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Supplementation with Selenium Augments the Functions of Natural Killer and Lymphokine-Activated Killer Cells

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ABSTRACT

This study examined the effects of dietary (2.0 ppm for 8 wk) and in vitro ($1 \times 10^{-7}M$) supplementation with selenium (Se, as sodium selenite) on the activity of spleen natural killer (NK) cells and plastic-adherent lymphokine-activated killer (A-LAK) cells from C57Bl/6J male mice. Dietary supplementation with Se resulted in a significant increase in the lytic activity of activated NK cells, and cells from these highly lytic effector cell populations expressed significantly higher numbers of intermediate affinity interleukin-2 receptors (Il-2R)/cell. In the presence of high concentrations of Il-2 and $1 \times 10^{-7}M$ Se, resting populations of spleen NK cells developed into A-LAK cells that had a significantly enhanced ability to proliferate, as indicated by the significantly higher amounts of nuclear ^{3}H-thymidine incorporation, and a significantly augmented cytolytic activity against both NK-sensitive and NK-resistant target cells. Se appears to enhance the lytic activity of activated NK cells and to augment the proliferation, expansion, and lytic activity of A-LAK cells in the presence of high concentrations of Il-2 through its ability to enhance the expression of intermediate affinity Il-2R on these cells.

Index Entries: Selenium; immunostimulation; NK cells; LAK cells; nutrition.

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INTRODUCTION

Natural killer (NK) cells are a heterogeneous population of large granular lymphocytes (LGL) that can lyse tumor cells in the absence of MHC-restriction and without previous sensitization *(1,2)*. Lymphokine-activated killer (LAK) cells, which can be induced in vitro by stimulation of peripheral blood or splenic lymphocytes with high doses of interleukin-2 (Il-2), also show MHC-unrestricted tumor cytolysis, but are capable of lysing a broader spectrum of tumor cells, including NK-resistant cells. When purified populations of LGL/NK cells are stimulated with Il-2 in vitro, a subpopulation of cells that adhere to a plastic surface can be isolated. These cells have an activated NK cell phenotype and LAK cell cytolytic function, and are termed A-LAK cells *(3)*. The activation of NK cells and the generation of highly cytotoxic A-LAK cells is initiated by the binding of Il-2 to its receptor (Il-2) on the surface of these cells and the signal for the resulting molecular interactions is mediated through the β chain (p [protein] 70/75) of the functional Il-2R *(4)*.

Selenium (Se) is an essential nutrient required for optimal growth of mammalian cells *(5)*. A number of studies have documented that Se affects the immune functions of a host in vivo and that Se deficiency and supplementation correlate, respectively, with decreased or increased resistance of a host to challenge with foreign antigens *(6)*. Utilizing a mouse model system and healthy human volunteers, we have shown that Se enhances the capacity of mouse cytotoxic lymphocytes and macrophages and human NK cells to destroy tumor cells *(7–10)*. In the mouse system, the effect was related to the ability of Se to enhance the expression of both the α (p55) and β (p70/75) subunits of the Il-2R on the surface of activated lymphocytes, which resulted in a greater number of high affinity Il-2R/cell and in enhanced proliferation and differentiation into cytotoxic effector cells *(11,12)*. The purpose of the present study was to determine whether supplementation with Se would result in: (1) enhanced NK cell activity that can be related to an enhanced expression of Il-2R and (2) in augmented LAK cell proliferation and activity.

MATERIALS AND METHODS

Animals and Diet

Male C57Bl/6J mice (The Jackson Laboratory, Bar Harbor, ME), 5 wk old, were housed as described previously *(7)*. The mice were maintained on normal (0.20 ppm Se) or Se-supplemented (2.00 ppm Se) *Torula* yeast-based diets for 8 wk. The diets were prepared commercially (Teklad, Madison, WI) and Se was added as sodium selenite *(7)*. The Se content of the diet was determined fluorometrically according to the method of Spallholz et al. *(13)*, as described previously *(12)*.

