

Combined Administration of L-Cystine and L-Theanine Enhances Immune Functions and Protects against Influenza Virus Infection in Aged Mice

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ABSTRACT. Cell-mediated and humoral immune responses are attenuated with aging. Intracellular glutathione (GSH) levels also decrease with aging. Previously, we have reported that combined administration of L-cystine and L-theanine enhances antigen-specific IgG production, partly through augmentation of GSH levels and T helper 2-mediated responses in 12-week-old mice. These findings suggest that combined administration of L-cystine and L-theanine to aged mice improves immune responses via increase of GSH synthesis. Here, we examined the effects of combined administration of L-cystine and L-theanine on antigen-specific antibody production and influenza virus infection in aged mice. Combined administration of these amino acids for 14 days before primary immunization significantly enhanced the serum antigen-specific IgM and IgG levels in 24-month-old mice. Furthermore, 13-month-old mice co-treated with these amino acids orally for 10 days had significantly lower lung viral titers than controls at 6 days after influenza virus infection. In addition, this co-treatment also significantly prevented the weight loss associated with infection. Enhancement of anti-influenza-virus IgG antibodies by combined administration of L-cystine and L-theanine was seen 10 days after infection. The significantly elevated serum interleukin-10/interferon- γ ratio and γ -glutamylcysteine synthetase mRNA expression, which is the rate-limiting enzyme of GSH synthesis, in the spleen 3 days after infection may have contributed to the observed beneficial effects. These results suggest that combined administration of L-cystine and L-theanine enhances immune function and GSH synthesis which are compromised with advanced age, and may become a useful strategy in healthy aging.

KEY WORDS: aged mice, antibody production, cystine, influenza virus infection, theanine.

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With aging, the human body's defense capability weakens, including production of antibodies and cytotoxic T lymphocytes (CTL) and cellular immune function [12, 28]. These age-related alterations seem to result from oxidative stress [9]. Diet supplementation with antioxidants has been used to prevent or delay the onset of age-related immune impairment [7]. For example, a diet that contains the antioxidant 2-mercaptoethanol, which reduces cystine to cysteine and increases the intracellular concentration of glutathione (GSH; L- γ -glutamyl-L-cysteinyl-glycine), improves several types of immune response in aged mice [17]. Dietary supplementation with GSH, another antioxidant, has also been shown to increase cell-mediated immunity in aged mice [11]. In addition, GSH levels and the gene expression of γ -glutamylcysteine synthetase (GCS), the rate-limiting enzyme in GSH synthesis, are known to decrease with aging [24]. These results suggest that GSH antioxidant therapy may aid in preventing or delaying the onset of age-related immune impairment.

A recent study we performed indicated that combined administration of L-cystine and L-theanine (γ -glutamylethylamide, a specific amino acid found in green tea) to young mice increases the GSH level in the liver and also enhances the serum antigen-specific IgG antibody level in comparison with those of control mice [22]. Administration of either L-cystine or L-theanine alone has no significant effect. However, combined administration of L-cystine and L-theanine shows a significant effect on the ratio of T helper (Th) 1/Th 2 cytokines in the serum.

The incidence of infectious diseases increases with age and is associated with increased morbidity and mortality. Influenza virus is a globally important respiratory pathogen, which annually causes a high degree of morbidity and mortality. In addition, it has been reported that recovery from influenza infection is primarily mediated by several factors, including anti-influenza CTLs, interferon (IFN)- γ and serum neutralizing antibody [1, 6, 27]. The influenza virus itself is involved in direct generation of reactive oxygen species (ROS), and oxygen stress can have indirect effects on the infectivity of the influenza virus [25, 26]. Furthermore, Hack *et al.* have reported low plasma cystine levels in the elderly and in various diseases, such as human immunodeficiency virus (HIV) infection [15].

Taken together, these reports suggest that combined oral administration of L-cystine and L-theanine increases intracellular GSH synthesis and is effective for augmenting anti-

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body production and the anti-infection response in aged mice. Here, we report that combined administration of L-cystine and L-theanine enhanced antigen-specific anti-dinitrophenyl (DNP) IgM and IgG antibody titers in the sera of 24-month-old mice. Moreover, we found that 13-month-old mice orally co-treated with L-cystine and L-theanine had significantly lower lung viral titers and weight loss than control mice following influenza virus infection. We also investigated the effects of combined administration of L-cystine and L-theanine to 13-month-old mice on the plasma cystine and glutamic acid concentrations, mRNA expression of γ -GCS, the rate-limiting enzyme in GSH synthesis, also known as an antioxidant gene product [31], serum cytokine balance and anti-influenza virus antibody production after infection.

MATERIALS AND METHODS

Animals: Female C3H/HeN mice were purchased from Japan SLC Inc. (Shizuoka, Japan) and used throughout the experiments. The animals were housed under specific pathogen-free conditions, given food and water *ad libitum* and kept under a 12-hr:12-hr light:dark cycle until 13 or 24 months of age. The experimental protocol was approved by the Ethical Commission for Animal Research of Osaka Prefectural Institute of Public Health.

