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In vitro Effects of L-Ascorbic Acid and Acrylamide on Lymphocyte Proliferation in Young and Aged Mice

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Abstract

This study examined the effects of Acrylamide (ACR) and L-ascorbic acid (AsA) on the proliferation of splenocytes and the mitogen-stimulated lymphocyte proliferation in young (8 weeks) and aged (82 weeks) C57BL/6 male mice *in vitro*. AsA increased splenocyte proliferation in both groups; however, this effect was higher in old mice, while the proliferation of lymphocyte was decreased except for treatment at 1 μg/mL low concentration in both mice. In addition, ACR treatment resulted in decreased LPS-induced B lymphocyte proliferation and ConA-induced T lymphocyte proliferation in both groups. However, AsA increased LPS/ConA-induced lymphocyte proliferation in young groups and had no effects in old mice except at 0.5 μg/mL. Thus, the present data indicate that there is no difference effect of ACR and AsA on lymphocyte proliferation, whereas the effect of AsA on mitogen-induced cell proliferation was reduced in old mice. Overall, our results suggest that various immunomodulators have differing effects of lymphocytic proliferation on young versus aged mice.

Key words: aging, immunomodulator, lymphocyte proliferation, acrylamide, L-ascorbic acid

INTRODUCTION

Aging is a natural phenomenon, accompanied by a motor and memory dysfunction, and is also believed to occur concomitantly with immune dysfunction (1,2). Even though there have been several reports on the age-related phenotype changes in immune cells, altered activities of immunomodulators in aged cells have not been extensively studied. Therefore, it was of interest to examine the proliferative effect of immunomodulators on lymphocytes in young and aged mice. Previous studies have shown that acrylamide (ACR; CH2=CHCONH2), a widely used industrial chemical, impairs the central and the peripheral nervous system in humans. In addition, acrylamide has been shown to be immunotoxic, genotoxic, and carcinogenic, and, as well, is capable of inducing harmful developmental and reproductive effects (3). L-ascorbic acid (AsA), also known as vitamin C, is an essential cofactor for the hydroxylation of proline and lysine residues in collagen, which is the most abundant protein in the body (4). AsA is a crucial element for the proliferation and differentiation of various cell types in culture and plays a role in myogenesis, chondrogenesis, and osteogenesis through its action on the collagen matrix formation (5). However, the effect of ACR and AsA on lymphocyte proliferation in young and aged mice remains unclear. The aim of present study was to determine whether an aged animal's response to ACR and AsA is different from that of a young animal.

MATERIALS AND METHODS

Animal care procedures

Male C57BL/6 mice (7 weeks old) were obtained from the Charles River Breeding Laboratories (Japan). The animals were randomly distributed into five per group. Aged (82 weeks) and young (8 weeks) C57BL/6 male mice were used in this study. The mice were provided with water and food ad libitum and quarantined under a 12 hr light: 12 hr dark photoperiod in the animal care facility of the Sungkyunkwan University, Suwon, Korea. Animal care was performed following the Institute for Laboratory Animal Research (ILAR) guideline.

Reagents

Unless stated otherwise, all chemicals were purchased from the Sigma Chemical Co. (St Louis, MO, USA). The RPMI 1640 medium and fetal bovine serum (FBS) were purchased from GIBCO (Grand Island, NY, USA). The XTT {2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt} cell viability assay kit was purchased from WelGENE (Daegu, Korea).