Each animal was provided with 5 g/d of the respective diet; there were no statistically significant differences in the weight of animals maintained for 8 wk on the diets. The average concentrations of serum Se in the Se-normal and Se-supplemented mice were 34.1 and 49.0 µg/dL, respectively. Animals maintained on the diets were used for all experiments involving NK cells.

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. All cultures were incubated at 37°C with 5% CO_2. The fetal bovine serum (FBS; Hyclone Labs, Logan, UT) used was selected for its low content of Se (1 µg/dL). In media supplemented to 5 or 10% with FBS, this represented $6 \times 10^{-9}M$ and $1.2 \times 10^{-9}M$ Se, respectively.

Isolation and Purification of NK Cells

To stimulate NK cell activity, the mice were injected ip with 100 µg of poly I:C (Boehringer Manheim, Indianapolis, IN) in 1 mL of saline 24 h before the assays. Pooled spleen cells from three animals were layered over lymphocyte separation medium (Organon Teknica, Durham, NC) to separate the mononuclear cells, which were then washed, plated in 75 cm^2 flasks in the basic medium supplemented to 10% with FBS, and cultured for 90 min to allow adherent cells to attach. The nonadherent cells were passed through a nylon-wool column *(14)*, to remove remaining monocytes and B cells, and the nylon-wool nonadherent cells were collected and further fractionated by centrifugation on a discontinuous gradient of 30 and 40% Percoll (Pharmacia Fine Chemicals, Piscataway, NJ), as described by Roncarlo et al. *(15)*. The lymphocytes in the interface fraction, which were highly enriched for cells with large granular lymphocyte morphology *(15)*, were used for the NK cell cytotoxicity assays. For the Il-2R determination assays, these cells were further purified by panning to remove residual T lymphocytes. Briefly, to block nonspecific binding, the cells were incubated for 15 min at room temperature in a 0.5% solution of rat IgG (Sigma, St. Louis, MO) prepared in Ca- and Mg-free phosphate buffered saline (PBS) containing 1 m*M* EDTA (Fisher Scientific, Springfield, NJ). The cells were washed, suspended in 0.4 mL of basic medium supplemented to 0.5% with FBS and containing 1 µg each, of anti-CD4 and anti-CD8 (Becton Dickinson, San Jose, CA), and incubated for 45 min at 0°C with constant shaking. The treated cells were then transferred to 60-mm, plastic culture dishes pretreated with 0.1% poly-*l*-lysine hydrobromide (Sigma) in PBS and Protein A (100 µg/mL in 0.1*M* sodium barbital buffer, pH 9.2; Pierce, Rockford, IL) and incubated

for 1 h at room temperature. The nonadherent cells ($CD4^-/CD8^-$) were collected, washed, and used for the assay.

A-LAK Cell Generation and Proliferation Assay

Spleen lymphocytes from unstimulated (i.e., not injected with poly I:C) animals maintained on the normal diet were prepared as described for cells used in the NK cell cytotoxicity assay. A-LAK cells were generated as described by others *(3,16)*, using four spleens per experiment. Briefly, the purified lymphocyte populations were plated in 75-cm^2, plastic, culture flasks at 0.5×10^6 cells/mL in 10 mL basic culture medium supplemented with $5 \times 10^{-5}M$ 2-mercaptoethanol, 1000 U/mL human recombinant Il-2 (Biological Response Modifiers Program, NCI), to 10% with FBS, and in the presence or absence of $1 \times 10^{-7}M$ Se (as sodium selenite). The cells were incubated for 3 d, the nonadherent cells were discarded, and the adherent cells were washed with warm medium. The plastic-adherent cells were cultured for another 4 d in the same medium diluted (1:1) with fresh Il-2 supplemented medium, with or without Se. A-LAK cells were collected *(16)*, washed, and used for the cytotoxicity assays.

For the A-LAK proliferation assay, the plastic-adherent cells were separated from the nonadherent cells after 2 d in culture and incubated for an additional 3 d as above. The cells were harvested, plated in the same medium in 96-well plates at 5×10^4 cells/well (10 replicates/sample), cultured for 24 h, and pulsed with 1 μCi/well of [methyl-^{3}H] thymidine (sp. act. 6.7 Ci/mmol; New England Nuclear, Boston, MA). The cultures were incubated for an additional 24 h, 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. The results from three experiments/group, i.e., ± Se, are reported.

