lation will be older than 65 years of age at the beginning of the twenty-first century. Accompanying this increased percentage of elderly people will be an increased incidence of geriatric disorders and infectious diseases associated with the decrease of cellular immunity that occurs during aging. If this decrease of cellular immunity can be diminished, it may improve the health state of elderly people and prolong their life expectancy. Meydani *et al.* have found that a higher intake of vitamin E is needed to maintain immune functions in the aged people [*1*] and improves the decreased cellular immune functions that occur with aging [*2*]. This finding is related to the decreases in the serum vitamin E level during aging and the actions of vitamin E as an antioxidant and immunostimulator. This review focuses on the effect of both vitamin E deficiency and supplementation on the immune responses in animals and humans, and the immunomodulating effects of vitamin E in aging. In addition, as recent topics in the research on vitamin E and the immune response, the actions of vitamin E on both T cell differentiation in the thymus and abnormal increases of immune functions such as allergy and autoimmune disease are discussed.

I. Effects of Vitamin E Deficiency and Supplementation on Immunity

Vitamin E Deficiency and Immunity

As summarized in Table 1, most studies have shown that vitamin E deficiency induces the impairment of both humoral and cellular immunity. These immunosuppressive effects of vitamin E deficiency are associated with increased production of free radicals, which results in increased lipid peroxidation in cell membranes. In fact, macrophages and neutrophils from vitamin E deficient rats had higher O_2 consumption, H_2O_2 release, and peroxidized lipids in their membranes[*3*, *4*]. Although vitamin E deficiency appears to induce the decreased cellular immunity as described above, the phagocytic function of alveolar macrophages (AM) from rats fed a vitamin E deficient diet for 4 months was 2 times higher than that of control rats (Fig. 1) [*5*]. In addition, those AM did not respond to the *in vitro* treatment with macrophage-activating factor (MAF) prepared from Con A-activated rat splenic lymphocytes. Since MAF production from splenocytes activated *in vitro* with Con A-Sepharose beads decreased in vitamin E deficient rats, the higher phagocytic activity of AM from rats fed the vitamin E deficient diet does not appear to be induced by MAF. Sharp and Colston demonstrated the presence of highly activated macrophages in nude mice and considered that hyperactivity seen in the macrophages of nude mice resulted from a lack of T cell-mediated suppression [*6*]. As described above, vitamin E deficiency causes immunodepression, including T and B cell responses, which may result in inducing the higher phagocytic activity of AM.

Table 1. Vitamin E deficiency and the immune response.

Immune response	Result	Species
T-cell mitogenesis	Decreased	Humans, mice, rats, pigs, dogs
Interleukin-2 production	Decreased	Humans, rats
B-cell mitogenesis	Decreased	Mice, rats
Natural killer cell activity	Decreased	Mice, rats
Plaque-forming cell	Decreased	Mice
Antibody titer	Decreased	Mice, chickens
Macrophage phagocytosis	Decreased	Rats
	Increased	Rats
Polymorphonuclear leucocyte phagocytosis	Decreased	Humans, rats
Polymorphonuclear leucocyte chemotaxis	Decreased	Humans, rats

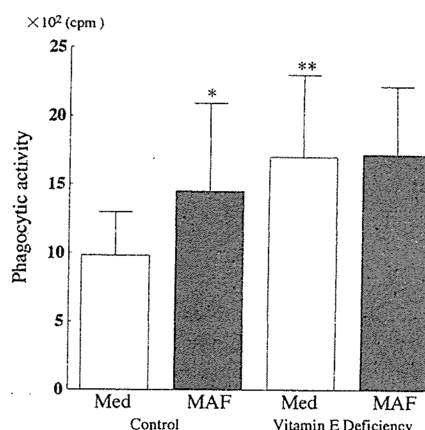


Fig. 1. Phagocytosis of ^{51}Cr-labeled opsonized SRBC by AM of rats fed a control diet or vitamin E-deficient diet. AM (2×10^5) were preincubated for 4 h in a medium consisting of RPMI 1640 with 5% fetal bovine serum (Med) or in a medium with macrophage-activating factor (MAF, 1/162 dilution). They were then incubated with ^{51}Cr-labeled opsonized SRBC for 2 h. Each value is the mean± SD for triplicate cultures. * Significantly different from cultures with medium ($p<0.05$); ** significantly different from cultures with medium in control rat ($p<0.01$). (Moriguchi *et al.*: *J. Nutr. Sci. Vitaminol.*, **35**, 419–430, 1989)

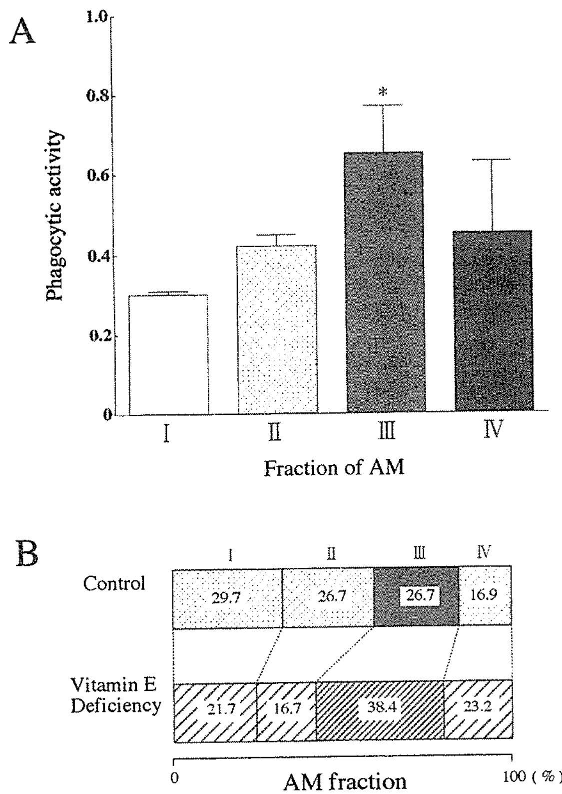


Fig. 2. Phagocytic activity of fractionated AM (A) and the proportions of fractionated AM (B) in rats fed a control or vitamin E-deficient diet. After AM were collected by tracheopulmonary lavage with warm saline, separation of the AM fraction was performed by using discontinuous Percoll gradient centrifugation. Discontinuous Percoll solutions were prepared by diluting a stock of Percoll with sterile saline to specific gravities of 1.050, 1.060, 1.070, and 1.080 and subsequently layered into centrifuge tubes. AM suspensions were carefully layered on the top of the diluted Percoll solutions and centrifuged at 400 g for 30 min. The cells localized at each interface area were then carefully collected with a Pasteur pipette. The fractions were designated Ito IV in order of increasing density. In this experiment, the phagocytic activity of AM was assessed by measuring the absorbance of SRBC phagocytosed within AM. * Significantly different from other fractions (p<0.05).

In a recent study, we separated AM into four fractions (I to IV) by discontinuous Percoll density-gradient centrifugation. The degree of AM maturation is high from Fraction IV to Fraction I. As shown in Fig. 2A, the phagocytic activity of AM is the highest in Fraction III. We have found that vitamin E deficiency decreased the number of AM and increased the percentage of AM in Fraction III having the higher phagocytic ability against opsonized sheep red blood cells (SRBC) (Fig. 2B) [7]. These results suggest that vitamin E deficiency appears to induce the destruction of highly matured AM (Fraction I), which results in a decreased number of AM and a higher phagocytic activity of AM.

Table 2. Vitamin E supplementation and the immune response.

