Supplementation with Selenium Restores Age-Related Decline in Immune Cell Function (43909)

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Abstract. **This study examined the effect of dietary (2.00 ppm for 8 weeks) supplementation with selenium (as sodium selenite) on the ability of lymphocytes from aged (24-month-old), male, C57BL/6JNIA mice to respond to: (i) stimulation with mitogen (phytohemagglutinin) or alloantigen; (ii) develop into cytotoxic effector cells; and (iii) destroy tumor cells. Supplementation with selenium resulted in a significant increase in the ability of spleen lymphocytes from aged animals to undergo blastogenesis, as indicated by significantly higher amounts of nuclear incorporation of ^{3}H-thymidine after stimulation with mitogen. The dietary regimen restored the age-related deficiency of the cells to respond to stimulation by nuclear DNA synthesis and cell proliferation, at least, to the level of cells from unsupplemented young adult animals. Furthermore, populations of *in vivo*, alloantigen-activated lymphocytes from Se-supplemented aged animals contained significantly higher numbers of cytotoxic lymphocytes than those from Se-normal aged animals, which resulted in an enhanced capacity to destroy tumor cells. The significant increase in the number of cytotoxic effector cells within these activated T-lymphocyte populations was probably the result of an enhanced clonal proliferation of cytotoxic precursors cells, followed by the differentiation of greater numbers of cytotoxic effector cells. This effect occurred in the absence of changes in the ability of the cells to produce IL-2, which confirmed our earlier observation that dietary supplementation with selenium does not affect the production of IL-2. The data suggested that selenium restores the age-related defect in cell proliferation through an increase in the number of high-affinity IL-2 receptors.**

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The normal process of aging is associated with a general decline in the functions of immune cells which primarily affects T cell-dependent immune responses (1). T lymphocytes from aged individuals often show signs of decreased responsiveness to stimulation with antigen or mitogen, as a result of defects in early signal transduction (2, 3) and reduced production of interleukin-2 (IL-2), expression of IL-2 receptors (IL-2R), and internalization of IL-2 (4, 5). Consequently, the number of cells entering and progressing through the cell cycle (6) and the number of cells differentiating into cytotoxic effector cells (7, 8) are reduced. Age-related decline in the activity of natural killer and lymphokine activated killer cells has also been reported (9, 10). While the mechanism/s underlining these age-associated changes in T cell function are still unresolved, the deterioration of immune functions among aged individuals results in an increased incidence of inflammatory and malignant disease and increased morbidity and mortality.

A number of studies have documented that the availability of selenium (Se) in the diet affects the immune functions of a host *in vivo* and that Se deficiency and supplementation correlate, respectively, with a decreased or an increased resistance of a host to chal-

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lenge with foreign antigens (11). Our previous studies with a mouse model and normal human subjects showed that cells from individuals maintained on Se-supplemented diets have an augmented capacity to proliferate in response to stimulation with antigen or mitogen and to differentiate into cytotoxic effector cells (12–15). This effect was related to the ability of Se to enhance the expression of high affinity IL-2R on activated T lymphocytes (15–17). The purpose of the present study was to determine whether dietary supplementation with Se would restore the age-related decline in the ability of T lymphocytes to: (i) proliferate in response to stimulation with antigen or mitogen; (ii) to produce IL-2; and (iii) differentiate into tumor cytotoxic cells.

Materials and Methods

Animals and Diet. Male C57BL/6JNIA mice, 18 months old, were purchased from Charles River Laboratories (Wilmington, MA) under contract with the National Institute of Aging and maintained until 24 months of age. The age was selected to be near the mean life span of this strain (~28 months), and the animals were designated as *aged*. Three months old, male, C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were designated as *young adults*. All animals were housed in a temperature- and humidity-controlled animal facility, with a 12:12-hr light:dark cycle, given water *ad libitum*, and inspected by a veterinarian for signs of disease. Animals were fed at midday and sacrificed between 9:00 AM and 12:00 noon to maintain uniformity and avoid circadian variations.