NK Cell and A-LAK Cell-Mediated Cytotoxicity Assay

For the NK-mediated cytotoxicity assay, the effector cells were tested in quadruplicate for activity against 1×10^4 YAC-1 (NK cells-sensitive) or P815 (NK cell-resistant) target cells labeled with ^{51}Cr (400 mCi/mg, New England Nuclear) at effector to target cell ratios of 100:1 to 3.1:1. For the A-LAK cell-mediated cytotoxicity assay, the effector cells were tested in quadruplicate against 5×10^3 ^{51}Cr-labeled YAC-1 or P815 cells at effector to target cell ratios of 16:1 to 1:1. The cells were plated in 96-well U-bottom plates in 0.2 mL/well of basic medium supplemented to 5% with FBS and incubated for 4 h *(10)*. Control wells (nonspecific release) consisted of 2×10^4 ^{51}Cr-labeled target cells incubated in the presence of medium alone. Total release was determined by incubating an equal number of target 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:

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

where the cpm values represent the means of four replicates/sample. The total number of effector cells needed to kill 20% of the target cells were calculated by regression analysis of the log-linear plots of the mean percentage of cytotoxicity at the various effector to target cell ratios for each experiment. The results were expressed in lytic units (LU_{20})/10^7 effector cells, where 1 LU is defined as the number of effector cells that mediate the lysis of 20% of the target cells. The results from 5 experiments/group with NK cells and 7 experiments/group with A-LAK cells are reported.

^{125}I-Il-2 Binding Assay

Purified populations of NK cells were washed in 5 mL of binding buffer (RPMI-1640 with 25 m*M* HEPES and 10 mg/mL of fraction V of bovine serum albumin, Sigma). The cells were resuspended in 5 mL of binding buffer and incubated in a Dubnoff shaker at 37°C for 45 min to remove bound Il-2; washed cells were used for the assay. The assay was performed as described previously *(11,12)*. Briefly, serial 2/3 dilutions (2 n*M*-12 p*M*) of ^{125}I-labeled human recombinant Il-2 (New England Nuclear), with and without 500 times molar excess of cold Il-2 (murine recombinant Il-2; a gift from Dr. Gerard Zurawski, DNAX Research Institute of Molecular and Cellular Biology, Inc., Palo Alto, CA), were incubated at 37°C with 5×10^5 NK cells. The amounts of free and cell-bound ^{125}I-Il-2 were determined as described previously *(11,12)*. Specific binding was calculated by subtracting nonspecific binding (in the presence of cold Il-2) from total binding. Computer-assisted Scatchard analyses were used to determine the number of intermediate affinity binding sites and dissociation constants (K_d). The results from 6 experiments/group are presented.

Statistical Analysis

The results are presented as the arithmetic means ± standard error of the means (SEM) for each control and experimental group. The K_d are presented as the geometric mean ± SEM *(17)*. Differences between means of groups were determined using the correlated *t*-test; P-values ≤ 0.05 were considered to be significantly different.

RESULTS

NK Cell-Mediated Cytotoxicity

Activated NK cells isolated from animals maintained on the Se-supplemented diet for 8 wk had a significantly enhanced ability to