Immune response	Result	Species
T-cell mitogenesis	Increased	Humans, mice, rats, pigs
	No change	Humans
Interleukin-2 production	Increased	Humans, mice, rats
T-cell helper activity	Increased	Mice
B-cell mitogenesis	Increased	Mice, rats
Natural killer cell activity	Increased	Mice, rats
Plaque-forming cell	Increased	Mice, rats
Antibody titer	Increased	Mice, rats, sheep
Serum antibodies	No change	Humans
Macrophage phagocytosis	Increased	Rats
Polymorphonuclear leucocyte phagocytosis	Increased	Humans
Delayed type hypersensitivity	Increased	Humans
	No change	Humans

Vitamin E supplementation and immunity

Contrary to the effect of vitamin E deficiency on the immune response, most studies have shown that vitamin E supplementation enhances the immune response as summarized in Table 2. As its mechanism, Meydani *et al.* found that vitamin E supplementation suppresses Prostaglandin E_2 (PGE_2) synthesis in spleen homogenate from old mice [*2*] and Baehner *et al.* showed that the *in vitro* administration of vitamin E prevents autoxidative damage to the membrane of polymorphonuclear leukocytes by scavenging H_2O_2 [*8*]. Because both PGE_2 and H_2O_2 are potent inhibitors of several lymphocyte functions, including mitogenesis, cytolysis and antibody production [*9–11*], vitamin E supplementation decreases the formation of PGE_2 and H_2O_2, which may result in the enhancement of mitogenic response and natural killer cell activity in splenic lymphocytes.

Studies on the effect of vitamin E supplementation on macrophage function are exceedingly few compared with those on lymphocyte functions. In particular, studies concerning AM, which play an important role in the defense against bacterias and neoplasms in the lung, are very limited. As shown in

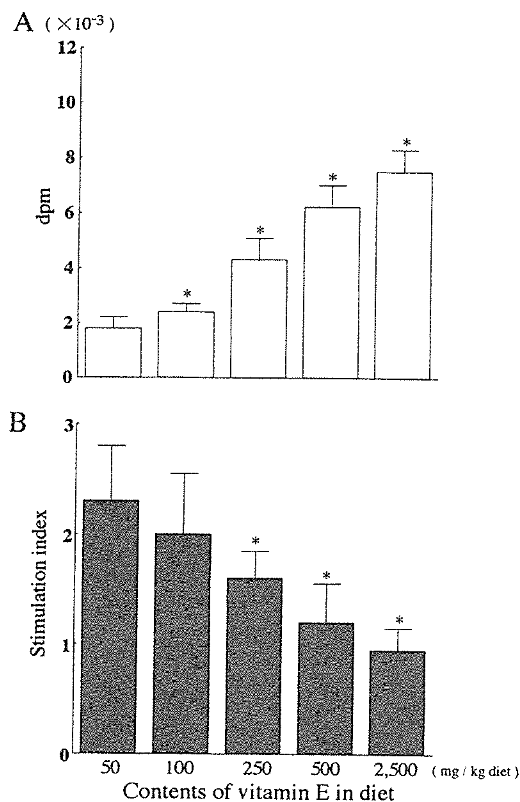


Fig. 3. Phagocytosis of ^{51}Cr-labeled opsonized SRBC by AM of rats fed high vitamin E diets. AM (2×10^5) were preincubated for 4 h in medium or in medium with macrophage-activating factor (MAF, 1/162 dilution). They were then incubated with ^{51}Cr-labeled opsonized SRBC for 2 h. (A) Phagocytic activity of AM incubated with medium only. Each value represents the radioactivity of ^{51}Cr-labeled opsonized SRBC within AM and is the mean±SD for 10 rats. (B) The enhancement of phagocytic activity following *in vitro* treatment with MAF. Each value represents the stimulation index and is the mean±SD for 10 rats. The stimulation index was calculated by assigning a value of 1 to the phagocytic activity of control AM incubated with medium only and by comparing this to the phagocytic activity of AM from each group treated with MAF. * Significantly different from the controls (50 mg of vitamin E/kg of diet) ($p<0.05$). (Moriguchi *et al.*: *J. Nutr.*, **120**, 1096–1102, 1990)

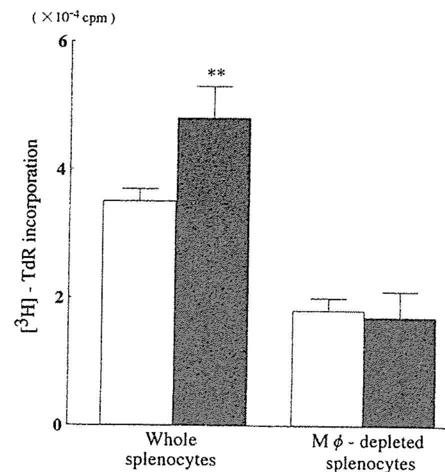


Fig. 4. *In vitro* effect of vitamin E (2 μg/ml) on the proliferation of whole and Mφ-depleted splenocytes with Con A (5 μg/ml) for 72 h. Unshaded, medium; shaded, vitamin E. ** Significantly different from whole splenocytes incubated with medium alone ($p<0.01$). (Oonishi *et al.*: *J. Nutr. Sci. Vitaminol.*, **41**, 445–453, 1995)

Fig. 3A, the ability of AM to phagocytose opsonized SRBC was significantly and dose-dependently enhanced by feeding high vitamin E diets [*12*]. In addition, those AM from rats fed high vitamin E diets exhibited decreasing responses to MAF, prepared from splenic lymphocytes *in vitro* activated with Con A for 48 h, with increasing contents of vitamin E (Fig. 3B). These results suggest that AM from rats fed high vitamin E diets had already been activated by MAF.

Macrophages are known to be major prostaglandin-producing cells and also play a critical role in the regulation of immune responses by releasing cytokines that affect lymphocyte functions [*13*, *14*]. As described previously, it has been reported that a high vitamin E diet decreases the production of PGE_2 from macrophages and enhances cellular immune functions [*12*, *15*]. However, the precise mechanism by which vitamin E stimulates lymphocyte proliferation remains unclear. Specifically, it is not known whether vitamin E first stimulates macrophage function or lymphocyte functions. Oonishi *et al.* investigated the *in vitro* effect of vitamin E on the proliferation of both whole splenocytes and macrophage-depleted splenocytes [*16*]. They found that the proliferation of whole splenocytes was significantly higher than that of macrophage-depleted splenocytes at all concentration of Con A (0.5–10 μg/ml). In addition, when whole and macrophage-depleted splenocytes were preincubated with vitamin E (2 μg/ml) for 24 h, the proliferation of whole splenocytes was significantly enhanced compared with that of whole splenocytes preincubated with medium alone. In contrast, macrophage-depleted splenocytes did not show any increase of splenic lymphocyte proliferation following *in vitro* pretreatment with vita-

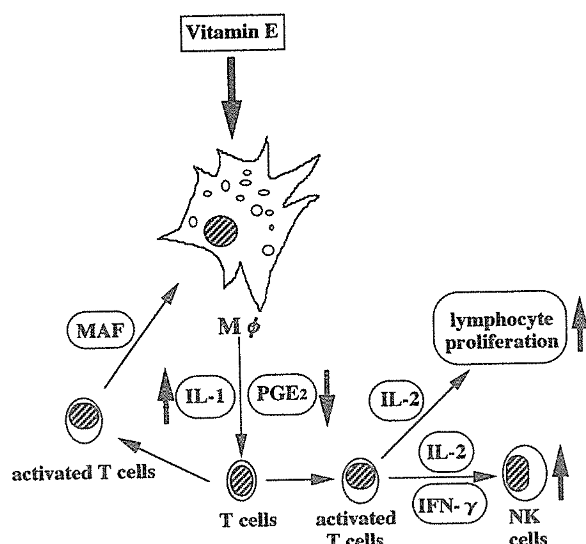


Fig. 5. Scheme on the activation of macrophages or natural killer (NK) cells by vitamin E.

min E (Fig. 4). This result suggests that vitamin E has the ability to stimulate splenic macrophages directly. In these *in vitro* experiment, the culture medium contained 2-mercaptoethanol (2-ME; 50 μM), which is also a potent antioxidant. The addition of 2-ME to culture media has been found to enhance the DNA synthetic response of lymphocytes to mitogens [*17*]. Oonishi *et al.* also found that the addition of 2-ME to the culture media induces a higher proliferation of splenic lymphocytes, comparable to that of vitamin E (2 μg/ml) [*16*]. This suggests that the action of vitamin E to enhance lymphocyte proliferation may be masked by the addition of 2-ME to the culture media. Furthermore, they investigated whether or not macrophages pretreated with both vitamin E (2 μg/ml) and Con A (5 μg/ml) *in vitro* for 24 h had the ability to enhance the proliferation of splenic lymphocytes. As its result, it was found that the addition of macrophages pretreated with both vitamin E and Con A to splenic lymphocyte cultures could induce even higher proliferation of splenic lymphocytes compared with the addition of splenic macrophages pretreated with Con A alone. Taken together, vitamin E may first activate macrophage function as an immunostimulator rather than an antioxidant, and then activate lymphocytes, including T cells and NK cells, as summarized in Fig. 5.