Measurement of organ weight/body weight ratios

Mice were weighed and then sacrificed via ether. The

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spleen was prepared for cell culture and liver, kidney and thymus were rinsed in two washes of ice-cold saline. Spleen samples were collected aseptically for lymphocytic proliferation assays. Other organ weights were determined and weight to total body weight ratios calculated.

$$\%\ \text{Organ/Body weight} = \frac{\text{organ weight}}{\text{body weight}} \times 100$$

Mitogen-induced lymphocyte proliferation

A modification of the method reported by Mosmann was used (6) to determine mitogen-induced lymphocyte proliferation. The spleens were aseptically removed and dissociated into a single-cell suspension in a culture medium. The concentration was adjusted to 2×10^6 cells/mL. The culture medium was RPMI 1640 (GIBCO) containing 10% heat-inactivated FBS, penicillin (100 IU/mL) and streptomycin (100 μg/mL) (RPMI-FBS). Cells (5×10^5 cells/well) were incubated in the presence or absence of the mitogens as follows: concanavalin A (ConA) at 4 μg/mL for T cell activation and lipopolysaccharide (LPS) at 10 μg/mL for B cell activation. After incubation of cells for 24 hr, 20 μL of phenazine methosulphate (PMS; electron-coupling reagent) and 25 μL of XTT was added to each well. The cells were further incubated for 3 hr to allow XTT formazan production. The absorbances were determined with a microplate reader at a test wavelength of 450 nm and a reference wavelength of 690 nm.

Statistical analysis

Each experiment was repeated two times, and the results of one representative experiment are shown. The results were expressed as means ± SEM and analyzed via ANOVA. A statistical probability of $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

Effect of aging on weight of thymus, liver, and kidney

The thymus represents the major site of the production and generation of T cells. Though these white blood cells begin from stem cells in bone marrow, they differentiate into subsets of specialized T lymphocytes. Age-related involution may affect the thymic atrophy and the ability of the thymus to reconstitute T cells (7,8). In addition, Li et al. has been reported that there is a significant difference of gene expression in murine thymic aged-related genes (9). Expression of some genes was exclusive in thymus from 4 or 40 weeks old mice, while other genes were expressed at different ages (1,9). These results suggest that differential expression of thymic genes might be participated in thymic atrophy with age.

Table 1. Effect of aging on weight of thymus, liver, and kidney in young and aged C57BL/6 male mice

Organ	Average of weight (g per group)	
	Young	Aged
Thymus	0.21	0.05*
Liver	6.91	6.32
Kidney	1.64	1.59

The organs from young group and aged group are isolated and weighed.
*$p<0.05$, significantly different from young group.

To confirm the aged state with normal healthy in our experimental animal model, organ (liver, kidney and thymus) weight/body weight ratio of the young and aged mice were measured and are listed in Table 1. None of the mice showed any abnormal sign of illness to the naked eye. The ratio of thymus weight to body weight decreased 42% in aged mice as compared to that of young mice, whereas liver and kidney weights and ratios were not significantly different between age groups and young groups (Table 1). These results indicate that the loss of thymus weight was caused by the senescence.

Effects of AsA and ACR on lymphocyte proliferation

The spleen has the largest accumulation of lymphoid tissue in the body. Because of its abundance of lymphocytes and some phagocytic cells, the spleen is an important defense against antigens that reach the blood circulation.

To examine the effect of AsA and ACR on splenocyte proliferation, XTT assays were performed by treating spleen cells with these compounds after 48 hr of cultivation. AsA increased splenocyte proliferation in both groups and this effect was higher in old mice, while the proliferation of ACR was decreased, except for treatment at 1 μg/mL low concentration in both mice. ACR induced a transient increase in the proliferation of lymphocytes at 1mM, whereas cell proliferation was markedly decreased at 3 and 5 mM in both young and aged groups (Fig. 1).

