Inhibition of Premature Death by Isothiocyanates through Immune Restoration in LP-BM5 Leukemia Retrovirus-Infected C57BL/6 Mice

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The purpose of this study was to determine the effect of isothiocyanates (ITCs) in delaying the progression of the murine immunodeficiency virus to murine AIDS, resulting in increased life span. Furthermore, we investigated the role of ITCs in modulating immune dysfunction caused by LP-BM5 retrovirus infection. Among the tested ITCs, oral administration of sulforaphane (SUL), benzyl isothiocyanate (BITC), and phenethyl isothiocyanate (PEITC) showed the inhibition of premature death caused by LP-BM5 retrovirus infection, while indole[3,2-b] carbazole (ICZ) and indole-3-carbinol (I3C) did not delay the progress of the LP-BM5 retrovirus to murine AIDS. Inhibition of premature death by BITC, PEITC, and SUL could be explained by restoration of the immune system and down regulation of free radicals. Dysfunction of T and B cell mitogenesis caused by retrovirus infection in primary cultured splenocytes has been partially recovered with administration of BITC, PEITC, and SUL. There was a shift from imbalanced cytokine production (increased Th2 and decreased Th1 cell cytokine production) into balanced Th1/Th2 cell secretion of cytokines under administration of these ITCs during the development of murine AIDS. Hepatic vitamin E level was significantly restored by administration of these ITCs, in accordance with reduced hepatic lipid peroxidation levels. This study suggests that certain types of ITCs have beneficial effects in preventing premature death during progression to murine AIDS by restoration of immune dysfunction and removal of excessive free radicals, implying that selective usage of ITCs would be helpful in retarding the progression from HIV infection to AIDS.

Key words: isothiocyanates; murine AIDS; immune dysfunction; oxidative stress; Th1 and Th2 cytokines

Infection with HIV has spread around the world to an estimated more than 30 million people during the last few years.1) Although a combination of antiretroviral drugs has dramatically improved the quality of life expectancy of those infected with HIV, life-long suppressive treatment is required, and a cure for HIV infection remains elusive. According to mathematical models, a patient’s life expectancy can become nearly normal if antiretroviral therapy is started at the early stage of HIV infection.2) Recently, Antiretroviral Therapy Cohort Collaboration (ART-CC) reported that the projected life span of a 20-year-old HIV-positive man is 70 years if the helper cell count is over 200/μL at the start of antiretroviral therapy, but only 52 years for a count under 100/μL.3) However, the treatment of patients with HIV poses a special challenge, as the virus replicates very rapidly and mutates frequently without any major loss of function for most of its constituent proteins.4) Thus HIV treatment can succeed over the long term only through lasting suppression of the viral burden, which not only limits viral replication, but also prevents the development of resistance to the antiviral drugs used. Since resistance develops rapidly when a single drug is given, antiretroviral drugs are usually given in combination with other drugs, nutritional elements, and natural products. The nutritional status of patients with HIV infection is an important topic, because many nutritional elements are involved in the progression of HIV to AIDS.5) Although nutritional status and minor drugs are not likely to be the most important etiological determinants, they can alter immune function to facilitate disease progression, influence viral expression, and have a significant impact on morbidity and mortality.

Numerous studies confirm that isothiocyanates (ITCs) protect against cancer, inflammation, and certain viral infections.6,7) ITCs are abundant in cruciferous vegetables such as broccoli, watercress, cabbage, cauliflower, and Brussels sprouts. It has known that some ITCs such as sulforaphane (SUL), benzyl isothiocyanate (BITC), and phenethyl isothiocyanate (PEITC), are highly effective in preventing or reducing the risk of several diseases, especially cancer induced by carcinogens in...
animal model. Also, recent studies show that they inhibit the disease process by affecting multiple pathways, including apoptosis, MAPK signaling, oxidative stress, and cell cycle progression. But there is only limited information of ITCs on viral pathogenesis such as HIV/AIDS. Thus our study investigated the association of ITCs with delay of premature death during disease progression and immune dysfunction induced by the LP-BM5 mouse leukemia retrovirus. This animal model can provide a useful means of studying survival and the relation between treatment by ITCs and HIV infection in humans.