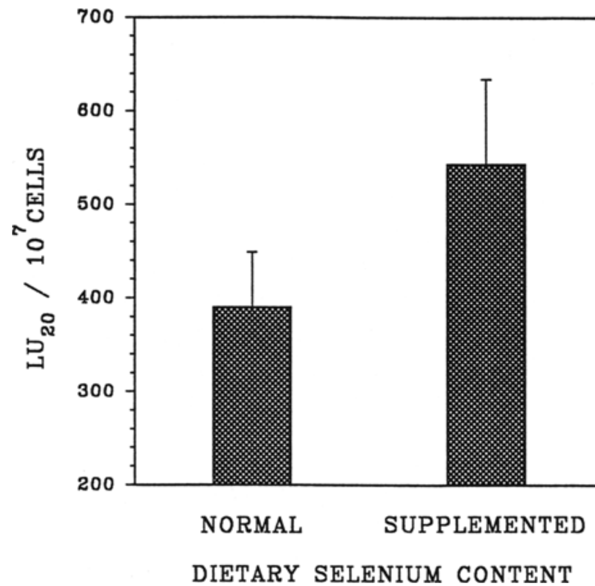

Fig. 1. Effect of dietary supplementation with Se (as sodium selenite) for 8 wk on NK cell activity. The data are presented in lytic units ($LU_{20}/10^7$ cells), where one LU is defined as the number of effector cells that mediate the lysis of 20% of the target (YAC-1) cells.

destroy tumor cells. There was a 39.2% increase ($P < 0.02$) in lytic activity as compared with the activity of cells from animals maintained on the normal diet (Fig. 1). These results are consistent with other reports on Se-mediated increases of NK cell activity in rats *(18)* and humans *(10)*.

Expression of Il-2R on NK Cells

Dietary supplementation with Se for 8 wk exerted a significant effect on the number of Il-2R/cell expressed on activated NK cells. Scatchard plots of the ^{125}I-Il-2 binding data (0.2 m*M*–12.0 p*M*) on NK cells isolated from Se-normal and Se-supplemented animals indicated that Se exerted its effect on the expression of the intermediate affinity Il-2 binding sites (mean K_d = 3.0 and 6.2 × 10^{-10}M, respectively; Fig. 2). Activated cells from Se-supplemented animals showed a 63.6% increase ($P < 0.05$) in the number of intermediate affinity Il-2R/cell as compared to cells from animals maintained on the normal diet (Fig. 3).

Proliferation and Cytotoxic Activity of A-LAK Cells

Cells from cultures of A-LAK precursor cells stimulated with Il-2 for 7 d in the presence of 1 × 10^{-7}*M* Se (as selenite) showed a 29.0% increase in [methyl-^{3}H] thymidine incorporation ($P < 0.005$) as compared with control cultures (Fig. 4). This indicated that Se enhanced the ability of A-LAK precursor cells to proliferate and develop into cytotoxic effector cells.

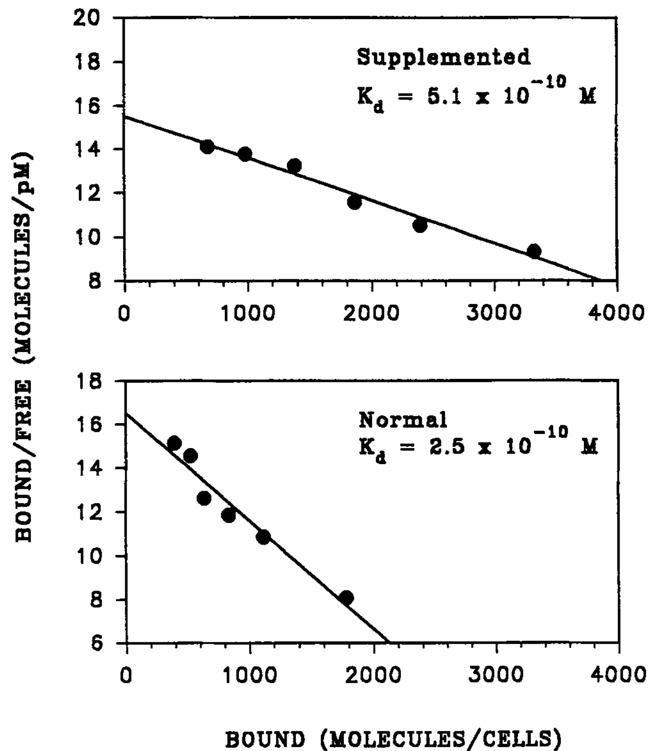

Fig. 2. Scatchard plots of the ^{125}Il-2 binding data on NK cells from Se-supplemented (2.0 ppm of Se as sodium selenite for 8 wk) and Se-normal animals. To stimulate NK cell activity, the animals were injected ip with 100 μg of poly I:C 24 h before the assays. Data represent the binding patterns from a typical experiment.