The aged mice were maintained on either normal (0.20 ppm Se) or Se-supplemented (2.00 ppm Se) Torula yeast-based diets for 8 weeks. The young adult mice were maintained on the normal diet. The diets were prepared commercially (Teklad, Madison, WI), and Se was added as sodium selenite (12). The Se content of the diets was determined fluorometrically according to the method of Spallholz *et al.* (18), using standard solutions of Se (25–200 ng Se/ml, as selenomethionine) to calibrate the assay; the sensitivity of the assay was 10 ng Se. The Se content of the normal and supplemented diets was determined as 0.21 and 2.02 ppm, respectively.

Each animal was provided with 5 g/day of the respective diet, and body weights were determined on the day of experimental use. There were no statistically significant differences in the weight of animals maintained for 8 weeks on the two diets. The concentrations of Se in pooled serum samples from five animals from each group were determined fluorometrically, as described above (18). The average concentrations of Se in the serum of Se-normal and Se-supplemented mice were 29.9 and 51.8 μg/dl, respectively.

Since the effect of Se supplementation on young adult mice has been extensively studied in our laboratory (11, 12), we did not include this experimental group in the present study in order to reduce the number of experimental animals used.

Culture Media. All cells were cultured in a basic medium of RPMI-1640 supplemented with 25 m*M* HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 m*M* nonessential amino acids, and 2 m*M* glutamine (GIBCO, Grand Island, NY), with additional supplementation as specified for each experiment. The fetal bovine serum (FBS; Hyclone Labs, Logan, UT) used contained 1 μg/dl of Se, which, in media supplemented to 10% with FBS, resulted in 1.2×10^{-8} *M* Se.

Cell Preparation. Ten days before the end of the dietary treatment, all animals were immunized by ip injection with 5×10^6 freshly isolated, allogeneic, P815 mastocytoma cells. At the end of the dietary treatment, spleens were removed from each animal and the cells were prepared as described previously (19), washed once in the basic medium supplemented to 5% with FBS, counted, and used for the mitogen stimulation and IL-2 production assays.

For the cytotoxicity assay, the alloantigen-activated peritoneal exudate lymphocytes were collected by lavage with 10 ml of the basic medium supplemented with 1 U/ml sodium heparin and to 5% with FBS. The cells from each animal were washed once, resuspended in the basic culture medium, and cultured for 2 hr at 37°C and 5% CO_2. The nonadherent cells (lymphocytes) were collected, counted, and used in the cytotoxicity assay.

Mitogen Stimulation Assay. Spleen cells from five Se-supplemented and eight Se-normal aged animals and five young adult animals were plated in 96-well flat bottom plates at 2×10^5 cells/well in 200 μl of the basic medium supplemented with 5×10^{-5} *M* 2-mercaptoethanol (2ME), to 10% with Se-defined FBS, and with 1 μg/ml of phytohemagglutinin-P (PHA; Sigma Chemical Co., St. Louis, MO). The cultures (10 replicates/sample) were incubated for 48 hr, pulsed with 1 μCi/well of [methyl-^{3}H] thymidine (sp. act. 6.7 Ci/mmol; New England Nuclear, Inc., Boston, MA), and incubated for an additional 24 hr. The cells were then harvested with an automatic cell harvester (Skatron, Sterling, VA), lysed in distilled water, and the nuclei collected on filters. The amounts of radioisotope incorporated, in cpm, were determined by liquid scintillation counting.

IL-2 Production. Spleen cells from five Se-supplemented and nine Se-normal aged animals and five young adult animals were plated in 24-well plates at 4×10^6 cells/well in 2 ml of the basic medium supplemented with 5×10^{-5} *M* 2ME, 1 μg/ml of PHA, and to 10% with Se-defined FBS. The cultures were incubated for 24 or 48 hr, the supernatants collected, and

the samples stored at −70°C until used. The amount of IL-2 in each sample was determined using a mouse IL-2 ELISA kit (Endogen, Cambridge, MA) as specified by the manufacturer. The sensitivity of the assay was <3 pg/ml.