Materials and Methods

Animals and treatment: Female C57BL/6 mice, 4 weeks old, were obtained from Charles River Laboratories (Wilmington, DE). They were housed in transparent plastic cages with stainless wire lids (3–4 mice per cage) in the animal facility of Kyung Hee University. Animal use was approved by the Kyung Hee Committee on Animal Research. The housing facility was maintained at 20–22°C at 60–80% relative humidity with a 12-h light/dark cycle. For the study, mice were randomly assigned the uninfected control or the infected groups to be received by oral administration of ITCs. Oral administration began at 2 d post-infection with LP-BM5 murine leukemia retrovirus, and continued for 24 weeks for the survival test (24 mice/group) and 12 weeks for immunological analysis (10 mice/group). All the mice were fed a normal AIN93M diet (Dyets, Bethlehem, PA) during the experiment.

ITCs, including BITC, IND, indole[3,2-b]carbazole (ICZ), and indole-3-carbinol (I3C), and PEITC were purchased from Sigma (St. Louis, MO). Female C57BL/6 mice were administered one of the ITCs or the vehicle once daily by oral gavage at a dose of 1 mmol/kg in 100 μL ethanol per d. A dose of 1 mmol/kg was chosen since it was effective against several types of cancer in a previous study.

LP-BM5 murine leukemia retrovirus was administered intraperitoneally in 0.1 mL of minimum essential medium with an isotropic titer (XC) of 4.5 log10 plaque forming units × 107/L, which induced disease on a time course comparable with that previously published. Infection of female C57BL/6 mice with LP-BM5 retrovirus led to rapid induction of clinical symptoms with virtually no latent phase. The mice were infected 2 d prior to initiation of oral administration. The infection and treatment period for immunological analysis was 12 weeks for all groups.

When murine AIDS had developed, all the mice were sacrificed while ethyl ether anesthesia. The spleens were dissected and subjected for primary splenocyte culture, and the lymph nodes were kept at 4°C. Livers for nutrient analysis were collected and stored at -70°C until assayed.

Survival test. Female C57BL/6 mice, 4 weeks old, were randomly assigned to one of the groups (24 mice/group) and fed one of the ITCs or the vehicle for 24 weeks to assess the effects of ITCs intake on survival. The mice were monitored every day for survival and conditions while their drinking water and AIN93M diet were changed every 3 d. Median survival time has represented the day expected to cause 50% death in the treatment group.

ELISA assay for cytokines. The production of IL-2, IL-4, IL-6, IFN-γ, and TNF-α from mitogen-stimulated splenocytes was determined as described previously. Briefly, spleens were gently teased with forceps in culture medium (RPMI 1640 CM containing 10% fetal bovine serum, 2 mmol/L glutamine, 1 × 105 units/L of penicillin and streptomycin), producing a suspension of spleen cells. Red blood cells were eliminated by the addition of a lysis buffer (0.16 mol/L of ammonia chloride tri buffer, pH 7.2) at 37°C for 3 min. Then the primary cells were washed twice with the culture medium. Cell concentrations were counted and adjusted to 1 × 106 cells/mL. Cell viability was >95% as determined by trypan blue exclusion. The primary splenocytes, 0.1 mL/well (1 × 106 cells/mL), were cultured in triplicate on 96-well flat-bottom culture plates (3072 Falcon, Lincoln Park, NJ), and then stimulated with concanavalin A (Con A, 1 × 10-5 g/L, 0.1 mL/well, Sigma, St. Louis, MO) to determine their production of IL-2 and IL-4 after 24 h incubation and IFN-γ after 72 h incubation in a 37°C, 5% CO2 incubator. Splenocytes were also simultaneously incubated for 24 h after the addition of lipopolysaccharide (LPS, 1 × 10-5 g/L, 0.1 mL/well, GibcoBRL, Grand Island, NY) to induce IL-6 and TNF-α production. After incubation, the supernatants were collected and stored at -70°C until analysis. The cytokines were determined by sandwich ELISA.