Administration of L-cystine and L-theanine: L-Cystine (Ajinomoto Co., Tokyo, Japan) was suspended in distilled water. L-Theanine (Taiyo Kagaku Co., Ltd., Yokkaichi, Japan) was dissolved in distilled water. L-Cystine and L-theanine suspension [cystine:theanine = 5:2; 16 mg (cystine = 47.6 μ mol, theanine = 26.2 μ mol)/10 ml/kg body weight] was administered orally to the mice every morning, once a day for 10 days (for study of influenza virus infection using 13-month-old mice) or 14 days (for study of DNP-specific antibody production using 24-month-old mice). The control mice were administered distilled water (10 ml/kg body weight) orally during the same period. On the day following the final oral administration, the mice received either antigen immunization or influenza virus infection.

Immunization and evaluation of the DNP-specific IgM and IgG antibodies in serum: To evaluate specific antibody production, we used DNP conjugates as antigens. For analysis of IgM production, the mice received an intravenous inoculation of the antigen DNP-dextran (10 μ g/mouse). For analysis of IgG production, the mice were intraperitoneally immunized with the antigen DNP-keyhole-limpet hemocyanin (KLH) (100 μ g/mouse) in incomplete Freund's adjuvant as the primary immunization. Four weeks after immunization, the mice were intraperitoneally immunized with DNP-KLH (50 μ g/mouse) as a secondary immunization. At 0, 4, 6 and 10 days after antigen immunization, serum samples for IgM antibody determination were collected from the jugular vein with tuberculin syringes. At 2, 4, 7, 11, 14 and 21 days after secondary immunization, serum samples for determination of IgG production were collected using the same methods as used for IgM determination. All serum

samples were stored at -80°C until analysis. The levels of anti-DNP IgM and IgG antibodies were evaluated as described previously [22]. The antibody titer was defined as the dilution rate calculated from the intercept and slope of the measured value of each dilution sample at the optimal optical density. The final titer of IgM or IgG in each sample was calculated as the mean value of duplicate measurements.

Virus and infection: A mouse-adapted strain of influenza virus, A/Osaka/5/70 (H3N2), was used for infection. The influenza virus was isolated from clinical specimens from the 1970 influenza season in Osaka using 10-day-old fertile chicken eggs according to the standard procedure. A/Osaka/5/70 virus was passed 35 times in mice and four times in 10-day-old fertile chicken eggs. The titer of infectious virus was determined by limiting dilution in microcultures of Madin-Darby canine kidney (MDCK) cells and was expressed as the 50% tissue culture infection dose (TCID₅₀). Infectious stocks typically contained approximately $5 \times 10^{5.25}$ TCID₅₀/ml. The mice were infected with the virus by inhalation using a glass nebulizer, which sprayed 5 ml of a 1:10 dilution of allantoic fluid (approximately $2.5 \times 10^{5.25}$ TCID₅₀/cage) over a period of 20 min. The mice were placed in a rotating cage, into which the nebulizer was inserted. The mice were monitored for changes in total body weight for 10 days after infection.

Determination of virus titer in mouse lungs: The lungs of the infected mice were removed under ether anesthesia at 3, 6 and 10 days after infection. The lungs were homogenized using a glass homogenizer in 10% (w/v) PBS, and then centrifuged at $10,000 \times g$ for 30 min. Serial 10-fold dilutions of the supernatant were prepared, and aliquots of 1 ml of each dilution were inoculated into MDCK cells on 24-well plates. The virus titer of each specimen was expressed as the TCID₅₀ of the MDCK cells cultured for 5 days.

Determination of the plasma cystine and glutamic acid concentrations: Plasma samples were collected from the mice at 0, 3, 6 and 10 days after viral infection. The plasma was mixed with a two-fold volume of 5% trichloroacetic acid. Each sample was centrifuged at $10,000 \times g$ for 30 min and filtered to obtain the supernatant for amino acids analysis. The concentrations of amino acids in the plasma were determined using an automated amino acid analyzer (L-8500; Hitachi, Ltd., Tokyo, Japan) in accordance with the manufacturer's instructions.

Quantification of γ -GCS mRNA expression: Spleen samples were isolated from the mice at 0, 3, 6 and 10 days after influenza virus infection. The isolated samples (0.1 mg) were homogenized with 1 ml total RNA extraction buffer (Isogen; Nippon Gene, Toyama, Japan), and cDNA was synthesized using a first-strand cDNA synthesis kit with random primers (Amersham Biosciences, Piscataway, NJ, U.S.A.). Quantitative PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, U.S.A.) and analyzed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) with the following primers: for mouse GCS-heavy catalytic subunit

(HS; GenBank accession no. NM_010295), 5'-TTCAGC-CAGCAGAGAGAGAG-3' (forward) and 5'-TGCAGGTCATCTTGGTCTTC-3' (reverse); for mouse GCS-light regulatory subunit (LS; GenBank accession no. NM_008129), 5'-CTTCGCCTCCGATTGAAGAT-3' (forward) and 5'-GCTGCTCCAACCTGTGTCTTG-3' (reverse); and for mouse 18S ribosomal RNA (rRNA; GenBank accession no. X00686), 5'-GACACGGACAGGATTGACAG-3' (forward) and 5'-TCGCTCCACCAACTAAGAAC-3' (reverse). All primers were designed using free software, Primer3 [33]. The amplification protocol was 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Relative expression was calculated using 18S rRNA as the internal control for each sample. All measurements were performed in triplicate.