Cytotoxicity Assay. The ability of the allogenically-activated lymphocytes from eight Se-supplemented and 13 Se-normal aged animals and from six young adult animals to destroy tumor cells was tested in a 4-hr ^{51}Cr-release assay, as described previously (13, 20). Briefly, the activated lymphocytes were cocultured with 2×10^4 P815 cells labeled with ^{51}Cr (400 mCi/mg; New England Nuclear, Boston, MA) at effector to target cell ratios of 1.25:1 to 10:1 in 96-well round-bottom culture plates. The cells were cultured for 4 hr in 200 μl/well of the basic medium supplemented to 5% with FBS. Control wells (nonspecific release) consisted of 2×10^4 ^{51}Cr-labeled P815 cells incubated in the presence of medium alone. Total release was determined by incubating an equal number of P815 cells with 100 μl of 5% Triton X-100 (Research Products International, Mount Prospect, IL). Release of ^{51}Cr into the medium was measured by liquid scintillation counting, and the percentage of specific lysis was calculated as:

$$\% \text{ Lysis} = \frac{(\text{cpm}_{\text{Experimental}} - \text{cpm}_{\text{Control}})}{(\text{cpm}_{\text{Total}} - \text{cpm}_{\text{Control}})}$$

where the cpm values represent the means of four replicates/sample. The total number of lymphocytes required to kill 2×10^4 target cells was determined by regression analysis of the log-linear plots of the mean percentage of cytotoxicity at the various effector to target cell ratios for each sample at the point at which the regression line intercepted 63% specific lysis. The cytotoxic cell activity, A (which represents the relative efficiency of cytotoxic cells to kill a specific target tumor cell), for each sample was calculated as:

$$Y = A(1 - e^{-kx})$$

where Y is the percentage of specific lysis, x is the number of lymphocytes/well at the 10:1 effector to target cell ratio, and k is the reciprocal of the number of lymphocytes required to kill 2×10^4 target cells (21). The cytotoxic cell frequency was calculated as k times 2×10^4 target cells.

Statistical Analysis. The results are presented as the arithmetic means ± the standard error of the means (SEM) for each control and experimental group. Differences between the means of groups were determined with the Student's two-tailed t and the Wilcoxon ranked sums tests. P values ≤0.05 were considered to be significantly different.

Results

Mitogen Stimulation. The ability of spleen lymphocytes from aged, Se-normal animals to undergo blastogenesis in response to simulation with PHA was significantly lower (37.0%; $P < 0.05$) compared with cells from young adult animals (Fig. 1). Dietary supplementation with Se resulted in a 260.7% increase in the mean ability of cells from aged animals to incorporate [methyl-^{3}H] thymidine after 72 hr of stimulation ($P < 0.02$) compared with the response of aged animals maintained on the normal diet. However, as shown in Figure 1B, there was a wide range in the responses. There was no significant difference in the mean incorporation of the radiotracer between cells from aged Se-supplemented animals and cells from young adult animals ($P < 0.20$).

IL-2 Production. The ability of spleen lymphocytes from aged animals to produce IL-2 was significantly lower ($P < 0.005$) than that of cells from young adult animals (Fig. 2). Supernatants from 48 hr-