Mitogenesis of splenocytes. Splenic T- and B-cell proliferation was determined by 3H-thymidine incorporation, as described previously. Briefly, splenocytes in 0.1 mL of culture medium (1 × 106 cells/mL) were cultured in 96-well flat-bottom cultured plates with Con A (10 μg/mL) and LPS (10 μg/mL), incubated at 37°C, 5% CO2 incubator for 24 h, and then pulsed with 3H-thymidine (0.5 μCi/well, New England Nuclear, Boston, MA). After 24 h incubation, they were harvested by a cell sample harvester (Cambridge Technology, Cambridge, MA). Radioactivity was determined by a liquid scintillation counter (Tri-Carb, 2200 CA, Packard, Laguna Hills, CA). Data were collected as counts per min (cpm).

Measurement of lipid peroxidation. Lipid peroxidation (LPO) in liver was measured by K-Assay™ LPO-CC Assay Kit obtained from Kamiya Biomedical Company (Seattle, WA). This method has been found to be a more sensitive lipid peroxide measurement (assay range: 2–300 nmol/mL) than conventional chemical analyses. Briefly, -0.2 g of liver tissue was homogenized in 3 mL of CHCl3/methanol (2:1, v/v). NaCl (0.9%, 0.6 mL) was added to clarify and the mixture was centrifuged at 3,000 × g for 10 min. The supernatant was discarded while bottom layer that includes CHCl3 was evaporated under nitrogen gas. Isopropanol (100 μL) was added to dissolve lipid residue, and 20 μL of the sample was used to measure lipid peroxides with the LPO Kit. In the presence of hemoglobin, lipid hydroperoxides are reduced to hydroxyl derivatives (lipid alcohols), and the MCDP (10-N-methylcarbamoyl-3,7-dimethylamino-10-H-phenothiazine) chromagen is oxidatively cleaved to form methylene blue in an equal molar reaction. Lipid peroxides are quantitated by measuring colorimetrically methylene blue at 675 nm. The lipid peroxidation values were calculated by the equation given from the manual. The LPO value was converted to the percent unit for illustration.

Determination of vitamin E. Hepatic vitamin E levels were measured by HPLC, as described previously. Briefly, -0.2 g of tissues was homogenized in 1 mL of water. Butylated hydroxytoluene (BHT) was added to prevent oxidation of α-tocopherol. Pentane, ethanol, and sodium dodecyl sulfate were used to extract α-tocopherol from the homogenate. Extracts were evaporated under a steady flow of nitrogen gas at 20°C and then redissolved in 0.5 mL of methanol injection on a C18 column (3.9 × 150 mm NovaPak, Millipore, Bedford, MA). A mobile phase composed of methanol and 1 mol/L sodium acetate in the ratio of 98.2 (v/v) at a flow rate of 1.5 mL/min was used. α-Tocopherol, with a retention time of about 5 min, was monitored by a fluorescence detector (Millipore, Bedford, MA) at 290 nm excitation and 320 nm emission wavelength.

Statistics. All data are presented as mean ± standard deviation (SD). Statistical analyses were performed by Duncan’s multiple range tests after one-way ANOVA using SAS software. Differences were considered statistically significant at p < 0.05.

Results

Body weights, dietary intake, and organ weights. As shown Table 1, the mice with the LP-BM5 infection significantly lost body weight (20.9 ± 2.87 g/mouse, p < 0.05) as compared to those with no infection (29.6 ± 1.74 g/mouse). There was no significant difference in body weight between the mice administered one of ITCs, except for those administered I3C (24.3 ± 1.54 g/mouse, p < 0.05). Spleen and lymph
Table 1. Body and Organ Weights for Female C57BL/6 Mice after 12 Weeks of Administration of Isothiocyanates