Analysis of the serum cytokine concentration: Serum samples were collected from the mice at 0, 3, 6 and 10 days after viral infection. The interleukin (IL)-10, IFN- γ and IL-12 concentrations were analyzed using mouse anti-IL-10, anti-IFN- γ and anti-IL-12 antibody bead kits, respectively, in accordance with the manufacturer's instructions (BioSource International, Camarillo, CA, U.S.A.). The IL-10/IFN- γ ratio was calculated from the determined concentrations of these cytokines.

Evaluation of the influenza-virus-specific IgM and IgG antibodies in serum: The amounts of anti-influenza-virus IgM and IgG in the serum were detected by the same procedure as described above for evaluation of anti-DNP IgM or IgG, except that the inactivated mouse-adapted strain of the influenza A/Osaka/5/70 (H3N2) virus was used to coat the plates at a concentration of 18 $\mu\text{g/ml}$ in coating buffer at 4°C overnight.

Statistical analysis: All statistical analyses were performed using the SigmaStat 3.1 software (Systat Software, Inc., Richmond, CA, U.S.A.). The data concerning the effects of L-cystine and L-theanine combined administration on the lung virus titer and serum cytokine ratio were analyzed by *t* test or Mann-Whitney rank sum test. Data that indicated the effects of combined administration of L-cystine and L-theanine on spleen γ -GCS expression were analyzed by one-way ANOVA followed by the Tukey test for multiple comparisons. Other data were analyzed by two-way ANOVA followed by the Tukey test for multiple comparisons. $P < 0.05$ was considered significant.

RESULTS

Enhancement of antigen-specific IgM antibody production by combined oral administration of L-cystine and L-theanine to aged mice: Previously, we reported that combined oral administration of L-cystine and L-theanine induces significant enhancement of antigen-specific anti-DNP IgG antibody production in 12-week-old BALB/c mice [22]. It has been reported that antigen-specific anti-DNP IgG antibody production in female C3H/HeN mice decreases with age [35]. Therefore, we initially examined whether combined oral administration augmented antigen-

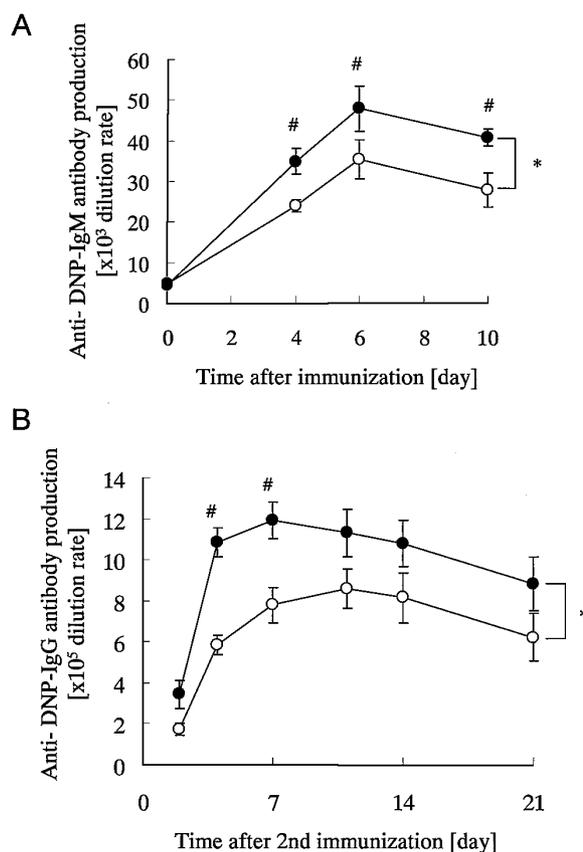


Fig. 1. Effects of combined administration of L-cystine and L-theanine on anti-DNP-IgM and -IgG antibody production. Twenty-four-month-old mice were orally administered distilled water as a control (open circles) or administered a combination of L-cystine and L-theanine at a dose of 16 mg/kg (closed circles) for 14 days followed by immunization. Serum was collected on the indicated days after immunization for IgM (A) or after 2nd immunization for IgG (B), and antigen-specific antibody production was analyzed by ELISA. Each value represents the mean \pm SEM ($n=5$). Differences among the groups were analyzed by two-way ANOVA followed by the Tukey test for multiple comparisons. * $P < 0.05$ compared with the control groups. # $P < 0.05$ compared with the control mice on each day after immunization.

specific anti-DNP IgM antibody production in aged 24-month-old C3H/HeN mice. These mice were orally administered a combination of L-cystine and L-theanine for 14 days and then immunized with DNP-dextran to determine antigen-specific IgM antibody production. As shown in Fig. 1A, a significant increase in serum antigen-specific IgM antibody production was observed in the combined administration group ($F=15.676$, $P < 0.001$). Moreover, combined administration significantly increased IgM production at 4, 6 and 10 days after immunization in comparison with controls, as revealed by a multiple comparison test.