II. Vitamin E and Decreased Cellular Immunity with Aging

Animal Studies

Even though the life expectancy of experimental animals such as mice and rats is very short, it needs at least 1 to 2 years to investigate the effect of vitamin E on immune functions with aging. A good model for studying aging has been established in mice, which are called as the senescence-accelerated mouse (SAM) [*18*]. It is known that this mouse strain shows a marked decrease of splenic lymphocyte proliferation with mitogens from 6 months of age [*19*]. Muraga *et al.* have tried to investigate whether the decreased mitogen response of splenic lymphocytes in SAM is associated with the nutritional status of vitamin E and whether a high vitamin E diet can restore the decreased mitogen response of splenic lymphocytes in SAM. They found that there was no significant difference in serum vitamin E concentration between old SAM-P1 and SAM-R1 as control. Since a high vitamin E diet induced a similar increase of serum vitamin E concentration in both old SAM-P1 and SAM-R1, the vitamin E status in SAM-P1 appears not to be impaired and maintain a normal level [*20*]. In addition, a high vitamin E diet did not restore the decreased mitogen response of splenic lymphocytes in SAM-P1. Because Muraga *et al.* found that the percentage of adherent cells, mainly macrophages, in splenocytes was remarkably decreased in old SAM-P1 and their macrophages did not have the enhancing effect on proliferation of splenic lymphocytes from young SAM-R1, they came to conclusion that the decreased mitogen response of splenic lymphocytes in SAM-P1 is not associated with vitamin E status and is due to the decreases of both number and function of macrophages.

In rats, since spontaneously hypertensive rats (SHR) were derived from Wistar Kyoto rats (WKY) by Okamoto and Aoki [*21*], they are now widely used as an animal model for the study of human essential hypertension. Because SHR exhibit an accelerated decrease in, and abnormalities of, cellular immune functions with aging, Takeichi *et al.* proposed that SHR are a good model for the study of not only human essential hypertension but also the mechanism of aging [*22*]. It has been found that SHR show the remarkable decreases in mitogenesis and NK activity of splenic lymphocytes in the early stage of life (3 months old), and vitamin E levels in

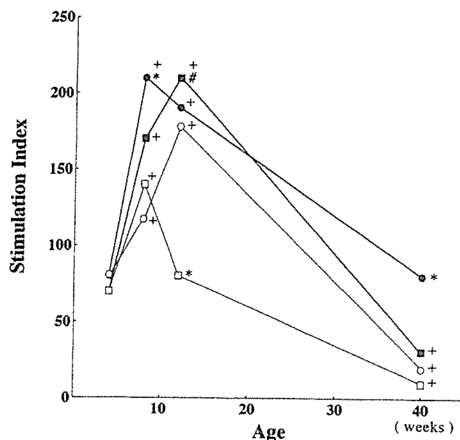


Fig. 6. Proliferation of thymocytes with Con A in WKY and SHR fed a control or high vitamin E diet for 40 weeks. (○) WKY; (●) WKY+vitamin E; (□) SHR; (■) SHR+vitamin E. +Significantly different from thymocyte proliferation with Con A in WKY or SHR fed a control diet at 4 weeks old ($p<0.05$). * Significantly different from WKY fed a control diet at the same age ($p<0.05$). # Significantly different from SHR fed a control diet at the same age ($p<0.05$). (Moriguchi *et al.*: *Nutr. Res.*, **15**, 401–414, 1995)

serum and thymus of SHR decreased significantly more during aging in SHR than in WKY[*23*]. It was considered that there are several explanations for decreased vitamin E levels in serum and thymus of SHR. First, because levels of vitamin E in the serum and thymus of SHR were increased when the rats consumed a high vitamin E diet, the decreased vitamin E level of SHR does not appear to be due to malabsorption of vitamin E. Second, the corn oil used in this experiment contained a considerable quantity of PUFA, which may also further promote the wastage of vitamin E in SHR. In addition, vitamin E supplementation (500 IU/kg of diet) prevented the decrease in proliferation of thymocytes observed in SHR after Con A stimulation (Fig. 6). However, NK activity of splenocytes from SHR was not affected by the high vitamin E diet and was significantly lower than that of WKY. In contrast, phagocytic activity of AM against opsonized SRBC was significantly higher in SHR than in WKY. As it is known that macrophages are nonspecifically activated in athymic, nude mice [*24*], AM in SHR, which have decreased functions of T cells, also may be nonspecifically activated. In short, this activation of AM may represent compensation for the defect in T cell-mediated immune system of SHR with aging.

The decrease in T cell function in SHR is also associated with increased production of natural thymocytotoxic autoantibody (NTA) with aging [*25*]. We have also found that NTA titer in the serum of SHR steadily increased with aging and that vitamin E supplementation suppressed this increase [*26*]. The increase of NTA in SHR is relevant to the impairment of T cell differentiation in the thymus. In other words, the decrease of NTA titer in the serum of SHR may be a mechanism in which the high vitamin E diet restores cellular immune responses decreased with aging. This is related to normalize T cell differentiation in the thymus of SHR. In fact, we have found that vitamin E supplementation improves the decreased expressions of CD4 and CD8 antigens on the membrane of thymic lymphocytes of SHR [*26*]. These data support that SHR is a good model for aging, especially for studying the effect of vitamin E supplementation and the possibility exists that high vitamin E diet has a potent effect on T cell differentiation in thymus. The effect of vitamin E on T cell differentiation in thymus will be described in the following head.

Human studies

Recent reviews on the beneficial effect of vitamin E for the immune system in the elderly have been summarized by Landmark [*27*], and Meydani and Beharka [*28*]. The effects of vitamin E supplementation on immune responses have been epidemiologically investigated on free-living elderly people. Goodwin and Garry failed to find any correlation between vitamin E intake and immune responses such as mitogen response, delayed cutaneous hypersensitivity, and serum antibodies in a population of healthy adults (65–94 years old) [*29*]. As its reason, they proposed the possibility that some of the previously reported immunoenhancing properties of megadose vitamins may be due to a nonspecific adjuvant effect disappearing with time. On the other hand, Chavance *et al.* found that plasma vitamin E levels were positively correlated with delayed type hypersensitivity response to diphtheria toxoid, candida, and trichophyton in 100 healthy subjects (>60 years old) [*30*]. They have also found that blood vitamin E concentrations were negatively correlated with the incidence of infectious disease episodes. Payette *et al.* also found that a negative correlation between dietary vitamin E level and IL-2 production in free-living elderly people [*31*]. However, because this study was performed by using dietary vitamin E

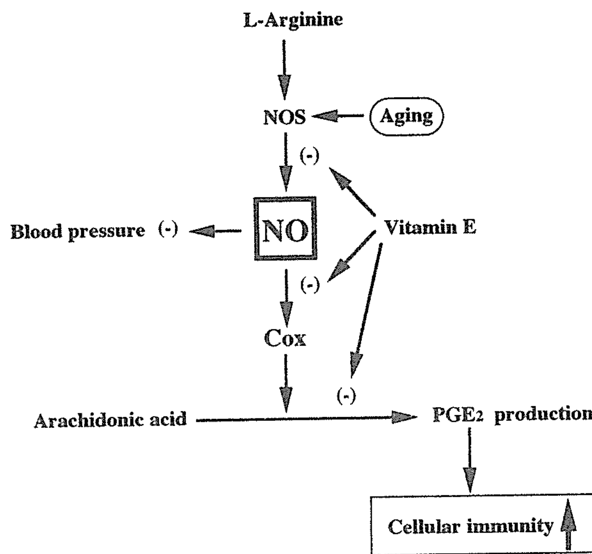


Fig. 8. A mechanism of vitamin E action on the improvement of cellular immunity decreased with aging.