The results from the A-LAK mediated cytotoxicity assay indicated that A-LAK cells generated in the presence of Se have a significantly higher (36.7% $P < 0.005$) lytic activity against YAC-1 cells than cells generated in the absence of Se (Fig. 5A). When tested for cytolytic activity against the NK cell-resistant P815 cell line, the A-LAK precursor cells (purified NK cell populations) showed no significant activity (mean of 0.08 $LU_{20}/10^7$ cells). However, A-LAK cells generated in the presence of Se had a 25.3% higher ($P < 0.05$) lytic activity against P815 cells as compared to cells generated in the absence of Se (Fig. 5B). The results from these studies indicated that Se significantly augments the cytotoxic activity of A-LAK cells against both NK cell-sensitive and NK cell-resistant target cells.

DISCUSSION

Although mature, resting NK cells have the ability to lyse certain malignant target cells without previous sensitization, exposure to Il-2

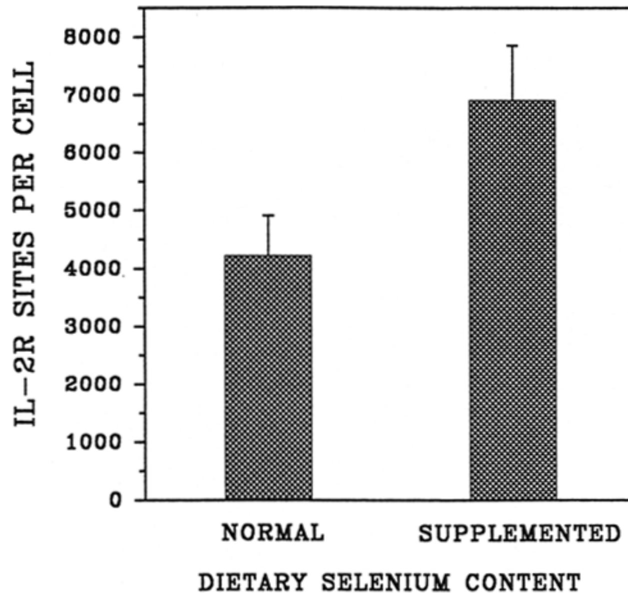

Fig. 3. Effect of dietary supplementation with Se (as sodium selenite) for 8 wk on the ability of spleen NK cells to express intermediate affinity Il-2R. To stimulate NK cell activity, the animals were injected ip with 100 μg of poly I:C 24 h before the assays.

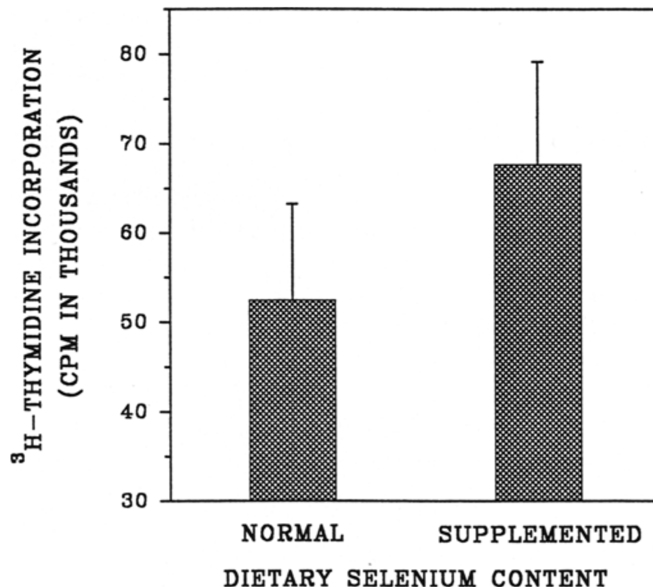

Fig. 4. Effect of supplementation with Se in vitro (1×10^{-7} *M*, as sodium selenite) on the ability of A-LAK cells to proliferate in response to stimulation with Il-2 (1000 U/mL). The data are presented as cpm of nuclear ^{3}H-thymidine incorporation.