Enhancement of antigen-specific IgG antibody production by combined oral administration of L-cystine and L-

theanine to aged mice: We examined whether the combined administration of L-cystine and L-theanine also augmented antigen-specific anti-DNP IgG antibody production in 24-month-old mice. The anti-DNP IgG response in the 24-month-old mice was reduced to 17% of that in the 3-month-old mice [35]. The mice were orally administered a combination of L-cystine and L-theanine for 14 days and then immunized with DNP-KLH as the primary immunization. On the indicated days after secondary immunization by DNP-KLH, sera were collected, and production of antigen-specific IgG antibody was measured by ELISA. Figure 1B shows that combined administration of L-cystine and L-theanine significantly increased antigen-specific IgG production after secondary immunization, as compared with the controls ($F=31.123$, $P<0.001$). Furthermore, a multiple comparison test revealed that combined administration significantly increased IgG production at 4 and 7 days after the second immunization, as compared with the controls.

Effects of combined oral administration of L-cystine and L-theanine on protection against infection with H3N2 influenza virus: As serum anti-influenza antibody is known to mediate recovery from influenza virus infection [27], we next investigated the effects of combined administration of L-cystine and L-theanine on the course of influenza virus infection. The clinical manifestations (lung viral titers and weight loss) of influenza virus infection were examined. We used 13-month-old C3H/HeN mice in the viral infection studies, as our influenza virus infection was found to be lethal in 24-month-old mice. The mice were orally administered a combination of L-cystine and L-theanine for 10 days and then infected intranasally with H3N2 influenza virus. At 3, 6 and 10 days after infection, the influenza viral titers in the lungs were investigated. As shown in Fig. 2A, although the highest lung viral titer in the control mice was observed at 6 days after infection, co-treatment significantly decreased the lung viral titer. At 10 days after infection, almost all of the infected mice with or without combined administration of L-cystine and L-theanine showed clearance of the virus from the lungs. In addition, the mice were weighed daily as a general measure of disease severity. The body weight before infection (day 0) was 30.5 ± 0.8 g (mean \pm SEM, $n=15$) in the control group and 31.1 ± 1.2 g (mean \pm SEM, $n=15$) in the administration group; the difference between the two groups was not significant. Oral administration of L-cystine and L-theanine for 10 days did not affect the body weight. As shown in Fig. 2B, the body weights of the control and co-treated mice decreased gradually after influenza virus challenge ($F=2.988$, $P=0.002$). However, combined administration of L-cystine and L-theanine significantly prevented the weight loss associated with viral infection, as compared with the controls ($F=5.881$, $P=0.016$).

Effects of combined oral administration of L-cystine and L-theanine on the plasma cystine and glutamic acid concentrations after influenza virus infection: To clarify the mechanism by which combined administration of L-cystine and L-theanine inhibited virus propagation in the lungs, we initially examined the effects of co-treatment on the plasma

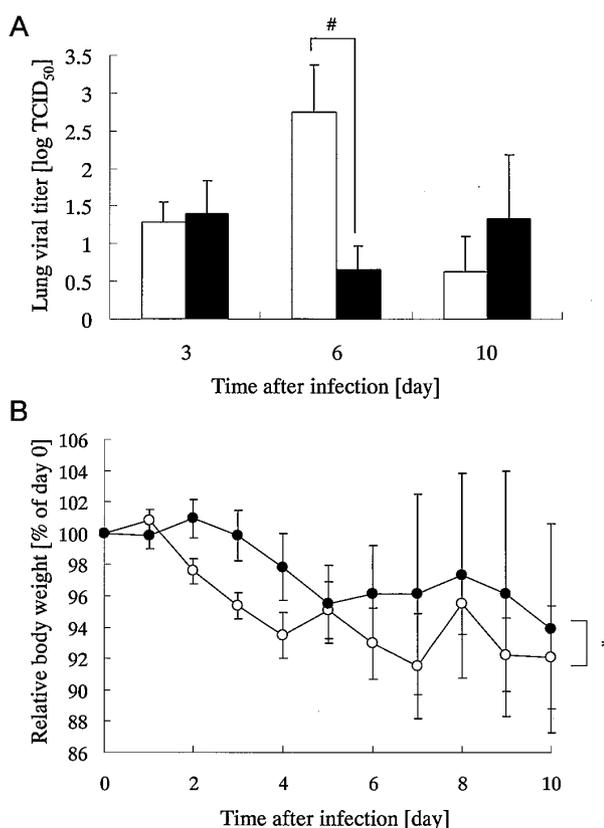


Fig. 2. Effects of combined administration of L-cystine and L-theanine on clearance of the virus in lungs and weight loss after infection with influenza virus. Thirteen-month-old mice were orally administered distilled water as a control (open bars or circles) or administered a combination of L-cystine and L-theanine at a dose of 16 mg/kg (closed bars or circles) for 10 days, and then aerosol influenza virus infection was performed. (A) The lungs were obtained at 3, 6 and 10 days after infection, and the lung viral titers (TCID₅₀) were determined using MDCK cells. Each value represents the mean \pm SEM ($n=4-5$). Differences among the groups were analyzed by *t* test. # $P<0.05$ compared with the control mice on each day after infection. (B) The body weights were determined daily from 0 to 10 days after infection. Each value (% of day 0) represents the mean \pm SEM ($n=4-15$). Differences among the groups were analyzed by two-way ANOVA followed by the Tukey test for multiple comparisons. * $P<0.05$ compared with the control groups.