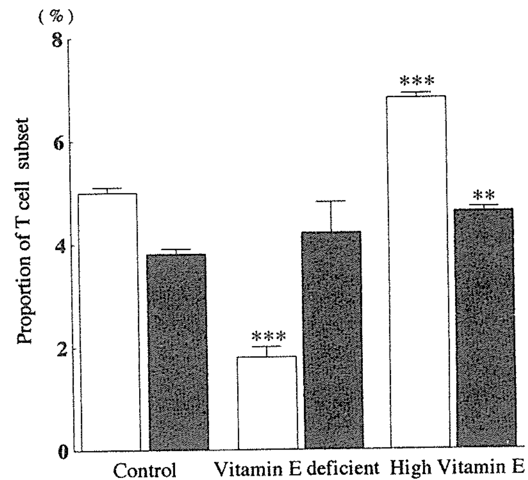
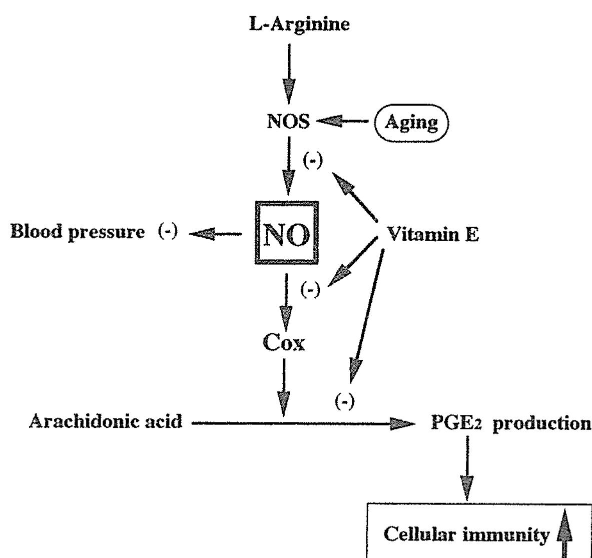


Fig. 9. Proportions of $CD4^+CD8^-$ (unshaded) and $CD4^-CD8^+$ (shaded) T cells in thymocytes of rats fed control, vitamin E free, and high vitamin E diets for 7 weeks. Significantly different from the control group (** $p<0.01$, *** $p<0.001$). (Moriguchi *et al.*: *J. Nutr. Sci. Vitaminol.*, **39**, 451–463, 1993)

blood lymphocytes with Con A ($p<0.05$) (Fig. 7B) [*35*]. This evidence suggests that the correction using plasma lipids needs to assess the effect of vitamin E on immune functions in the aged. In addition, although Meydani *et al.* found that PGE_2 level in blood was significantly increased in the aged, which associated with decreased cellular immunity in the aged [*36*], nitric oxide (NO) produced from macrophage and other cells has also an inhibitory effect on T cell proliferation with mitogen [*37*]. NO activates cyclooxygenase (Cox), leading to the production of PGE_2. Age-related increase in the production of PGE_2 may be due to increased NO production with aging, which appears to cause the decreased cellular immunity in the aged as shown in Fig. 7A. In other words, the decreased cellular immunity in the aged with the increase level of blood PGE_2 is closely associated with the increased NO production and the action of vitamin E improving the decreased cellular immunity in the aged is due to inhibit NO production from macrophages and other cells (Fig. 8). In fact, a negative correlation between plasma NO concentration and α-tocopherol/VLDL-cholesterol was seen as shown in Fig. 7C. These data support that it is important for elderly to take an enough intake of vitamin E for preventing the decrease of cellular immunity.

III. Vitamin E and T-Cell Differentiation in the Thymus

From the experimental results in SHR described previously, Moriguchi *et al.* speculated that vitamin is an important nutrient in T-cell differentiation in thymus. To test this hypothesis, 6-week-old Fisher rats were separated into three groups and fed diets containing various levels of α-tocopherol acetate: 0 (vitamin E free), 50 (regular), and 500 (high vitamin E)/kg of diet for 7 weeks. In this experiment rats fed the vitamin E free diet showed not only a decreased number of thymocytes but also a significant decrease of the percentage of $CD4^+CD8^-$(helper/inducer) T cells in thymocytes compared with rats fed the regular diet (Fig. 9) [*38*]. In rats fed a high vitamin E diet, the number of thymocytes was not altered, but the percentages of both $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells in their thymocytes increased significantly. Furthermore, IL-2 production by thymocytes was significantly greater than that in rats fed the regular diet. As Moriguchi *et al.* [*12*] have previously noted that AM can be greatly activated by high vitamin E diets, it is conceivable that an increase of macrophage function as antigen-presenting cells (APC) in the thymus may bring about the increased production of IL-2. In addition, the marked increases of $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells in thymocytes may be directly related to the increased production of



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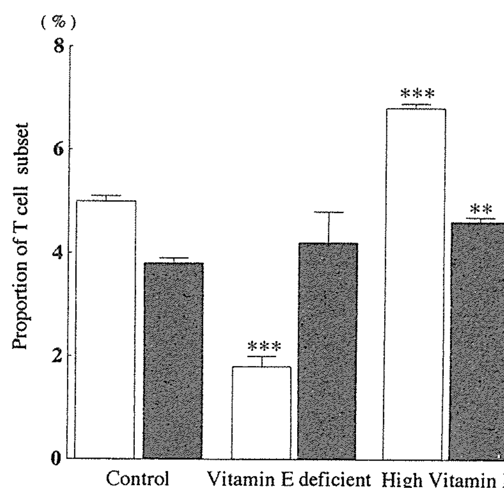

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IL-2 by thymocytes in the thymic medulla of rats fed the high vitamin E diet.

It is known that PGE_2 has an inhibitory effect on IL-2 production by activated T cells [*39*]. A marked decrease of IL-2 production following consumption of vitamin E free diet and an increase after consumption of a high vitamin E diet may be closely related to the degree of PGE_2 synthesis. In fact, Moriguchi *et al.* have reported an increase in PGE_2 production in vitamin E deficiency and a decrease after high vitamin E supplementation [*38*]. These changes in PGE_2 production by thymocytes may affect production of IL-2 by T cells in thymocytes and result in a decreased percentage of $CD4^+CD8^-$ T cells in thymocytes of the vitamin E free group and increased percentage in those of the high vitamin E group. In fact, since addition of indomethacin, an inhibitor of PGE_2 synthesis, to the thymocyte cultures enhanced IL-2 production by thymocytes of rats fed a vitamin E free diet, the increased production of PGE_2 in thymocytes appears to be closely related to the decreased production of IL-2 in the vitamin E free group.

Thymic epithelial cells (TEC) play an important role in the differentiation of T cells [*40*]. Moriguchi and Itoh have found that the ability of TEC isolated from thymocytes of rats fed a high vitamin E diet to bind to immature T cells was greater than that of TEC from rats fed a regular diet [*41*]. Anderson *et al.* showed that the contact of immature T cells to TEC is more important than soluble factors from TEC in positive selection of $CD4^+CD8^-$ T cells [*42*]. Moriguchi and Itoh have also found that the supernatant of a TEC culture does not affect the differentiation of T cells *in vitro.* These results suggest that vitamin E enhances T cell differentiation through increased binding of immature T cells to TEC in the thymic cortex. Although it is not known how vitamin E increases the binding of immature T cells to TEC, vitamin E may enhance the expression of adhesion molecules. Moriguchi and Itoh have found that vitamin E supplementation or *in vitro* addition of vitamin E to the culture medium enhances the expression of intracellular adhesion molecule-1 (ICAM-1) in TEC. However, they have failed to find that *in vitro* incubation with macrophages isolated from rats fed a high vitamin E diet induces a significant change in T cell subsets in immature T cells. These results suggest that vitamin E enhances T-cell differentiation not through macrophage function (negative selection) but through an increased binding capacity of TEC to immature T cells *via* increased expression of ICAM-1 (positive selection).

IV. Vitamin E and Immune Diseases

Vitamin E and acquired immune deficiency syndrome (AIDS)

Acquired immune deficiency syndrome (AIDS) is a clinical disorder caused by the human immunodeficiency virus (HIV). It represents the end point in a progressive sequence of immunosuppressive changes that render the body highly susceptible to tumors and opportunistic infections. Currently, AIDS is one of the most serious public health problems in the world. Although many studies have been done from the viewpoints of prevention and treatment for AIDS, there are still few studies on the effect of nutrition against the development of AIDS.