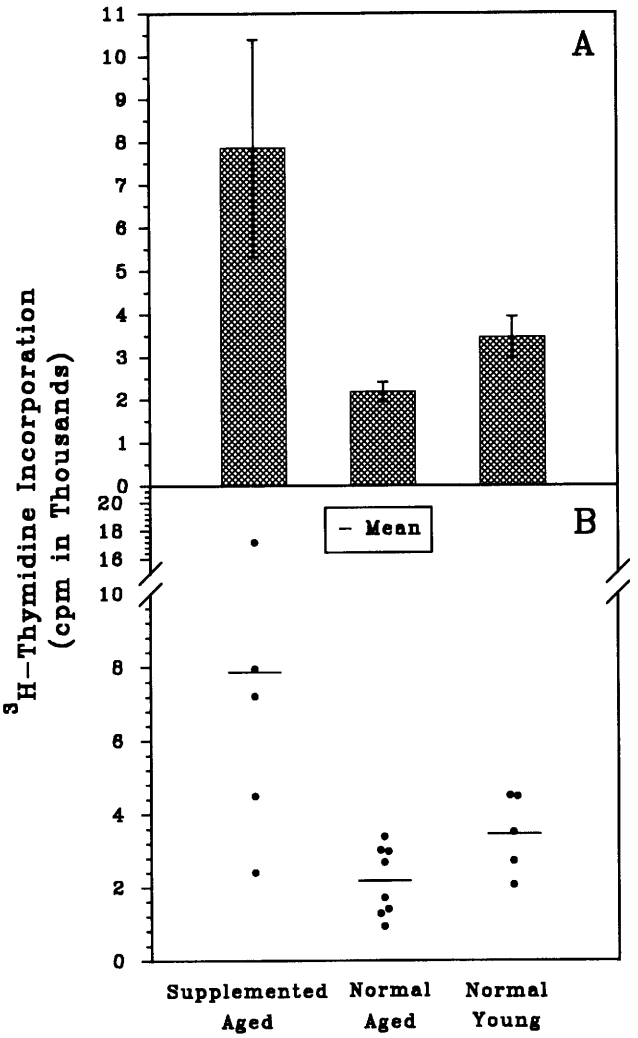

Figure 1. Proliferation of spleen lymphocytes from Se-supplemented and Se-normal aged animals and Se-normal young adult animals in response to stimulation with phytohemagglutinin. (A) Radiotracer incorporation after 3 days in culture. (B) Range and distribution of responses. Direct comparative data showing enhancement of proliferation by Se in young adult mice are given in Ref. 12.

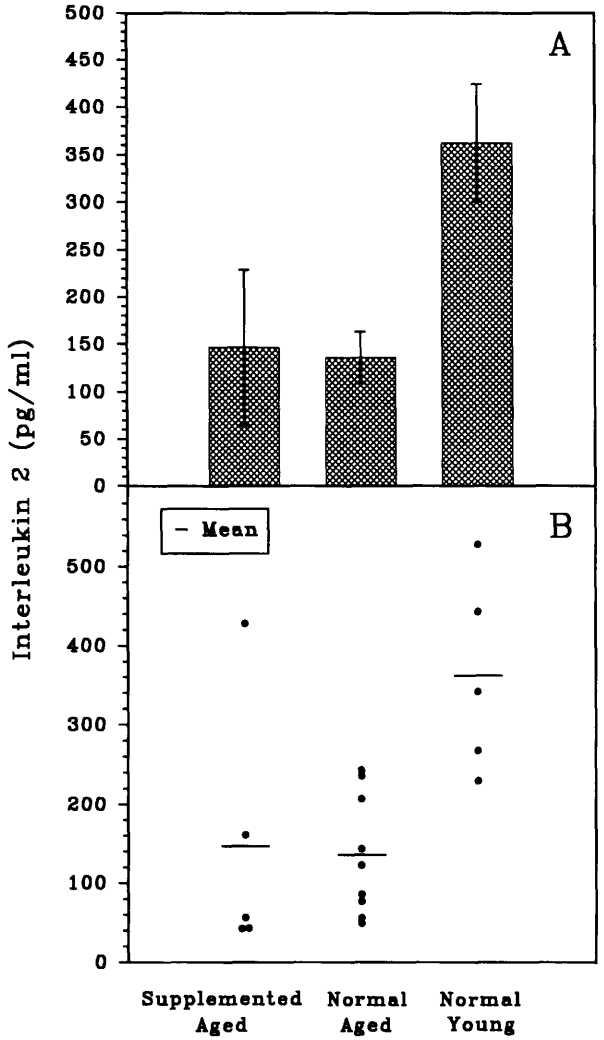

Figure 2. Production of interleukin-2 by spleen lymphocytes from Se-supplemented and Se-normal aged animals and Se-normal young adult animals in response to stimulation with phytohemagglutinin. (A) Amount of interleukin-2 produced after 48 hr in culture. (B) Range and distribution of responses.