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body (g)</th>
<th>Spleen (g)</th>
<th>Liver (g)</th>
<th>Lymph node (g)</th>
<th>Heart (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP-BM5 Samples</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>29.6 ± 1.74a</td>
<td>0.08 ± 0.01b</td>
<td>1.26 ± 0.29d</td>
<td>0.01 ± 0.01d</td>
</tr>
<tr>
<td>+</td>
<td>–</td>
<td>20.9 ± 2.73a</td>
<td>0.68 ± 0.23a</td>
<td>1.42 ± 0.33a</td>
<td>0.53 ± 0.14a</td>
</tr>
<tr>
<td>+</td>
<td>I3C</td>
<td>24.3 ± 1.54a</td>
<td>0.66 ± 0.17a</td>
<td>1.34 ± 0.17a</td>
<td>0.42 ± 0.09a</td>
</tr>
<tr>
<td>+</td>
<td>ICZ</td>
<td>27.4 ± 2.71a</td>
<td>0.59 ± 0.25a</td>
<td>1.43 ± 0.27a</td>
<td>0.42 ± 0.13a</td>
</tr>
<tr>
<td>+</td>
<td>BITC</td>
<td>28.2 ± 1.93a</td>
<td>0.23 ± 0.11b</td>
<td>1.38 ± 0.13a</td>
<td>0.33 ± 0.07b</td>
</tr>
<tr>
<td>+</td>
<td>PEITC</td>
<td>29.9 ± 2.01a</td>
<td>0.32 ± 0.23b</td>
<td>1.29 ± 0.25a</td>
<td>0.25 ± 0.11b</td>
</tr>
<tr>
<td>+</td>
<td>SUL</td>
<td>29.3 ± 2.37a</td>
<td>0.29 ± 0.15b</td>
<td>1.35 ± 0.19a</td>
<td>0.15 ± 0.05c</td>
</tr>
</tbody>
</table>

*Indicates the significant difference as determined by Duncan’s multiple range test after one-way ANOVA at p < 0.05.

Table 2. Median Survival Times of Mice under LP-BM5 Retrovirus Infection and Isothiocyanate Administration

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Median survival time (ds)*</th>
<th>Number of mice (%)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP-BM5 Samples</td>
<td></td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>&gt;168</td>
</tr>
<tr>
<td>+</td>
<td>–</td>
<td>99</td>
</tr>
<tr>
<td>+</td>
<td>I3C</td>
<td>116</td>
</tr>
<tr>
<td>+</td>
<td>ICZ</td>
<td>129</td>
</tr>
<tr>
<td>+</td>
<td>BITC</td>
<td>&gt;168</td>
</tr>
<tr>
<td>+</td>
<td>PEITC</td>
<td>&gt;168</td>
</tr>
<tr>
<td>+</td>
<td>SUL</td>
<td>&gt;168</td>
</tr>
</tbody>
</table>

*Median survival time is represented by the number of d expected to show 50% death in the treatment group.
**Indicates the number of mice surviving after 24 weeks, converted to a percentage.

Survival test for 24 weeks

In the infected mice with the LP-BM5 retrovirus, the median survival time was 29 d, while for the uninfected control mice were over 168 d (Table 2). In the infected mice administered I3C and ICZ, the median survival time was 116 d and 129 d, respectively. No significant difference in median survival time was observed between the infected mice administered BITC, PEITC, and SUL, indicating that more than 50% of the mice survived during 24-week experiment.

Mitogenesis of primary splenocytes

For the LP-BM5 retrovirus-infected group, in vitro T-cell proliferation stimulated by Con A showed a significant difference as compared to the uninfected control (Fig. 1A). Administration with PEITC and SUL, followed by BITC, increased the ability of the T-cells to proliferate near the normal level. However, administration with I3C and ICZ did not show any significant difference as compare to the infected control. As shown in Fig. 1B, LP-BM5 retrovirus infection reduced B-cell proliferation in all groups as compared to the uninfected group with statistical differences (p < 0.05). Not surprisingly, the severity of impairment in B-cell proliferation has been partially recovered by treatment of BITC, PEITC, and SUL, indicating that a certain type of ITCs may influence on the ability of B-cells to proliferate beyond retrovirus infection. Similar results were obtained on B-cell proliferation in the retrovirus-infected group with administration of I3C and ICZ, which showed no significant difference as compared to the infected group alone.
Cytokine production by splenocytes

*In vitro* production of IL-2 by Con A-stimulated splenocytes was significantly (*p* < 0.05) reduced by retrovirus infection (Fig. 2). I3C, BITC, PEITC, and SUL increased the IL-2 level close to that of the uninfected control, but ICZ partially increased. Similar results were observed in IFN-γ production, which is significantly increased by administration of all the ITCs in the study as compared to the retrovirus-infected group. But, there was no significant difference among treatment of ITCs, except for I3C in IFN-γ production.