cystine and glutamic acid concentrations after influenza virus infection, as theanine is a derivative of glutamic acid. As shown in Fig. 3, influenza virus infection decreased plasma cystine level ($F=6.717$, $P=0.002$). However, no significant decrease was observed in the combined administration group, despite significant decreases at 3, 6 and 10 days after infection as compared with day 0 in the controls. Meanwhile, no significant change in the plasma glutamic acid level was observed in either group after infection (data not shown).

Effects of combined oral administration of L-cystine and L-theanine on expression of γ -GCS in the spleen after influ-

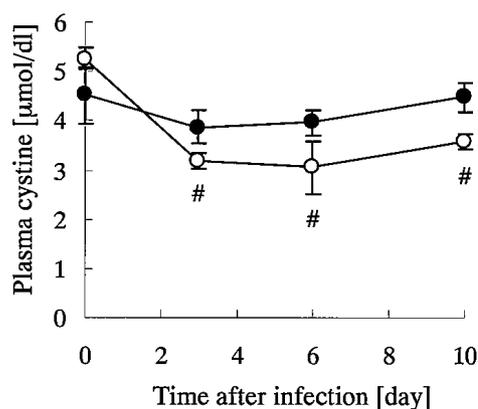


Fig. 3. Effects of combined administration of L -cystine and L -theanine on the plasma cystine concentration after infection with influenza virus. Mice were administered and infected by the same protocol as shown in Fig. 2. Open and closed circles indicate data for the control and combined administration groups, respectively. Plasma was collected at 0, 3, 6 and 10 days after infection, and the concentration of cystine was analyzed using an amino acid analyzer. Each value represents the mean \pm SEM ($n=4-5$). Differences among the groups were analyzed by two-way ANOVA followed by the Tukey test for multiple comparisons. # $P<0.05$ compared with day 0 in the control mice.

Table 1. Effects of combined administration of L -cystine and L -theanine on the mRNA expression of γ -GCS in the spleen after infection with influenza virus

	Virus (-)	Virus (+)	
		Control	L -Cystine + L -Theanine
GCS-HS	100 \pm 7.4	77.7 \pm 4.4	113.2 \pm 13.5*
CCS-LS	100 \pm 10.4	60.6 \pm 7.3#	92.2 \pm 11.3

Mice were administered and infected by the same protocol as shown in Fig. 2. At 0 and 3 days after infection, total RNA was extracted from the spleen, and the mRNA levels of GCS-HS (catalytic-subunit) and GCS-LS (regulatory-subunit) were quantified by quantitative PCR analysis using 18S ribosomal RNA as an internal control. Each value [% of virus (-)] represents the mean \pm SEM ($n=4-5$). Differences among the three groups were analyzed by one-way ANOVA followed by the Tukey test for multiple comparisons. * $P<0.05$ compared with the virus (+)/control mice. # $P<0.05$ compared with the virus (-)/control mice.

enza virus infection: The influenza virus itself is involved in direct generation of ROS, and oxygen stress can have indirect effects on virus infectivity [25, 26]. Therefore, we analyzed the effects of combined administration of L -cystine and L -theanine on mRNA expression for GCS-HS and GCS-LS in the spleens of influenza-virus-infected 13-month-old mice. As shown in Table 1, influenza virus infection induced a significant reduction in GCS-LS mRNA expression in the spleen compared with the uninfected control mice, and tended to reduce GCS-HS mRNA expression.

Combined administration of L -cystine and L -theanine significantly increased GCS-HS mRNA expression in the spleen at 3 days after infection, as compared with the virus-infected control mice, although there was only a non-significant increase in GCS-LS mRNA expression. At 6 or 10 days after infection, there was no significant effect on expression of γ -GCS mRNA in the combined administration group (data not shown).