Aging is a complex process that may consist of both environmentally stimulated and inherently programmed components. Phipps et al. have described that the *in vitro* aging cell culture system could be likened to a model of cell proliferation regulatory mechanisms that show age-associated changes (10,11). In addition, many studies have focused on specific age-related conditions that change cell growth (12-14). In the present study, we found that the cell proliferative effect of AsA and transient effect of ACR at 1 mM is higher in aged groups. Thus, it is plausible that cells could be changed by

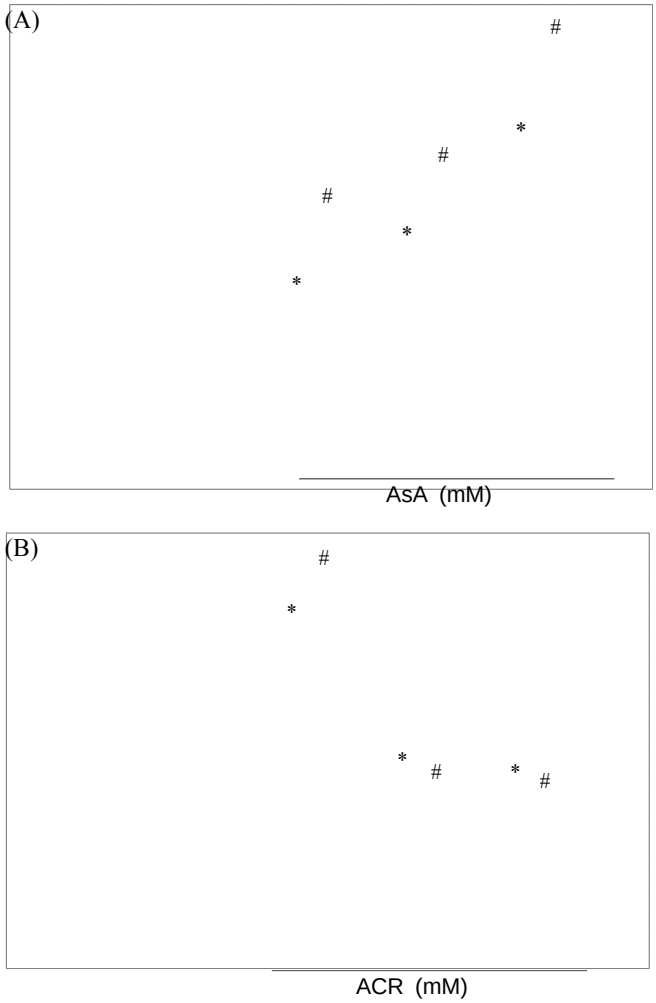

Fig. 1. The effect of AsA and ACR on the proliferation of splenocytes in young and aged C57BL/6 male mice. The effect of AsA (A) and ACR (B) on cell viability of splenocytes in young and aged C57BL/6 male mice. Splenocytes (1×10^7 cells/mL) were treated with various concentrations of AsA or ACR for 48 hr. Cell viability and splenocyte proliferation was assessed by XTT assay. Absorbance was measured at 450 nm and 650 nm. $^{*}p<0.05$, significantly different from the untreated young group. $^{\#}p<0.05$, significantly different from the untreated aged group.