Since reactive oxygen species promote HIV replication [*43*], previous studies have found that antioxidants such as vitamin C and *N*-acetyl-L-cysteine (NAC), the precursor of glutathione (GSH), are effective in inhibiting HIV replication [*44*]. Odeleye and Watson have reviewed the potential role of vitamin E in the treatment of immunologic abnormalities during AIDS [*45*]. They described that vitamin E is nontoxic over a wide range of intakes and a moderately high dose may be used to target and stimulate some specific immune cells destroyed by HIV infection. Moseson *et al.* have also reviewed the potential role of nutritional factors in the induction of immunologic abnormalities in HIV-positive homosexual men [*46*]. Since malnutrition and intestinal nutrient malabsorption have been found in AIDS patients, they have described that dietary manipulations might diminish the immune defects in HIV infection and enhance resistance to opportunistic infections.

Murine AIDS has often been used as an AIDS model instead of human AIDS. Although the etiology between human and murine AIDS, induced by LP-BM5 retrovirus infection, is different in some points, including the changes of the T cell subsets, both are closely similar in the functional changes following development of AIDS. Murine AIDS is characterized by lymphadenopathy, splenomegaly, hypergammaglobinemia, deficient B-cell response to mitogen, functional deficiency of T cells, and cytokine dysregulation as shown in human AIDS [*47*]. In addition, it is also known that azidodideoxythymi-

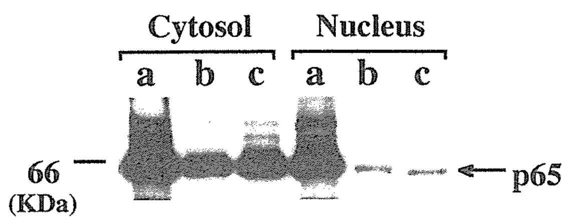

Fig. 10. Effect of vitamin E supplementation on the expression of NF-κB in cytoplasm and nucleus of splenic lymphocytes of rats infected with LP-BM5 retrovirus. Twenty microgram of each protein was analyzed by Western blotting. (a) Control diet; (b) high vitamin E diet before LP-BM5 retrovirus infection; (c) high vitamin E diet after LP-BM5 retrovirus infection. (Hamada *et al.*: *Nutr. Res.*, **20**, 1163–1171, 2000)

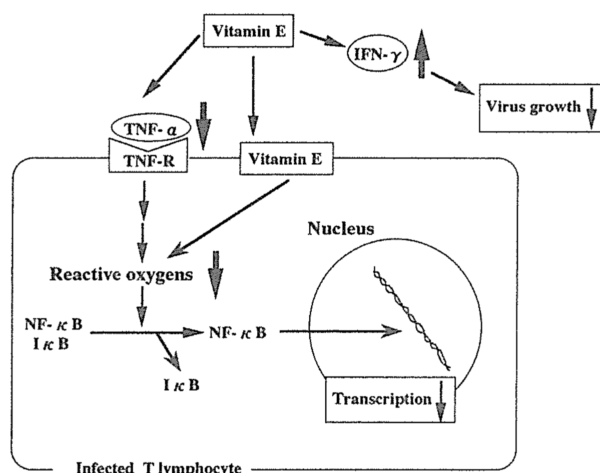

Fig. 11. Mechanism of the improvement of immune dysfunction in murine AIDS following vitamin E supplementation.

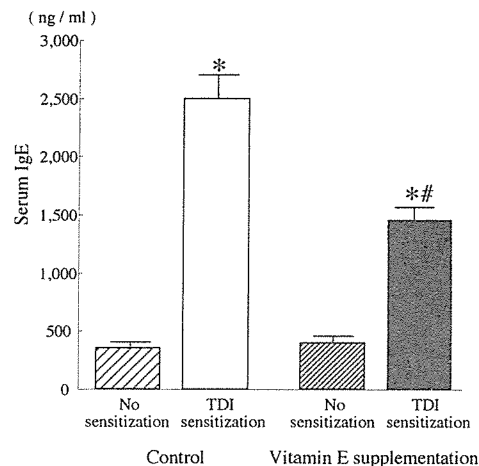

Fig. 12. Effect of vitamin E supplementation on serum IgE production from TDI-sensitized mice. Mice were sensitized by dropping 2 μl of 5% toluene diisocyanate (TDI) dissolved in ethyl acetate from an autopipette into the nostrils under slight ether anesthesia for consecutive days. This was repeated after a week of rest. No sensitization groups were similarly treated with the vehicle, ethyl acetate. The mice were again allowed 1 week of rest, after which all groups were provoked with 5 μl of 2.5% TDI without anesthesia to induce nasal allergy-like symptoms. One week after provocation, the mice were anesthetized with sodium pentobarbital and were exsanguinated by cardiac puncture. Each value is the mean±SE. * Significantly different from no sensitization mice fed a control or vitamin E-supplemented diet ($p<0.01$). # Significantly different from TDI sensitized mice fed a control diet ($p<0.01$). (Zheng *et al.*: *Am. J. Med. Sci.*, **318**, 49–54, 1999)

dine (AZT), a drug for treatment of human AIDS, is effective for treatment of murine AIDS [*48*]. These reports support that murine AIDS is a useful model for studying the treatment and prevention of human AIDS.

It is known that NK-κB enhances HIV replication through activation of the HIV long-terminal repeat (LTR) [*49*]. In addition, reactive oxygen species promote the dissociation of NF-κB from I-κB and subsequently increase the production of NF-κB [*43*, *44*]. As shown in Fig. 10, Hamada *et al.* have found that vitamin E supplementation suppresses the expression of NF-κB in splenic lymphocytes from mice infected with LP-BM5 retrovirus [*50*]. Especially, the suppressive effect of vitamin E is strongly shown in the nucleus rather than in the cytoplasm. This fact suggests that the suppressive action of vitamin E against NF-κB expression is not only the dissociation of NF-κB from I-κB but also the translocation of NF-κB to the nucleus. Figure 11 shows that there are two systems of vitamin E actions improving immune dysfunction in murine AIDS. First, vitamin E increases the production of IFN-γ, an antiviral cytokine produced from activated T cells. Vitamin E may inhibit the development of murine AIDS through the enhancement of host antiviral activity. Second, since vitamin E is a potent antioxidant and decreases the production of TNF-α, which enhances the production of reactive oxygen species and subsequently increases the expression of NF-κB, vitamin E may directly and indirectly inhibit the production of reactive oxygen species and NF-κB expression,

and result in inhibiting the development of murine AIDS.

Vitamin E and abnormal increase of immune system

In this part we describe effects of vitamin E supplementation on two types of diseases with immunoabnormality such as allergy and autoimmune disease. Zheng *et al.* have found that high doses of vitamin E supplementation suppress nasal allergy [*51*]. As shown in Fig. 12, groups B [control+toluene diisocyanate(TDI) sensitization] and D (vitamin E+TDI sensitization) had higher ($p<0.01$) serum IgE concentrations compared with groups A (control) and C (vitamin E). However, serum IgE concentrations in group D were significantly lower than those in group B ($p<0.05$). As its mechanism it has been shown that vitamin E has a direct inhibitory effect on serum IgE formation in mice [*52*]. In addition, vitamin E acts as an antioxidant in cellular membranes and scavenges free radicals by blocking the peroxidation of PUFA, modifying prostaglandin formation, and thereby enhancing production of PGI_2 [*53*]. It is known that PGI_2 has an inhibitory effect on the formation of histamine [*54*], which is a major chemical mediator in the events of TDI-induced allergy, and causes itching, sneezing, and rhinorrhea. From these reports it appears that vitamin E may have reduced nasal allergic symptoms by inhibition of the production of histamine. Fogarty *et al.* have also investigated the relation between dietary vitamin E intake and serum IgE concentrations and atopy, measured as allergen skin sensitization, in a random sample of 2,633 adults [*55*]. As its result they found that higher concentrations of vitamin E intake were associated with lower serum IgE concentrations and a lower frequency of allergen sensitization.