Effects of combined oral administration of L -cystine and L -theanine on the serum IL-10/IFN- γ ratio after influenza virus infection: We further investigated the effects of combined administration of L -cystine and L -theanine on the balance of infection-associated Th2/Th1 cytokines using the serum IL-10/IFN- γ ratio because the balance of pro-inflammatory (Th1, IFN- γ) and anti-inflammatory (Th2, IL-10) cytokines in respiratory virus infection is thought to be critical in determining disease pathogenesis and the outcome of infection [23]. Serum samples were collected at 0, 3, 6 and 10 days after infection, and the concentrations of IL-10 and IFN- γ in the serum were analyzed using a fluorescent microbeads array system. As shown in Fig. 4A, the peak production of IL-10 after infection in the co-treated mice was observed at 3 days after infection, although that in the control mice was observed at 6 days. On the other hand, the IFN- γ concentration in the co-treated mice decreased 3 days after infection compared with day 0 and then gradually increased, but not significantly (Fig. 4B). The ratio of IL-10/IFN- γ increased gradually after infection (Fig. 4C). Combined administration of L -cystine and L -theanine significantly increased the serum IL-10/IFN- γ ratio at 3 days after infection, as compared with the controls, and the ratio then decreased rapidly to the pre-infection level. In addition, the serum IL-10/IFN- γ ratio at 10 days after infection was significantly decreased as compared with that in the control mice. Furthermore, we investigated the serum IL-12 concentrations. As shown in Table 2, the serum IL-12 concentration at 6 days after infection was significantly increased as compared with that in the control mice.

Effects of combined oral administration of L -cystine and L -theanine on influenza-virus-specific antibody production: Finally, we examined the effects of combined administration of L -cystine and L -theanine on production of influenza-virus-specific antibody. Sera were collected at 0, 3, 6 and 10 days after infection. The serum levels of influenza-virus-specific IgM and IgG antibodies were measured by ELISA. As shown in Fig. 5A, the anti-influenza-virus IgM antibody titer increased in both groups after infection ($F=51.805$, $P<0.001$), although no significant increase was observed in the combined administration group as compared with the controls. Figure 5B shows that the anti-influenza-virus IgG antibody titer increased gradually in both groups after infection ($F=46.130$, $P<0.001$). Furthermore, a multiple comparison test revealed a significant increase in IgG antibody production by combined administration at 10 days after infection as compared with the controls.

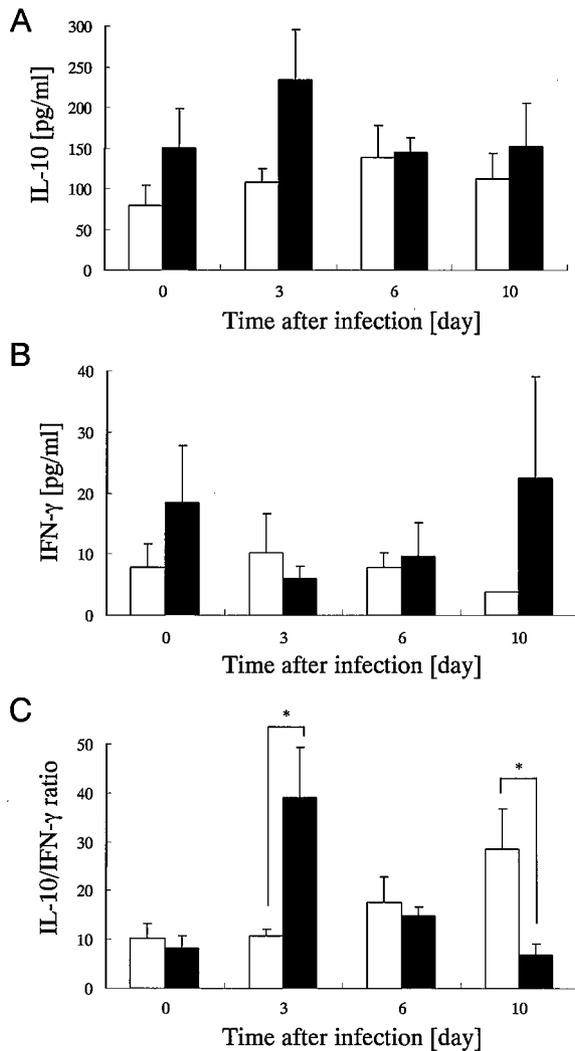


Fig. 4. Effects of combined administration of L-cystine and L-theanine on the serum IL-10/IFN- γ ratio after infection with influenza virus. Mice were administered and infected by the same protocol as shown in Fig. 2. Open and closed bars indicate data for the control and combined administration groups, respectively. Serum was collected at 0, 3, 6 and 10 days after infection, and the IL-10 (A) and IFN- γ (B) concentrations were investigated using a fluorescent micro-bead array system. The IL-10/IFN- γ ratio (C) was calculated from the determined concentrations of these cytokines. Each value represents the mean \pm SEM (n=4–5). Differences among the groups were analyzed by Mann-Whitney rank sum test. * $P < 0.05$ compared with the control mice on each day after infection.