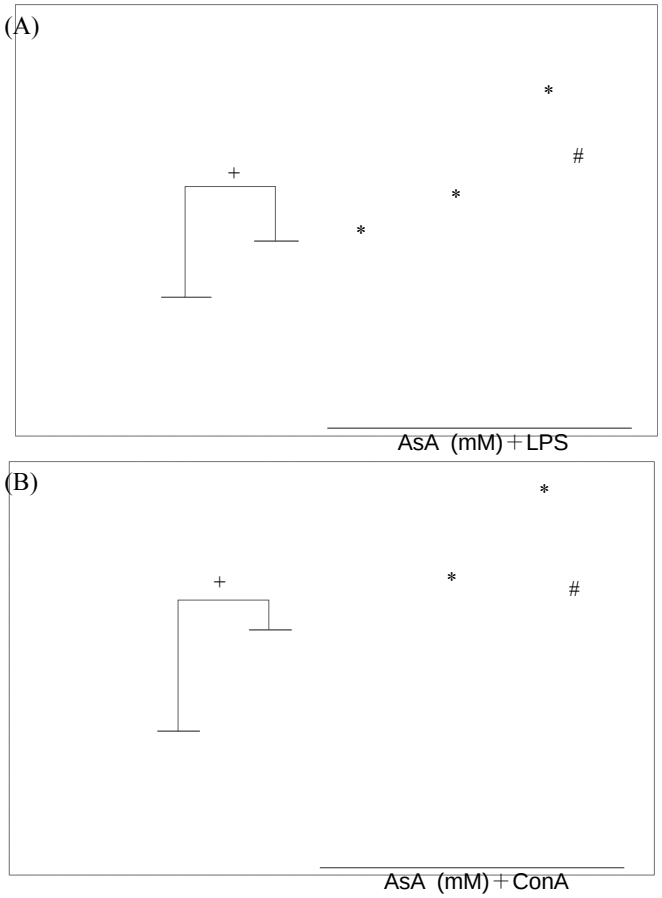

Fig. 2. The effects of AsA on the proliferation of LPS-induced B lymphocytes (A) and ConA-induced T lymphocytes (B) in young and aged C57BL/6 male mice. (A) The effect of AsA on the proliferation of LPS-induced B lymphocytes in the spleen. Splenocytes (1×10^7 cells/mL) were treated with various concentrations of AsA and LPS for 48 hr. (B) The proliferative effect of AsA on ConA-induced T lymphocytes in the spleen. Splenocytes were treated with various concentrations of AsA and ConA for 48 hr. $^{*}p<0.05$, significantly different from the ConA- or LPS-treated young group. $^{\#}p<0.05$, significantly different from the ConA- or LPS-treated aged group. $^{+}p<0.05$, significantly different from untreated group.

age-associated protein and metabolic needs, which subsequently causes the different cellular proliferative response to immunomodulators such as AsA.

Differential effects of AsA and ACR on mitogen-induced lymphocyte proliferation

Next, the effects of AsA and ACR on the mitogen-stimulated lymphoblastogenic responses were examined. XTT assays were performed by stimulating spleen cells with LPS or ConA, after exposure to AsA and ACR for 48 hr. As shown in Fig. 2, LPS- and ConA-treated lymphocyte proliferation was significantly increased by AsA in young mice, whereas there is no effect in aged mice except at 0.5 μg/mL. Furthermore, this increasing proliferation responsiveness to AsA at 0.5 μg/mL was slightly altered in mitogen-treated cells of aged mice as compared to young mice.

As shown in Fig. 3, ACR treatment resulted in decreased lymphocyte mitogenic activity in both young and aged groups. In contrast, AsA significantly increased the mitogenic response induced by LPS or ConA in splenocytes from young mice, whereas effect is attenuated in aged mice. This decreasing LPS-treated lymphocyte proliferation indicates the possible suppressive effect of ACR on B cell proliferation, leading to the reduction of antibody production in aged states. Moreover, we found that ACR was able to cause a dose-dependent inhibition of murine lymphocyte responsiveness to ConA