By using MRL/MP-lpr/lpr (MRL/lpr) mice Weimann and Hermann have found that vitamin E beneficially affects the development of the SLE-like (systemic lupus erythematosus) autoimmune disease [*56*]. In addition, Kuemmerie *et al.* investigated the effects of fish oil, with and without α-tocopherol, on the course of IgA nephropathy [*57*] and found that vitamin E, more so than fish oil, mitigates the injury and promotes repair in experimental IgA nephropathy. Bae *et al.* investigated on plasma antioxidant/oxidant status and the dietary nutrient intake of 97 consecutive patients with SLE and 97 age- and sex-matched healthy controls [*58*]. They have found that plasma antioxidant status is impaired and dietary antioxidant intake is decreased in patients with SLE. In particular, they found the plasma α-tocopherol concentration was lower in those patients. As described above, vitamin E supplementation may be effective for treatment of those patients having vitamin E deficient status. Further studies are necessary to define the exact mechanism of vitamin E modulating the immunoabnormalities in allergy and autoimmune diseases.

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Beneficial effects of the vegetable juice Aojiru on cellular immunity in Japanese young women

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Abstract

This study was performed to investigate the effect of vegetable juice, Aojiru, on cellular immune functions of healthy young women (n = 19, aged 18–22 yr). They drank 180 mL of Aojiru daily for 4 weeks, and then their blood samples were collected at 2 and 4 weeks after study onset. Aojiru intake had no effect on the proportion of either natural killer cells (NK) or T-lymphocytes in their peripheral blood. There was a positive correlation between NK activity and the proportion of $CD16^+$ $CD56^+$ (NK) cells in their peripheral blood lymphocytes (PBL). In addition, NK activity per cell increased with prolonged Aojiru intake. Serum interleukin-2 (IL-2) concentration in subjects was significantly higher after 4 week-feeding of Aojiru compared to levels at the onset of this experiment. This result suggests that Aojiru has an ability to enhance NK activity, possibly through the increased production of IL-2. Therefore, it may be a useful supplement to promote health. © 2004 Elsevier Inc. All rights reserved.

Keywords: Aojiru; Kale; NK activity; Interleukin-2

1. Introduction

In Japan, lifestyle-related diseases such as cancer, heart disease, cerebrovascular disease, diabetes, hyperlipidemia, and hypertension have increased in recent years. Among these

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diseases, the most common cause of death is cancer. However, the rate of death from stomach cancer has dramatically decreased and the rates of death from lung and colon cancers steadily increase every year in both men and women [1–3]. The increase in colon cancer appears to be related to changes in dietary habits in Japan. Traditional Japanese dietary habits have changed to the Western style, which increases intakes of animal protein and fat; this results in the increased ratio of fat-derived energy compared to carbohydrate-derived energy. This change of dietary habits in Japan involves decreased intakes of both vegetable and rice, which is closely associated with a decreased intake of dietary fiber. To reduce the incidence of colon cancer, we Japanese have to change our dietary habits and eat more vegetables and rice to increase the intake of dietary fiber and vitamins. Aojiru is a juice prepared from kale, a plant akin to cabbage, and is named for its green color. At present, 400,000 households periodically purchase and drink Aojiru daily. The effects of Aojiru on health promotion were investigated by replies to a questionnaire sent to 1582 subjects drinking Aojiru daily. It was shown that Aojiru has a beneficial effect on constipation (20.7%), the common cold (13.3%), and hypertension (12.6%) [4]. Although the mechanism of health promotion by Aojiru is not fully understood, we have previously found that NK activity was significantly increased by feeding a diet with freeze-dried Aojiru to male rats [4]. In this study, we tried to investigate whether Aojiru has an ability to enhance NK activity of PBL in young women.

2. Methods and materials

2.1. Subjects

The study subjects consisted of 19 healthy young women aged 18–22 years. Women with disease or engaged in heavy exercise training were excluded from the experiment. The selected subjects agreed to drink 180 mL/day of Aojiru for 4 weeks. There was no limitation placed on their food intake during the experiment. No subjects took any medication during this study. Subjects received oral and written information about the study and gave their written consent. The study was approved by the Clinic Research Ethics Committee of Yamaguchi Prefectural University, Yamaguchi, Japan.

2.2. Contents of Aojiru

Aojiru, named for its color, is a juice prepared from kale, which is a plant akin to cabbage. Aojiru was obtained from Q'sai Co. (Fukuoka Japan). As shown in Table 1, the main component of Aojiru is water (~95%). The content of Ca in Aojiru is high and almost equal to that of a bottle (100mL) of milk. The contents of vitamins A and C are also high and are equivalent to levels of these vitamins in five and three tomatoes, respectively.

2.3. Isolation of PBL and preparation of serum

After overnight fasting, blood samples were obtained between 8:00 and 8:30 AM and collected in heparinized Vacutainer tubes (Becton Dickinson, San Jose, CA).

Table 1
Ingredients of Aojiru (100 g)

Protein (mg)	1313
P (mg)	30
K (mg)	289
Ca (mg)	146
Mg (mg)	23
Caroteinoids (mg)	1.05
Vitamin A (IU)	580
Vitamin C (mg)	196
Vitamin E (mg)	0.4
Fiber (mg)	200
Water (g)	94.8

Each value is the mean average in Aojiru produced from kale.

Peripheral blood mononuclear cells (MNC) were immediately isolated from whole blood by lymphocyte separation medium (LSM at 2000 rpm for 20 minutes). Cells were suspended in RPMI 1640 medium containing 5% heat-inactivated fetal bovine serum (FBS) (Cansera International Inc., Ontario, Canada), 100,000 U/L bicarbonate benzylpenicillin potassium (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and 1000 μg/L streptomycin sulfate (Wako Pure Chemical Industries, Ltd., Osaka, Japan). They were then counted, adjusted to 1×10^6/mL, and immediately used for measuring NK cell activity.

After blood samples were centrifuged at 3000 rpm for 20 minutes, serum from each subject was collected and stored at −70°C until use for measuring cytokine concentration [5].

2.4. Lymphocyte subpopulations

Whole-blood samples were stained with both fluorescein isothiocyanate (FITC)–conjugated anti-human CD3 monoclonal antibody (mAb), and phycoerythrin (PE) conjugated anti-human CD16 and CD56 mAb (Cosmo Bio Co., Tokyo, Japan). After washing with phosphate buffered saline (PBS) (Wako Pure Chemical Industries, Ltd., Osaka, Japan), stained cells were fixed with 0.5% paraformaldehyde in saline and were analyzed with a FACS Calibur flow cytometer and Cell Quest software program (Becton Dickinson, San Jose, CA) [6].

2.5. NK cell activity

NK cell activity was measured by using a fluorescence release assay which was a modification of the carboxy–fluorescein-diacetate (C-FDA) method [7].

In brief, K562, target cells, were labeled with C-FDA (100 μg/mL). They were prepared in triplicate at 20:1 (effector [peripheral blood mononuclear cells] to target cell ratio) in multiwell tissue culture plates (Asahi Techno Glass Co., Chiba, Japan). The plates were incubated at 37°C in 5% CO_2 incubator for 3 hrs. Then, 100μl of the supernatant was harvested from each well and their fluorescence intensity were measured by FL600 Micro-

plate Fluorescence Reader (BIO-TEK Instrument, Inc., Winooski, VT, USA). Spontaneous fluorescence release was determined from target cells incubated with medium alone. Maximum release was determined from target cells incubated with 100μL of 0.1 N NaOH. The percent lysis was calculated as follows:

$$\frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximum release} - \text{Spontaneous release}} \times 100.$$

2.6. Serum INF-γ and IL-2 concentrations

The serum samples before and 4 weeks after the onset of the experiment were placed in duplicate in multiwell tissue plates. IL-2 and IFN-γ concentrations were measured by using enzyme-linked immunosorbent assay (ELISA kit, Cayman Chemical Co., Michigan, USA for IL-2; Immunotech, Marseille, France for IFN-γ) [8].

2.7. Statistical analysis

Data are statistically evaluated by analysis of variance (ANOVA) with separation of treatment means (Duncan's multiple range test) using a statistical analysis program (Systat, Inc., Evanston, IL). A P value of < 0.05 was regarded as significant.