DISCUSSION

Our results demonstrated that combined oral administration of L-cystine and L-theanine increased serum antigen-specific anti-DNP IgM and IgG in 24-month-old mice. We also showed that combined administration of these amino acids to 13-month-old mice was effective in lowering the

Table 2. Effects of combined administration of L-cystine and L-theanine on the serum IL-12 concentration after infection with influenza virus

		Control	L-Cystine + L-Theanine
IL-12 [pg/ml]	0 days	91.2 \pm 49.1	156.1 \pm 40.6
	3 days	241.5 \pm 104.7	85.4 \pm 41.9
	6 days	62.1 \pm 15.0	192.6 \pm 15.0***
	10 days	163.2 \pm 45.8	231.2 \pm 117.7

Mice were administered and infected by the same protocol as shown in Fig. 2. Serum was collected at 0, 3, 6 and 10 days after infection, and the IL-12 concentrations were investigated using a fluorescent micro-bead array system. Each value represents the mean \pm SEM (n=4–5). Differences among the groups were analyzed by Mann-Whitney rank sum test. *** $P < 0.001$ compared with the control mice.

viral titer in the lung and suppressed weight loss after influenza infection. Furthermore, the mice co-treated with L-cystine and L-theanine had significantly elevated serum IL-10/IFN- γ ratios and γ -GCS mRNA expression in the spleen 3 days after infection, and these effects of combined administration might contribute to the protective effect against the infection.

L-Cystine and L-theanine enhanced the antigen-specific anti-DNP IgM and IgG antibody titers in the serum at a lower dose (16 mg/kg) in 24-month-old C3H/HeN mice than in 12-week-old BALB/c mice (280 mg/kg) [22]. Franklin *et al.* have reported that the peak GSH-induced proliferation is consistently fourfold greater in splenocytes from old rats as compared to young rats when cells are stimulated with concanavalin A. The low plasma levels of cystine, one of the precursors of GSH, in old age [15] may result in high responsiveness to exogenous cystine. Although the precise mechanisms responsible for this difference are obscure, our results suggest that the GSH peroxidase-dependent detoxification system is defective in T lymphocytes from aged animals and that exogenous GSH is more effective in augmenting proliferation of splenocytes from aged animals than of those from young animals [8]. GSH levels and γ -GCS gene expression are known to decrease with age [24]. In the present study, combined administration did not significantly increase significantly the GSH levels in the liver before infection (data not shown). However, the reduction of γ -GCS mRNA expression induced by the infection was inhibited in the administration group. These findings suggest that the effect of combined administration occurs only when the GSH level is decreased after viral infection. Furthermore, an abundance of substrate for GSH synthesis, such as cysteine and glutamate (metabolite of theanine), may be necessary for recovery of the reduced GSH levels induced by immune stimulation, such as viral infection.

Although the L-cystine and L-theanine induced decreases in influenza virus titer in the lung and inhibition of weight loss, further investigations are required to determine the mechanisms of these effects. Regulation of the plasma cystine level is disrupted in conditions with progressive skeletal muscle catabolism, including cancer, HIV infection and old age [15]. Virtually all diseases in which abnormally low

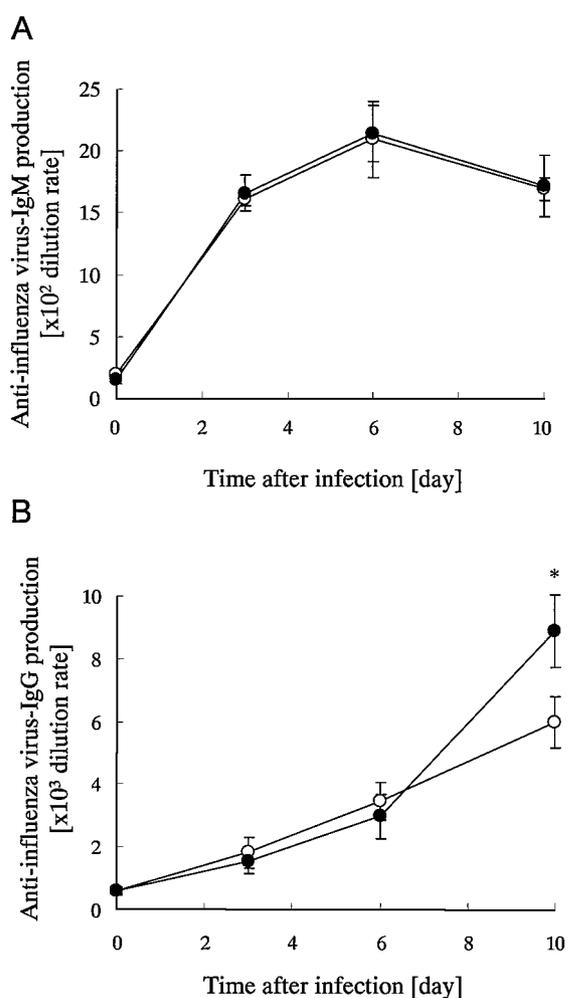


Fig. 5. Effects of combined administration of L -cystine and L -theanine on anti-influenza virus-specific antibody production. Mice were administered and infected by the same protocol as shown in Fig. 2. Open and closed circles indicate data for the control and combined administration groups, respectively. Sera were collected at 0, 3, 6 and 10 days after infection, and anti-influenza virus-IgM (A) or IgG (B) antibody production in the serum was analyzed by ELISA. Each value represents the mean \pm SEM ($n=4-5$). Differences among the groups were analyzed by two-way ANOVA followed by the Tukey test for multiple comparisons. * $P<0.05$ compared with the control mice on each day after infection.