The release of TNF-α, IL-4, and IL-6, by LPS-stimulated primary splenocytes was significantly (*p* < 0.05) increased by retrovirus infection (Fig. 3). Administration of BITC, PEITC, and SUL significantly reduced IL-4, IL-6, and TNF-α production after retrovirus infection. However, treatment of I3C and ICZ had less influence on IL-4, IL-6, and TNF-α production as compared to other ITCs.

Hepatic lipid peroxidation levels

As shown in Fig. 4, murine retrovirus infection significantly (*p* < 0.05) increased hepatic lipid peroxidation, while administration of ICZ, BITC, PEITC, and SUL reduced hepatic lipid peroxidation beyond LP-BM5 retrovirus infection. However, I3C showed no significant difference compared to the retrovirus-infected group.

Hepatic vitamin E levels

Hepatic vitamin E levels decrease during murine retrovirus infection due to increased free radicals, resulting in increased tissue lipid peroxidation. To estimate the effects of ITCs administration on the production of free radicals during murine retrovirus infection, the hepatic vitamin E level was measured at 12 weeks post-infection. As shown in Fig. 5, it was significantly reduced by retrovirus infection, while administration of BITC, PEITC, and SUL increased the cellular vitamin E content as compared to that of the retrovirus-infected group. Although I3C significantly...
increased hepatic vitamin E levels, the degree of increase was very slight. Otherwise, administration of ICZ did not show significant any difference as compared to the infected group.

**Discussion**

In the study of HIV infection and phytochemicals, it is interesting to consider whether viral infection or ITCs affect the life span. ITCs are produced upon cutting or chewing of cruciferous vegetables due to myrosinase-mediated hydrolysis of the corresponding glucosinolates. Recently, numerous studies demonstrated that intakes of ITCs can exhibit chemopreventive activity against several types of cancer by inducing apoptosis. It is hardly found whether ITCs influence on viral diseases such as AIDS or not. Thus, our study aimed to determine the effect of ITCs in delaying the progression of murine immunodeficiency virus to murine AIDS, resulting in increased life span. Furthermore, we investigated the roles of ITCs in modulating immune dysfunction caused by LP-BM5 retrovirus infection.

From the previous reports, it is not surprising that murine LP-BM5 retrovirus infection shortened life span in the C57BL/6 mouse model. Interestingly, our data revealed that BITC, PEITC, and SUL with retrovirus infection only inhibited premature death, assuming that they play critical roles in viral progress to murine AIDS and influence the survival of the mice. However, I3C and ICZ did not show any influence on premature death in the mice infected with the LP-BM5 retrovirus. These results can imply that not all of the ITCs in the study were effective on viral progress, although all of them exhibited critical roles in preventing several types of cancer. The prolongation of life span in infected animal with BITC, PEITC, and SUL can be explained by two possible mechanisms: improvement of immune dysregulation and inhibition of excessive free radical production. It is thought that these ITCs intake during the progression to murine AIDS ameliorates cytokine dysfunction, since it has been reported that ITCs can modify cytokine production or its receptor expression, which can result in a shift from imbalanced cytokine production (increased Th2 and decreased Th1 cell cytokine production) into balanced Th1/Th2 cell secretion of cytokines with administration of these ITCs during the development of murine AIDS. It was demonstrated by a study blocking Th2 cell activation and its excessive cytokine production, which should retard development of murine AIDS. In a study of IL-4 gene knockout mouse with suppressed Th2 cytokine production after LP-BM5 retrovirus infection, the usual lethality and the development of T-cell abnormalities were delayed. In another study, administration of anti-IL-4 monoclonal antibody in LP-BM5 retrovirus-infected mice also maintained the balance of Th1 and Th2 responses, preventing retrovirus-induced suppression of immune responses. Thus long-term administration of certain ITCs may independently modulate imbalanced cytokine secretion, a possible cofactor in perpetuating the cytokine imbalance and increasing the rate of murine AIDS progression. We observed that decreased production of Th1 cytokines due to murine retrovirus infection was increased by administration of BITC, PEITC, and SUL, while Th2 cytokines were decreased to near normal levels.