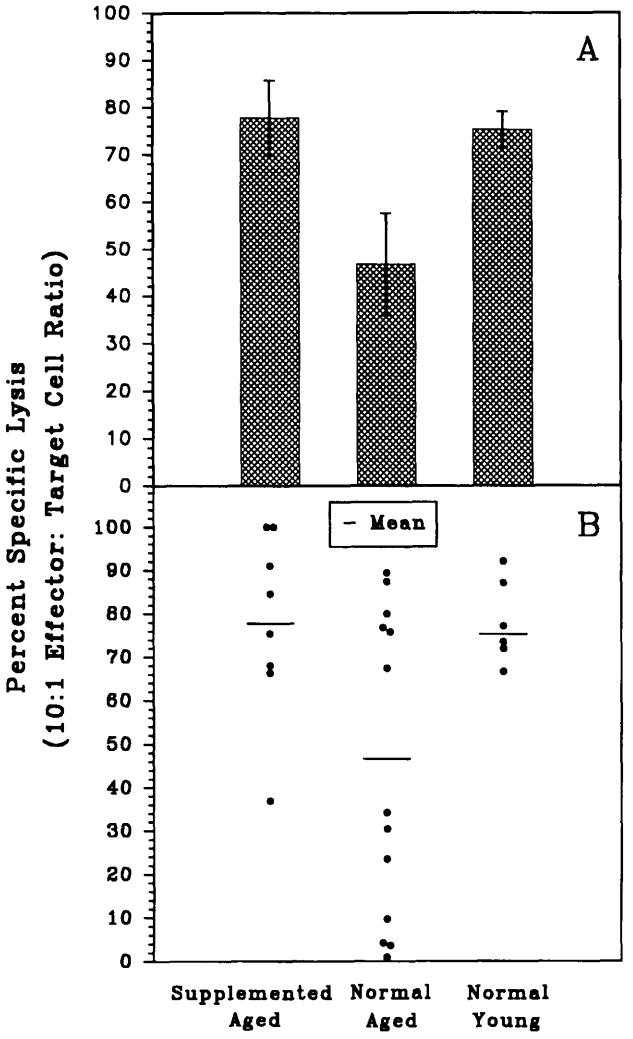

Figure 3. Cytolysis of P815 tumor cells by peritoneal cytotoxic lymphocytes from Se-supplemented and Se-normal aged animals and from Se-normal young adult animals. (A) Specific lysis after allogeneic activation *in vivo*. (B) Range and distribution of responses. Direct comparative data showing enhancement of cytotoxicity by Se in young adult mice are given in Ref. 13.

stimulated cultures of cells from young adult animals contained 166% higher amounts of IL-2 than supernatants from cultures of cells from aged, Se-normal animals. Supplementation with Se did not abrogate this deficiency, as the mean amounts of IL-2 in supernatants from 24- (not shown) or 48-hr cultures from both groups of aged animals did not differ significantly, and there was no significant effect on the range of responses (Fig. 2B). This confirmed our earlier observations that Se does not affect IL-2 production (12).

Tumor Cytotoxicity. Dietary supplementation with Se significantly enhanced tumor cytotoxicity mediated by *in vivo* generated, alloantigen-induced cytotoxic lymphocytes from aged animals. The ability of cytotoxic cell populations from young adult animals to destroy tumor cells was 61.1% higher ($P < 0.05$) than from Se-normal, aged animals (Fig. 3A). Supplementation with Se resulted in a 66.6% increase ($P < 0.05$) in the ability of cells from aged animals to destroy tumor cells and in a significant reduction in the range of responses (Fig. 3B). There was no significant difference in the ability of cells from Se-supplemented aged animals and cells from young adult animals to destroy tumor cells. Supplementation of aged animals with Se resulted in a significant increase in the number of cytotoxic cells within a given cell population, as reflected in the significant decrease ($P < 0.01$) in the number of cells from Se-supplemented aged animals required to destroy a fixed number of tumor cells (Fig. 4A). The calculated increase in cytotoxic cell frequency was from 3.38% to 22.91% ($P < 0.01$; Fig. 4B).

Discussion

The regulation of immune responses by clonal expansion of immunocompetent T lymphocytes involves the coordinated interaction between a number of cy-