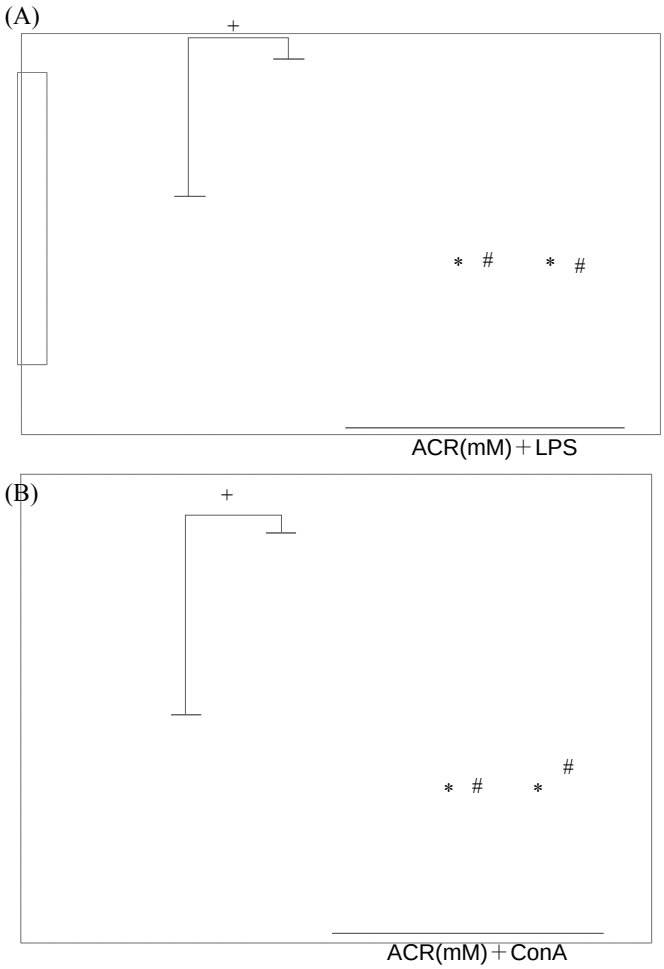

Fig. 3. The effects of ACR on the proliferation of LPS-induced B lymphocytes (A) and ConA-induced T lymphocytes (B) in young and aged C57BL/6 male mice. (A) The proliferative effect of ACR on LPS-induced B lymphocytes in the spleen. Splenocytes (1×10^7 cells/mL) were treated with various concentrations of ACR and LPS for 48 hr. (B) The proliferative effect of ACR on ConA-induced T lymphocytes in mice spleen. Splenocytes were treated with various concentrations of ACR and ConA for 48 hr. $^{*}p<0.05$, significantly different from the ConA- or LPS-treated young group. $^{\#}p<0.05$, significantly different from the ConA- or LPS-treated aged group. $^{+}p<0.05$, significantly different from untreated group.

and LPS, although T cell mitogenic response was less effective in aged animals. Based on these findings, it is possible that the inhibition of lymphocyte proliferation response to immunomodulators is due to shrinkage of thymus in aged mice.

As shown in Fig. 2(B) and 3(B), ConA-stimulated lymphocyte proliferative responses of T cells from spleens of aged mice were much lower than those of cells from young mice (Fig. 3B). Taub and Longo (7) have reported that aging-associated immunological alterations can be found in lymphoid organs as well as in cellular components of the immune system, and T cells are more susceptible to the effects of aging. This susceptibility of T cells to aging is believed to result from the progressive loss of thymus function during aging with the consequent decrease in the output of naive T cells into the peripheral T cell pool (7). In addition, Speziali et al. (15) demonstrated that although some antigen-specific antibody responses were decreased, total antibody production, frequency of antibody-producing cells and proliferative response of spleen T cells were enhanced in aged mice. Our results also showed that the proliferative response of non-stimulated spleen cells from aged mice by AsA were augmented and that LPS-stimulated B lymphocyte proliferation is not impaired. In aged mice, the susceptibility of B and T cell and their functions in specific humoral and cellular immune responses may be different.

CONCLUSION

The present data indicate that the effects of ACR and AsA on lymphocyte proliferation do not differ, but the responsiveness of AsA on mitogen-induced cell proliferation is significantly lower in aged mice. Moreover, AsA increased mitogen-treated lymphocyte proliferation in young mice, whereas, at same concentration, it had little effect on the T-lymphocyte proliferation in aged mice.

The current mode of thinking is that nutritional effects are similar for youth and the elderly, such that if something has a positive immunlogical effect in the young, it is likely also good for the elderly. However, our data suggests that certain compounds have differential immunomodulating effects and responsiveness in younger versus older mice, and may regulate different cytokines and growth factors in age-associated conditions. Thus, level of nutrition at various ages might need to be considered to select proper immunomodulators.

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