3. Results

3.1. Number of MNC and percentage of NK cells or mature T cells

The number of MNC significantly decreased with prolonging the period of Aojiru consumption (Fig. 1A). The percentages of NK cells and mature T cell in PBL of young women taking Aojiru for 2 or 4 weeks were not significantly different from those before the experiment (Figs. 1B and 1C).

3.2. Correlation between the percentage and activity of NK cells in PBL

There was a significant positive correlation between the percentage and activity of NK cells in PBL of young women ($y = 0.984x - 8.533$, $r = 0.797$) at the onset of the experiment (Fig. 2). Furthermore, a significant positive correlation at 4 weeks was also observed between the percentage and activity of NK cells ($y = 0.687x + 2.137$, $r = 0.591$). NK activity at 4 weeks after Aojiru intake was relatively higher than that at the onset of the experiment (Fig. 2).

3.3. Concentration of serum IL-2

Although we measured both IFN-γ and IL-2 concentrations in serum of each subject, IFN-γ levels were not within a detection range of the ELISA kit used in this experiment.

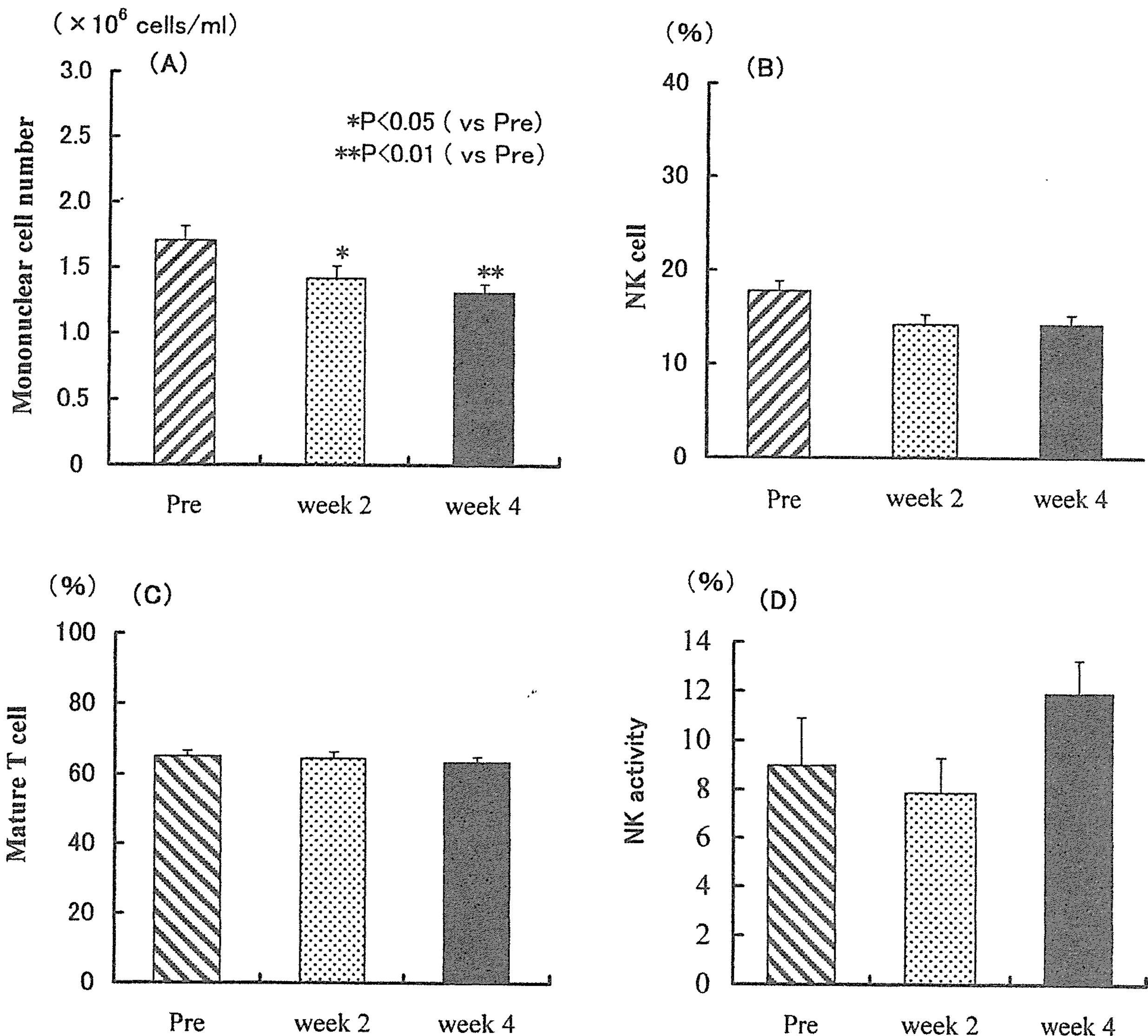

Fig. 1. Changes in number of mononuclear cells (MNC) (A), proportion of natural killer (NK) cells (B) and mature T cells (C), and change in NK activity (D) of peripheral blood lymphocytes (PBL) in young women ($n = 19$) after intake of the vegetable juice Aojiru pre-intake (open bars), at week 2 (striped bars), and at week 4 (filled bars). (*$P < 0.05$, **$P < 0.01$: significantly different from pre-experiment).

Concentration of serum IL-2, however, was significantly higher in the subjects taking Aojiru for 4 weeks compared to that at the onset of this experiment ($P < 0.01$) (Fig. 3).

4. Discussion

The purpose of this study was to clarify the influence of Aojiru intake on NK activity of PBL in healthy young women. It is known that NK activity is greatly depressed by various stresses such as cigarette smoking, exercise-induced increases in plasma epinephrine, and emotional stress [9–11]. As the taste of Aojiru juice is not pleasant, Aojiru intake itself may