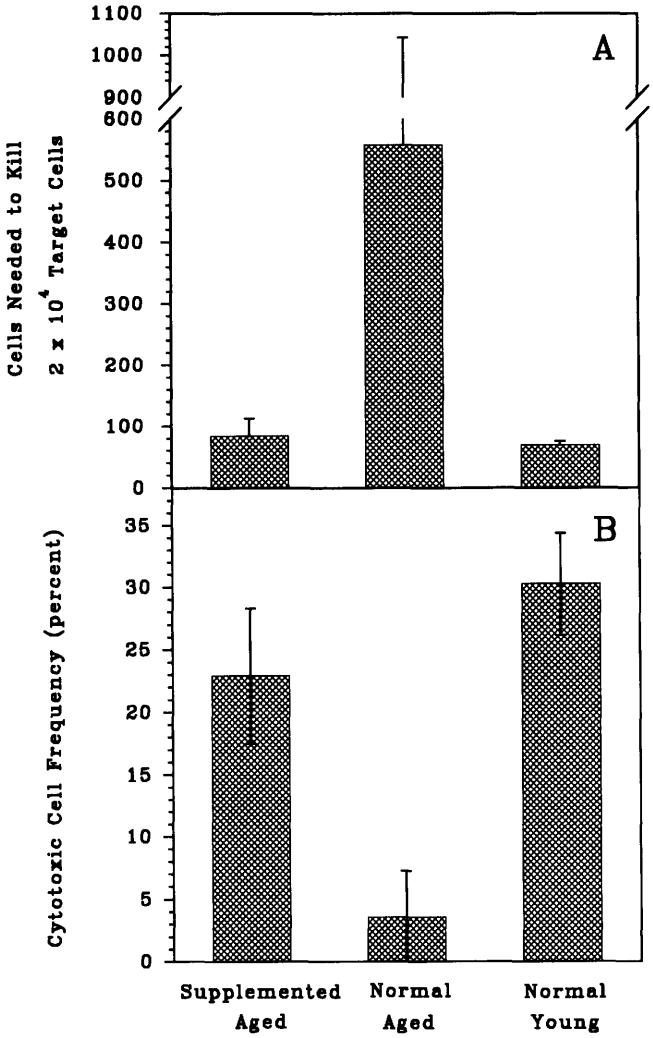

Figure 4. Cytolysis of P815 tumor cells by peritoneal cytotoxic lymphocytes from Se-supplemented and Se-normal aged animals and from Se-normal young adult animals. (A) Calculated number of cells within a given cell population needed to lyse a fixed number of tumor cells. (B) Calculated cytotoxic cell frequency. Direct comparative data showing the effect of Se supplementation in young adult mice are given in Ref. 13.

tokines (e.g., IL-1, IL-2, IFN-γ (22), IL-12 (23), IL-15 (24), and signals arising from the T-cell antigen-receptor complex (22). Data from numerous studies (reviewed in Ref. 22) have established that IL-2 plays a central role in this process, and that the interaction of IL-2 with a sufficient number of high-affinity IL-2R on the surface of the activated cells is necessary for the commitment of the cells to the cell cycle and the clonal expansion of activated T lymphocytes (25, 26).

Studies with mice have shown that the age-related decline in the proliferation of lymphocytes following stimulation with antigen or mitogen can be related to a significant decrease in the number of T lymphocytes that produce IL-2 (27), and to a decrease in the percentage of cells that express IL-2-mRNA (28). However, the amount of IL-2 produced per cell, as well as the level of expression of IL-2-mRNA per cell, did not show significant changes (27, 28), and exogenous IL-2 did not fully restore the decreased proliferative responses of cells from aged donors (29, 30). The decreased levels of IL-2 production were accompanied by a major deficiency in the expression of high-affinity IL-2R (31), and together they provided the basis for the inability of the cells from aged mice to respond adequately with proliferation. Thus, in the presence of limited amounts of IL-2 in the cellular environment of the aged animal, it can be expected that only cells capable of responding to stimulation by expression of a sufficient density of high-affinity IL-2R can be committed to enter the cell cycle (25, 26).

The failure of T lymphocytes from aged mice to express adequate numbers of high-affinity IL-2R has been linked to defective activation of protein kinase C (PK-C) that results from an impaired signal transduction after stimulation with mitogen (2, 31). Bypassing the membrane signaling mechanism with phorbol-12,13-dibutyrate resulted in restoration of IL-2R expression on aged T cells, presumably as a result of an increase in the activity of PK-C (31). This indicated that the mechanism of IL-2R production and expression in lymphocytes from aged animals was intact but the pathway of initial signal transduction was defective.