plasma cystine levels have been found are known to be associated with immunological dysfunction, including reduced $CD4^+$ T cell proliferation, reduced natural killer (NK) activity and skeletal muscle wasting or muscle fatigue [5]. In the present study, combined administration of L -cystine and L -theanine inhibited the decrease in plasma cystine level induced by influenza infection. Our results were compatible with a previous study in which treatment of HIV-infected patients with N -acetyl-cysteine resulted in strong increases

not only in plasma cystine levels, but also in plasma glutamine and arginine levels [4]. In that study, the researchers suggested that the plasma glutamine and cystine levels should be monitored at regular intervals to determine the optimal therapeutic dose for individual patients. Inhibition of the decrease in the plasma cystine levels by combined administration of L -cystine and L -theanine may contribute to its beneficial effects. Cystine is one of the precursors of the antioxidant GSH, and its supply is rate-limiting for GSH synthesis [32]. It has been reported that influenza virus infection results in a decrease in the total concentration of GSH in lung and liver tissues [18]. Oxidative stress has been shown to play a role in both the pathogenesis and infectivity of influenza virus [25, 26]. The increased mRNA level of γ -GCS as a result of combined administration of L -cystine and L -theanine in the spleen may contribute to the protective effect.

Many previous studies have indicated that intracellular GSH synthesized by γ -GCS modifies cytokine production in monocytes and macrophages [14, 29]. In addition, we have reported previously that combined administration of L -cystine and L -theanine increases the GSH level and regulates Th-cytokine balance [22]. In the present study, the combined administration group showed a significantly increased or decreased IL-10/IFN- γ ratio as compared with the control mice at 3 days or 10 days after influenza infection, respectively. We also investigated the plasma IL-12 concentration after an infection that induces Th1-skewed response via IFN- γ production. In the combined administration group, the plasma IL-12 concentration 6 days after infection was significantly higher than in the control group. As ethylamine, a metabolite of L -theanine, induces secretion of Th1 cytokines from $\gamma\delta$ T cells [2, 20], it is possible that this enhancement contributes to shift to the Th1 response. These findings suggest that combined administration augments Th2-skewed response 3 days after virus infection and then increases IL-12 production 6 days after infection to shift to the Th1 response and regulate the balance of Th1/Th2 immune responses. However, further analyses, such as flow cytometry to investigate the subpopulations of lymphocytes, are required to clarify these points. Stimulation of type 2 immunity is characterized by high antibody titers [34]. However, in the mice co-treated with L -cystine and L -theanine, a significant increase in anti-influenza virus IgG level was only seen later in the infection (day 10), while significantly different lung viral titers were found earlier (day 6). We also evaluated anti-influenza virus IgA production in nasal washes. However, nasal IgA production 3 days after infection in the combined administration group was not significantly increased compared with the control group [0.14 ± 0.04 in the combined administration group vs. 0.06 ± 0.01 in the control group, each value represents the mean \pm SEM (optimal density) of four or five mice]. These results suggest that anti-influenza virus antibody production may not contribute to the beneficial effect on lung viral titer induced by combined administration of amino acids.

Macrophages, NK cells and neutrophils are also known to

be significant effector cells in the host defense against viral infection [10, 16, 19, 21]. The protective effect of *Astragali radix* extracts against viral infection is based on a non-specific mechanism during the early stage of infection, before shifting to antibody production, and macrophages play an important role in this resistance to viral infection [19]. This report suggests that protection against viral or bacterial infections in the early stage is very important because these organisms can grow exponentially with time. The beneficial effects of vitamin E in lowering influenza virus titer have been attributed to a moderate increase in NK activity [16]. Fujisawa reported that infiltration and functional activation of neutrophils play significant roles in elimination of influenza virus from the site of infection [10]. In this regard, studies to examine the effects of combined administration of L-cystine and L-theanine on innate and anti-influenza cell-mediated immunity, including NK activity and CTL induction, respectively, are currently underway in our laboratory.

The increased susceptibility of the elderly to influenza virus infection can be attributed to several factors, including the age-associated decline of cell-mediated and humoral immune responses and increased oxidative stress [21]. In addition, as elderly people account for most cases of serious morbidity and mortality attributable to influenza virus epidemics [13], it is recommended that they be vaccinated on a yearly basis [3]. However, it has been reported that IgG responses to the influenza vaccine are reduced with advanced age [30]. In addition, poor nutritional status is a commonly observed phenomenon in the elderly and limits the efficacy of vaccination [36]. The enhanced virus-specific IgG antibody production seen in the present study suggests that combined use of L-cystine and L-theanine can improve the age-related decline in influenza vaccine efficacy with malnutrition.

Animal diseases related to the increased longevity of pets and infections in domestic animals have become an issue in recent years. Our results suggest that combined oral administration of L-cystine and L-theanine may become a useful strategy for achieving healthy aging. Our results also suggest that combined administration of these amino acids has important benefits for the elderly, offering protection against influenza virus infection through enhancement of GSH synthesis in immunocompetent cells. Attempts to define the effects of combined administration of L-cystine and L-theanine in the elderly are also currently underway in our laboratory.

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