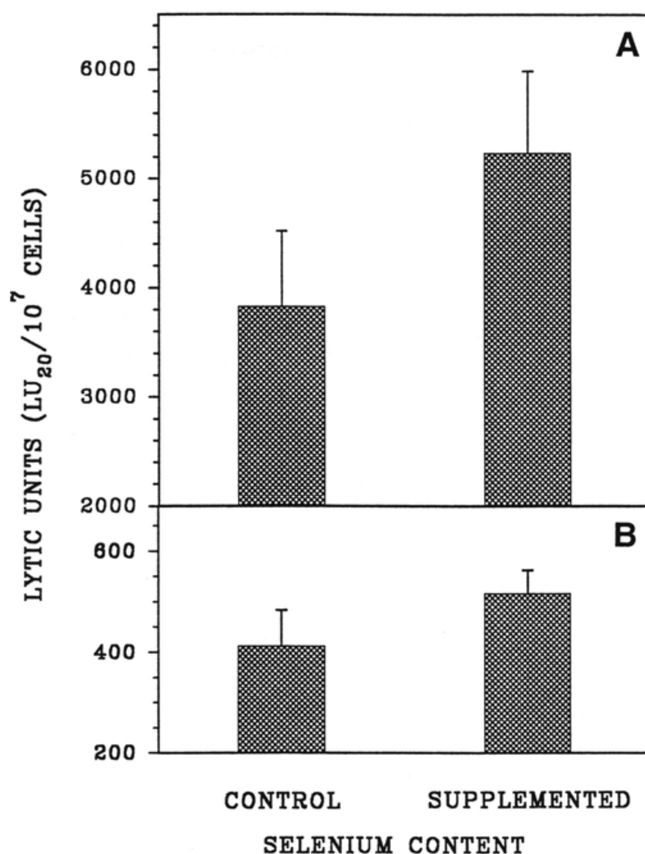

Fig. 5. Lytic activity of A-LAK cells generated in the presence or absence of 1×10^{-7} *M* Se, as sodium selenite. The data are presented in lytic units ($LU_{20}/10^7$ cells), where one LU is defined as the number of effector cells that mediate the lysis of 20% of the target cells. **(A)** Against YAK-1 cells. **(B)** Against P815 cells.

results in augmented "lymphokine activated killing" and renders the cells capable of killing targets that are relatively resistant to resting NK cells. The mechanisms by which Il-2 enhances NK cell-mediated cytolytic activity remain poorly understood because of the pleiotropic effects of the cytokine on NK cell function: e.g., Il-2 induces proliferation and expansion of NK cells both in vitro and in vivo by triggering the entry of the cells into the S and G2/*M* phases of the cell cycle *(19)*; induces expression of Il-2R *(20)*; upregulates multiple NK cell surface adhesion molecules *(21,22)*; induces the production of lymphokines *(20)*; and enhances mobilization of cytotoxic granules *(23)*. Central to all of these events, however, is the necessity for an interaction between Il-2 and its receptor. As with T lymphocytes, the extent of the resulting response is determined by the number of productive interactions between Il-2 and

the Il-2R *(4,24)* that can be modified by variations in either the rate of Il-2 synthesis and secretion, or by the number of functional Il-2R present on the surface of the cells *(25)*.

The induction of Il-2 and Il-2R are independent events, and the induction of Il-2R requires fewer and weaker signals than does the induction of Il-2 *(26)*. To exert its effect on T lymphocytes, Il-2 must interact with the high affinity Il-2R ($K_d = 10^{-11}M$), which is a stable complex of three, noncovalently associated subunits—α (p55), β (p70/75), and γ (p64)—each of which contributes to the binding of Il-2 *(27)*. The β/γ subunit binds Il-2 with an intermediate affinity (K_d of approx 10^{-9} to $10^{-10}M$) and participates in the internalization of Il-2 and the transduction of signals that initiate cell division *(28)*.