The results from our studies indicated that dietary supplementation with Se resulted in a significant increase in the ability of spleen lymphocytes from aged animals to undergo blastogenesis, as indicated by the significantly higher (260.7%) amounts of nuclear ^{3}H-thymidine incorporation after stimulation with PHA. However, because of the wide range in responses with cells from the Se-supplemented aged animals there was no statistical difference between the mean responses of these cells and cells from Se-normal, young adult mice. Our previous studies with spleen lymphocytes from young adult (14 weeks old) C57BL/6J mice maintained on the same Se-normal or Se-supplemented diets have shown that the supplementation regimen resulted in 110.6% increase in the ability of these cells to proliferate in response to stimulation with PHA (12). On this basis, it can be concluded that the increase in nuclear DNA synthesis and proliferation of spleen lymphocytes from aged animals observed after dietary supplementation with Se restores the ability of the cells to respond to stimulation, at least to the level of the Se-normal, young adult animals (Fig. 1B). Furthermore, populations of *in vivo*, alloantigen-activated lymphocytes from Se-supplemented aged animals contained significantly higher numbers of cytotoxic cells than those from Se-normal aged animals, which resulted in a significant increase (66.6%) in the cytolytic activity of these cell populations. However, no statistical difference in the lytic activity of these cells and cells from the Se-normal young adult

animals was demonstrated. As reported previously, under the same experimental conditions, dietary supplementation with Se resulted in a 22.3% increase in the cytolytic activity of spleen lymphocytes from young adult animals (13). This indicates that dietary supplementation with Se restores the capacity of cells from aged animals to destroy tumor cells, at least, to the level of cells from Se-normal, young adult animals (Fig. 3B). The significant increase in the number of cytotoxic effector cells within these activated T lymphocyte populations was probably the result of an enhanced clonal proliferation of cytotoxic precursor cells, followed by the differentiation of greater numbers of cytotoxic effector cells. Paradoxically, this effect was achieved in the absence of changes in the ability of the cells to produce IL-2, which confirmed our earlier observation that dietary supplementation with Se does not have an effect on IL-2 production (12).

The mechanism by which Se augments the proliferation and clonal expansion of cytotoxic effector cells in the aged animals is not known. Because Se has no effect on the production of IL-1 (12) and IFN-γ (32), and IL-12 increases the lytic activity of allostimulated lymphocytes in aged animals without an increase in the proliferation of the activated cells (23), it is not likely that these cytokines are involved in the mechanism of Se-mediated immunoenhancement. Recently, it was shown that IL-15 also augments the proliferation and differentiation of T lymphocytes and that the cytokine exerts its effect through the β-chain of the IL-2R (24). Therefore, it is also unlikely that IL-15 exerts a direct effect on the mechanism of Se-mediated immunoenhancement.

Our previous studies with mouse and human lymphocytes from Se-supplemented donors have shown that Se enhances the expression of both the α and β subunits of the high-affinity IL-2R on activated lymphocytes and that the higher number of high-affinity IL-2R/cell resulted in the internalization of greater amounts of IL-2 (15–17). As a result of the earlier expression of greater numbers of IL-2R/cell, greater numbers of cells replicated and expanded faster in the absence of any changes in the endogenous levels of IL-2. It is, therefore, likely that the enhancing effect of Se on the proliferation and clonal expansion of lymphocytes from aged animals may be related to a similar enhancement of IL-2R expression. Since cells from aged animals express decreased numbers of IL-2R/cell (31), and a critical threshold level of high-affinity IL-2R density is required before a cell is committed to enter the cell cycle (26), it can be postulated that Se restores the age-related defect in cell proliferation through an increase in the number of high-affinity IL-2R expressed per cell. Although the presented data do not provide direct evidence for the support of this hypothesis, our previous studies, and the fact that aging is associated with a defect in the expression of IL-2R (31), provide strong credence for this conclusion.

The results from these studies indicated that dietary supplementation with Se in aged individuals may result in enhanced T cell-mediated immune functions in response to challenge with foreign antigen. It appears that supplementation with Se restores the age-related decline in cellular proliferation and clonal expansion of effector cells, at least, to the level of cells from young, unsupplemented donors. In an elderly individual, this may translate to an enhanced ability to combat infections, resistance to tumor cell growth, and decreased morbidity and mortality.

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