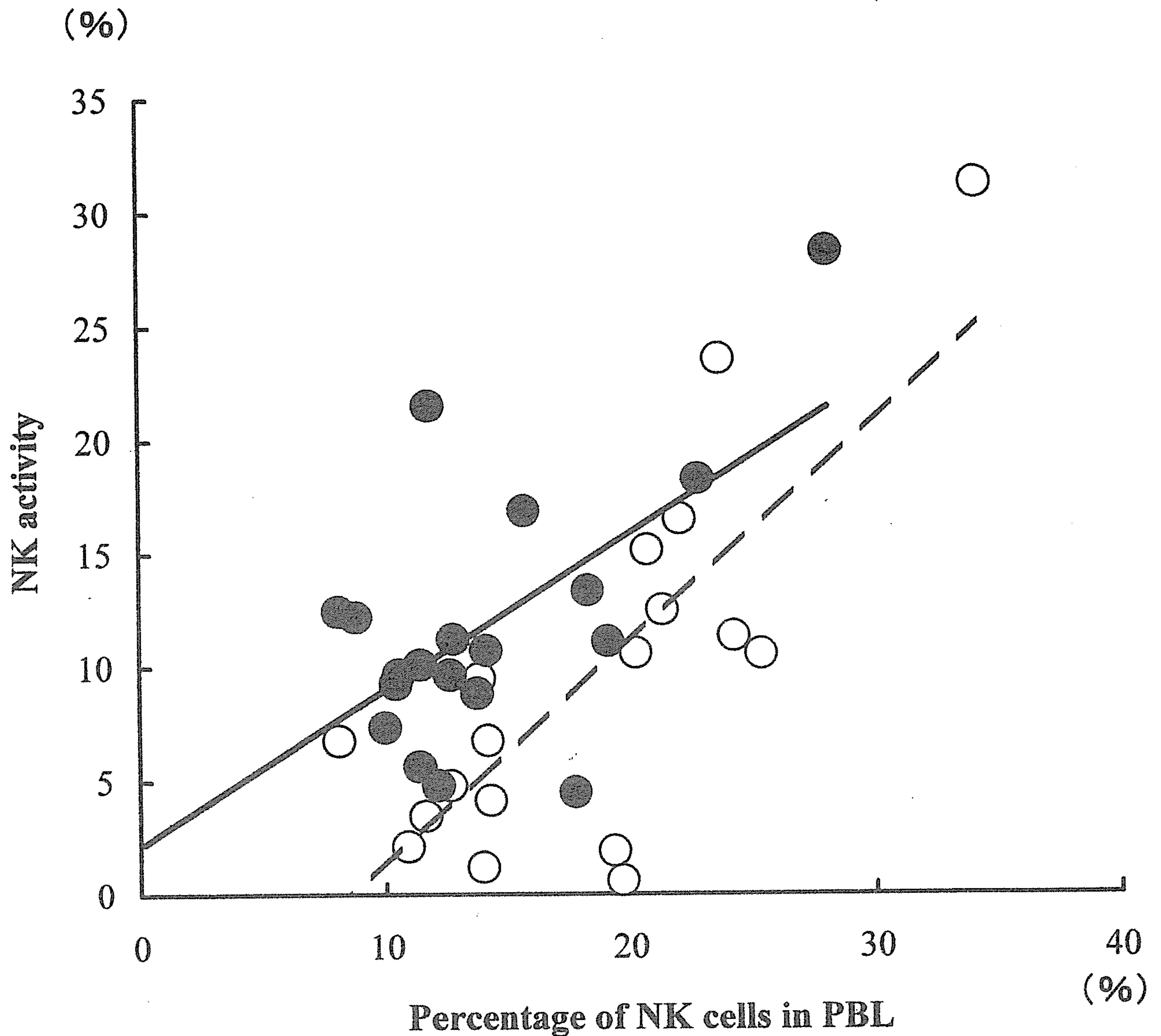

Fig. 2. Changes in the correlation between the proportion of natural killer (NK) cells and NK activity of peripheral blood lymphocytes (PBL) from young women ($n = 19$) after intake of the vegetable juice Aojiru for 4 weeks (onset of experiment [open circles]: y = 0.984x − 8.533, $r = 0.797$; 4 weeks after intake of Aojiru [filled circles]: y = 0.687x + 2.137, $r = 0.591$).

be a source of stress for some individuals and may induce a decrease in NK activity. To avoid this possibility, we selected subjects who already drank Aojiru juice and did not find this stressful. As a result, the number of subjects decreased from 70 to 19 at the onset of this study. Following this procedure it is considered that NK activity obtained in this study was little affected by the taste of Aojiru juice.

In the present study, Aojiru intake induced significant decreases in numbers of MNC (Fig. 1A). However, this does not appear to be due to the adverse effect of Aojiru juice, because the proportions of NK cells and mature T cells, and NK activity were not significantly decreased compared to values at the onset of this study, as shown in Fig. 1B, 1C and 1D. It

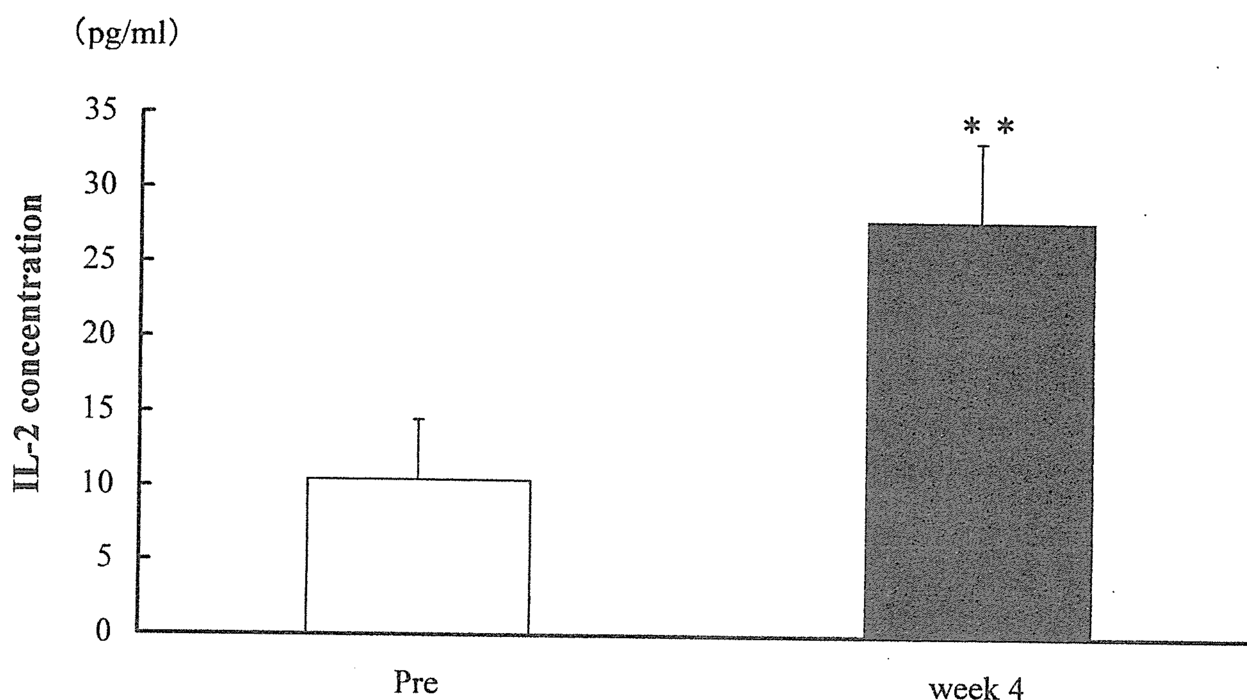

Fig. 3. Change in serum interleukin-2 (IL-2) concentration in young women (n = 19) after intake of the vegetable juice Aojiru for 4 weeks (pre-experiment [open bar]: 10.5 ± 4.0 pg/mL; at week 4 [filled bar]: 28.0 ± 5.3 pg/mL). **P < 0.01, significantly different from pre-experiment.

is unknown why Aojiru intake induces a decrease of MNC number. As shown in Fig. 1D, NK activity showed an upward trend (not significant) after Aojiru intake for 4 weeks. This result is in agreement with results of our previous study using splenocytes of rats fed a diet containing the freeze-dried Aojiru [4]. The mechanisms by which Aojiru intake enhances NK activity may be as follows: 1) Aojiru intake may increase the proportion of NK cells in MNC, or 2) Aojiru intake may induce the activation of NK cells. Given that Aojiru intake did not change the proportion of NK cells, this evidence supports the second hypothesis, i.e., that Aojiru intake may induce the activation of NK cells.

NK activity is closely connected with the proportion of NK cells in PBL and had a significant positive correlation at the onset of this experiment. This result also suggests that the assay of NK activity by using the C-FDA method was appropriate.

In addition, the intake of Aojiru for 4 weeks induced little change in the proportion of NK cells in PBL but did increase NK activity. This suggests that the increase of NK activity after Aojiru intake is not due to an increased proportion of NK cells in PBL but rather to an increase in individual activity of NK cells. The enhancement of NK activity after Aojiru intake was largely seen in the subjects with smaller proportions of NK cells in PBL. As a result, the regression line between the proportion of NK cells and NK activity in PBL at 4 weeks showed a gentle slope compared to that at the onset of this study.

We also investigated the mechanism by which Aojiru intake activates NK cell activity. It is known that the cytokines produced from activated T cells have an ability to activate NK cells. In particular, IFN-γ and IL-2 are known as the cytokines activating NK cells [12,13]. Therefore, we measured serum concentrations of both cytokines in subjects at the study onset

and after 4 weeks of Aojiru intake. Although no IFN-γ was detected in the serum of subjects at either stage, the concentration of serum IL-2 was 2.5-fold higher in the subjects after 4 weeks of Aojiru intake compared to that at the onset of this study ($P < 0.01$). Since activated T cells produce IL-2 [14], the hypothesis is advanced that Aojiru intake has an ability to induce the activation of T cells. The first step is that Aojiru intake induces an increase in IL-2 secretion from T cells. The second step is that IL-2 activates NK cells. However, this hypothesis has many unexplained aspects. For example, it is not known how Aojiru intake activates T cells and enhances IL-2 production. To clarify these points will require further study.

In conclusion, the vegetable juice Aojiru enhances NK cell activity, which may be related to increased IL-2 production. The results from this study suggest that Aojiru intake could indeed be useful for maintaining and promoting health.

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