Most resting murine and human NK cells constitutively express the isolated intermediate affinity p70/75 Il-2R, but a small subpopulation of human NK cells constitutively expresses both the p70/75 and p55 chains and thus forms the high affinity Il-2R *(29)*. In the presence of high concentrations of Il-2, the majority of human NK cells adhere to plastic, express mRNA for both the p70/75 and p55 chains of the Il-2R, proliferate, and develop into the highly cytotoxic A-LAK cell type *(30,31)*. In contrast, murine NK cells respond to Il-2 in the absence of the p55 Il-2R and are not induced to transcribe detectable levels of the p55 chain even when induced to proliferate in response to Il-2 in vivo or in vitro *(19,31)*. Thus, stimulation of murine NK cells with high doses of Il-2 results in the upregulation of the p70/75 gene, presumably through the direct interaction of Il-2 with the constitutively expressed intermediate affinity Il-2R *(4)*, adhesion to plastic, cell proliferation, and the induction of A-LAK cells that do not express the p55 Il-2R (31).

The results from our studies have shown that dietary supplementation with Se results in a significant increase in the lytic activity of activated murine NK cells, and that cells from these highly lytic effector cell populations express significantly higher numbers of intermediate affinity Il-2R/cell. In the presence of high concentrations of Il-2 and $1 \times 10^{-7}M$ Se, resting murine NK cell populations developed into A-LAK cells that had a significantly enhanced ability to proliferate, as indicated by the significantly higher amounts of nuclear ^{3}H-thymidine incorporation, and a significantly augmented cytolytic activity against both NK cell-sensitive and NK cell-resistant target cells. Thus, it appears that Se enhances the lytic activity and the expression of intermediate affinity Il-2R on activated NK cells, and in the presence of high concentrations of Il-2, Se causes the proliferation of greater numbers of plastic adherent cells and the development of A-LAK cells with significantly higher lytic activity.

The mechanisms by which Se enhances the cytolytic activity and expression of Il-2R on murine NK cells and the proliferation and lytic activity of A-LAK cells are not known. However, because each of these processes is mediated by the interaction of Il-2 with its receptor, the abil-

ity of Se to enhance the expression of intermediate affinity Il-2R on murine NK cells may be the basis for the immunomodulating activity of Se. As shown in our previous studies with mouse and human T lymphocytes, Se enhances the expression of both the p70/75 and p55 subunits of the Il-2R *(12)*, as well as the high affinity Il-2R complex *(9,11)* on activated cells. This results in enhanced proliferation and the generation of greater numbers of cytotoxic lymphocytes within a given cell population *(7,8,10)*. Inasmuch as Se does not upregulate the production of Il-2 *(7)*, and has no effect on nuclear ^{3}H-thymidine incorporation or the expression of Il-2R if the cells have not been stimulated by mitogen or antigen *(9,11)*, it is unlikely that Se affects gene activation/transcription directly. Since the expression of Il-2R has been shown to be regulated by posttranscriptional mechanisms *(32,33)*, and Se has been shown to regulate posttranscriptionally the production of several biologic molecules at the level of translation *(34,35)* or stabilization of m-RNA *(36)*, it may be possible that Se exerts its effect on the expression of p70/75 Il-2R on murine NK cells through a posttranscriptional mechanism(s).

The results from this study have indicated that dietary supplementation with Se (as selenite) results in enhanced NK cell function, which in a host challenged with viral infections or an early neoplastic growth, may result in an augmented capacity to destroy the offending agents. Furthermore, the ability of Se to enhance the proliferation, expansion, and lytic activity of A-LAK cells may have a direct application in the design of adoptive immunotherapy protocols, as addition of Se to the culture medium provides an effective and an inexpensive means for the generation of cytolytic effector cells with an enhanced lytic activity. However, as indicated in our studies with both the mouse model and human volunteers, the immunoenhancing effects of Se on the functions of T lymphocyte and NK cells require supplementation with Se above the replete levels produced by normal dietary intake.

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