants was assessed by quantitating cytolytic activity against the L929 target cell line. Plasma antioxidant status was studied by plasma malonyldialdehyde determination.

Toxicological Studies

A separate group of mice received once-daily doses of MMT (5–30 g/kg of body weight per day) or sterile water via oral gavage for 14 consecutive days, and signs of toxicity in 10 predefined tissues (brain, lungs, heart, liver, spleen, pancreas, stomach, small and large intestine, kidneys, bone marrow) were evaluated.

Statistical Analysis

Statistical comparisons between groups were made by "paired *t* test" and *P* values lower than 0.05 were accepted as statistically significant.

RESULTS

Infected mice developed a febrile response beginning about 12 h after infection and lasted approximately 48 h. Oral administration of MMT markedly blunted the nasal signs of viral infection as well as the decrease in motor activity and caused a 46% reduction in the area-under-the-curve measurement for the increase in body temperature over baseline values ($P < 0.05$ vs. untreated infected animals). Formazan-positive cells were increased by 80% during viral infection but decreased to 44% after supplementation ($P < 0.01$). Plasma SOD, catalase activities, and ascorbic acid were significantly decreased in the infected groups (9.8 ± 1.2, 23.7 ± 2.3, and 52.2 ± 8.2 vs. 14.2 ± 1.1, 29.7 ± 1.9, and 79.7 ± 7.9, respectively, $P < 0.05$); however, supplemented groups showed activities similar to those of the control group ($P < 0.05$). The levels of MDA in the lung extract and at the plasma level as well as of TNF-α in lung tissue significantly increased during viral infection when compared with the control group (>twofold and >sevenfold, respectively; $P < 0.01$ vs. healthy control). However, the supplementation with MMT enabled a significant reduction of all these parameters (nearly 50%, $P < 0.05$). Virus titer in the nasal wash rapidly increased 36 h after the virus inoculation and reached a maximum of 50% of tissue culture infective doses ($TCID_{50}$) on the third day of observation after infection (3.88 ± 0.33 $\log_{10}$ $TCID_{50}{}^{s}$/ml). MMT-treated animals did not show any significant difference. On the contrary, the total count of inflammatory cells in the nasal washing showed an early significant decrease that was maintained throughout the study period ($P < 0.05$). MMT was also found to yield a significant 37% reduction in either the expression or the production of RANTES in mice BAL-collected epithelial cells ($P < 0.05$ vs. untreated mice). Although viral activity in the lung homogenate was not as high as in the nasal washing, it significantly decreased in MMT-supplemented animals ($P < 0.05$ vs. untreated group, FIG. 1).

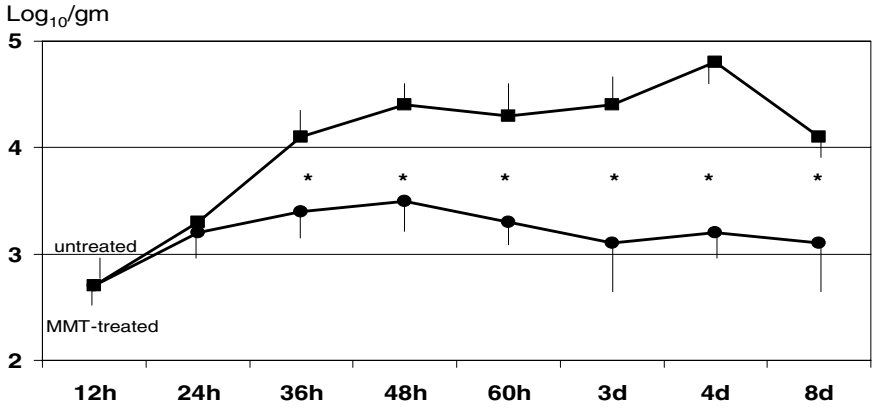

FIGURE 1. Lung virus titer in influenza model: effect of MMT (mean ± SD). $^*P < 0.05$ vs. untreated mice

MMT Toxicology Tests

MMT, at dosages up to 30 g/kg/day (i.e., over 50-fold higher than needed to protect mice against the effects of influenza virus infection) was not associated with any drug-related toxicity.

DISCUSSION

Elderly patients who often have an immunocompromised status while taking multidrug prescriptions are known to be at a higher risk of complicated viral infections. Accumulation in the lung of neutrophils and macrophages could play a role in the development of the disease. Indeed, early studies have reported that influenza infection impairs the levels of endogenous concentration of vitamin E and glutathione[6] and an increase in the levels of xanthine oxidase.[7] Accordingly, in our study a significant increase of superoxide radical production together with a decrease of SOD and catalase occurred in the BAL pellets. This process was paralleled by an increase of MDA in the bloodstream and, in particular, in the lung. Lung homogenates also showed a decrease in ascorbic acid and an increase in TNF-α, which is known to trigger the recruitment of leukocytes by the expression of IL-8 and intracellular adhesion molecules. All the above phenomena were significantly prevented, either partially or totally, when the phytocompound MMT was added to the diet. Although the precise mechanism of each single component cannot be clarified at the moment, many of its ingredients have either anti-inflammatory or antioxidant properties.[1–5] Such properties, together with the anti-asthmatic effect exerted by the alk(en)ylsulfinothioic acid alk(en)yl-ester component of *Allium fistolosum*[8] might also help in explaining the symptomatic improvement of

infected mice when administered MMT. As a matter of fact, dietary antioxidant deficiency can further impair the function of the lung immune system and vitamin E supplementation has been proven to improve Th1 cytokine production in influenza-infected mice.[9] Interestingly, MMT significantly decreased RANTES levels that are associated with a Th1-related immune response and this might have been the result of the inhibition of phosphorylation of the nuclear transcription NF-κB regulatory molecule IκB-α and the p38 MAP kinase, as recently suggested for one of MMT's ingredients.[10] Finally, while MMT did not alter the high virus titer in the nasal washing, it significantly decreased the viral load in the lung. One can speculate that, due to the lack of any specific antiviral activity, MMT could not interfere with the rapid viral growth into the nasal cavity soon after inoculation, although it decreased the local inflammatory cell recruitment. However, the improved endogenous and anti-inflammatory properties exerted at a systemic and lung level might have played a role in limiting viremia in the lungs. On the basis of the above experiment, it is suggested that a safe natural compound, that is, MMT, has the potential to be applied in clinical practice, while further studies are ongoing to elucidate its mechanism of action in more detail.

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