A recent report suggested that SUL restores the decrease in Th1 immunity by restoration of cellular redox equilibrium, indicating that certain ITCs directly restore immune-cell functions, particularly when T-cell numbers are reduced by oxidative stress due to viral infection. These results are in accord with our findings of increased proliferation under conditions of BITC, PEITC, and SUL. Decreased cell division with retrovirus infection was expected due to reduced release of IL-2, a major T-cell growth factor. We found that administration of ITCs during the development of murine AIDS augmented the ability of T- and B-cells to proliferate. Increased immunoglobulin production during murine retrovirus infection is due to increased numbers of B-cells stimulated by heightened IL-4 secretion. However, our data indicated increased B-cell proliferation and decreased IL-4 secretion by administration of ITCs during murine retrovirus infection. It is clear that B-cell proliferation was determined by stimulation of LPS while IL-4 secretion was induced by Con A. Thus, IL-4 production did not affect B-cell proliferation in our *in vitro* study. Therefore, in this study, administration of ITCs during murine retrovirus infection may have directly augmented the proliferative function without the help of IL-4, or indirectly activated the signal transduction required to stimulate B lymphocytes.

As another possible mechanism inhibiting premature death, excessive free radical induction during progress to murine AIDS might be removed as a result of ITC administration. In fact, it is known that ITCs act as a prooxidant, and target mitochondrial respiratory chain complexes to trigger the generation of reactive oxygen species (ROS) in many cancer cell lines. It is worth to notice that ITCs may selectively act on cancer cells as a prooxidant to cause apoptosis. This hypothesis is supported by recent studies indicating that normal cells are significantly more resistant to the proapoptotic and prooxidant effects of ITCs than cancer cells. In other studies, consumption of vegetables including ITCs or themselves reduced free radical-related molecular damage by activating nuclear factor kappa B (NF-κB) or Nrf2 to induce antioxidative enzymes. Therefore, mechanisms of the selectivity of ITCs for cancer cells as prooxidants or for other cells as antioxidative reagents still need the further explanation. As evidenced by the reduced production of IL-2 during excessive ROS production, most of the cytokine alteration in this study may have been due to cellular deprivation of antioxidant molecules. Pro-inflammatory cytokines (IL-1, IL-6, and TNF-α) and ROS are mutually stimulatory. Stimulation of cytokine production by ROS involves activation of NF-κB, which induces retrovirus replication. Attack by ROS results in detachment of the inhibitory component (iκB) from the NF-κB complex, which results in transcription of the genes for the synthesis of pro-inflammatory cytokines. Similarly, our data indicate that lipid peroxidation, a marker of the increased presence of ROS, was markedly increased under murine retrovirus infection. Since LP-BM5 virus infection alone increased both the cellular and plasma levels of oxidants and TNF-α, administration of ITCs
during murine retrovirus infection may block the production of these parameters. Oxidative stress due to reduced levels of antioxidants may be a potent inducer of murine retrovirus replication in addition to DNA damage in virus-infected cells, producing one of the long-term consequences of retrovirus infection, such as immunosuppression. Reduced levels of antioxidants correlated well with progression to murine AIDS. 29,30) Decreased free radicals produced during the administration of ITCs lowered the utilization of antioxidants, including membrane vitamin E, for increased lipid peroxidation. 31) In an animal study, vitamin E deficiency decreased IL-6 levels than animals consuming adequate amounts. 32) Lipid peroxidation was also found to be greater in the vitamin E-deficient animals in this study.

In conclusion, our results may represent that amelioration premature death in mice by administration of ITCs might be a result of immune restoration, increased levels of tissue antioxidants, and decreased oxidative stress during the progression to murine AIDS. However, the molecular mechanisms of ITCs activating T- and B-cells and regulating antioxidant response elements inducing phase II enzymes in immune function-related cells or tissues were still remained unclear.

Acknowledgments

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References