Selenium Supplementation Enhances the Expression of Interleukin 2 Receptor Subunits and Internalization of Interleukin 2

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Abstract. **Selenium (Se) is an essential nutritional factor that was shown previously by us to alter the kinetics of expression of high affinity (p55/p75) interleukin 2 receptors (IL-2R). This study shows that dietary (2 ppm for 8 weeks) or *in vitro* (1×10^{-7} *M*) supplementation with Se (as sodium selenite) results in a significant upregulation of the expression of both the p55 and p70/75 IL-2 binding sites on the surface of concanavalin A-stimulated lymphocytes from C57BL/6J mice. This resulted in the formation of significantly higher numbers of high affinity IL-2R/cell with preservation of the normal ratio of high affinity to total IL-2 binding sites/cell. The high affinity IL-2R on cells from Se-supplemented animals functioned normally in terms of ligand binding and kinetics of IL-2 internalization, but their greater numbers/cell resulted in the internalization of significantly larger amounts of IL-2/cell. As Se supplementation results in an earlier expression of greater numbers of high affinity IL-2R, the presence of Se in the cell environment can result in an accelerated clonal expansion of activated lymphocytes.**

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The process of T lymphocyte activation in response to stimulation with antigen or mitogen involves the coordinated expression of a number of genes and a complex sequence of highly cooperative molecular interactions (1). The commitment to cell division requires an interaction between interleukin (IL) 2 and its receptor (IL-2R), expressed on the surface of activated T lymphocytes, and the magnitude and duration of the resulting clonal proliferation depend on the density of IL-2R on the cell surface and the availability of IL-2 (2). The functional IL-2R complex consists of at least two distinct IL-2-binding subunits: the constitutively expressed p70/75 IL-2R_β chain that binds IL-2 with intermediate affinity (K_d of 0.5–1 n*M*) and the inducible p55 IL-2R_α chain that binds IL-2 with low affinity (K_d of 7–10 n*M*) (3–6). The coexpression of both chains results in their noncovalent association and the formation of the biologically functional high affinity IL-2R complex (p55/p75; K_d of 10 p*M*) (7).

We have shown previously that supplementation with low doses of selenium *in vivo* and *in vitro* significantly enhanced the ability of activated lymphocytes to proliferate and differentiate into cytotoxic cells (8, 9). The effect was exerted in the absence of changes in the endogenous levels of IL-2 or IL-1 and was apparently related to the ability of Se to induce an earlier and augmented expression of high affinity IL-2R (10, 11). The purpose of the present study was to determine the effect of Se supplementation on the surface expression of the IL-2R_α and IL-2R_β subunits and on the ability of the high affinity complex to internalize IL-2.

Materials and Methods

Animals and Diet. Male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME), 6 weeks old, were housed in a temperature- and humidity-controlled animal facility, with a 12:12-hr light:dark cycle, and given water *ad libitum*. Animals were fed at midday

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and sacrificed between 9 AM and 12 noon to maintain uniformity and avoid circadian variations.

The 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 diets were prepared commercially (Teklad, Madison, WI), and Se was added as sodium selenite (8). The Se content of the diet was determined fluorometrically according to the method of Spallholz *et al.* (12), using standard solutions of Se (25–200 ng Se/ml, as selenomethionine) to calibrate the assay. The sensitivity of the assay was 10 ng of Se, and the Se content in samples of the normal and supplemented diets was 0.17 and 2.13 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 (9) from five animals from each group were determined fluorometrically, as described above (12). The average concentrations of Se in the serum of Se-normal and Se-supplemented mice were 32.6 and 41.2 μg/dl, respectively.

For the *in vitro* Se-supplementation studies, 14-week-old male C57BL/6J mice were used. The mice were maintained as described above and fed Purina Lab Chow (standard laboratory diet) *ad libitum*. The DBA/2 mice (The Jackson Laboratory) used for the passage of P815 cells were maintained identically.

Culture Media. All cells were cultured in a basic medium of RPMI-1640 supplemented with 25 m*M* HEPES, 100 units/ml of penicillin, 100 μg/ml of 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, at 37°C with 5% CO_2. The fetal bovine serum ([FBS] Hyclone Laboratories, Logan, UT) used contained 1 μg/dl of Se, which, in media supplemented to 5% with FBS, resulted in 6×10^{-9} *M* Se.

Monoclonal Antibodies. 7D4 is a rat IgM that recognizes an epitope of the p55 subunit of the mouse IL-2R that is distinct from the ligand binding site (13). Hybridoma cells that produce 7D4 (ATCC No. CRL 1698) were incubated at 37°C (1×10^5 cells/ml) for 72 hr and the culture supernatants were collected. MAR 18.5, an IgG_{2a}, is a mouse monoclonal antibody to the rat κ-light chain (14). The hybridoma cells (ATCC No. TIB 216) were grown as above. Undiluted culture supernatants were used in all experiments.

Cell Preparation. Groups of 20 mice were maintained on each of the two diets for 8 weeks. Ten days before the end of the dietary treatment, all animals were immunized by intraperitoneal injection with 5×10^6 freshly isolated P815 mastocytoma cells. At the end of the dietary treatment, pooled spleen cells from two to three animals (eight experiments/group) were prepared as described previously (15), washed once in basic medium supplemented to 5% with FBS, and counted. The spleen cells were cultured in 24-well culture plates at 4×10^6 spleen cells/well in a total volume of 2 ml of basic medium supplemented with 5 μg/ml of concanavalin A ([Con A] Sigma Chemical Co., St. Louis, MO) and to 5% with FBS.

For the *in vitro* Se-supplementation studies, spleens were removed from animals maintained on standard laboratory diet. Pooled spleen cells from three animals/experiment (five experiments) were cultured, as described above, in medium supplemented with 0 or 1×10^{-7} *M* Se (as sodium selenite). The plates were incubated for 48 hr and the lymphoblasts were collected and separated from cellular debris by centrifugation over lymphocyte separation medium (Organon Teknica Corp., Durham, NC) and 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 two 1-hr periods, with extensive washes between incubations to facilitate removal of bound IL-2, and washed cells were used for the assays.

^{125}I-IL-2 Binding Assay. The assay was performed essentially as described by Robb *et al.* (16). Serial 2/3 dilutions (2 n*M*–6 p*M*) of ^{125}I-labeled human recombinant IL-2 (New England Nuclear, Inc., Boston, MA), 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 lymphoblasts in 150 μl of binding buffer in Eppendorf tubes underlain with 150 μl of a 3:1 mixture of dibutyl phthalate (Aldrich Chemical Co., Inc., Milwaukee, WI) to dinonyl phthalate (ICN Biochemicals, Cleveland, OH) (17). After 15 min, 350 μl of ice-cold binding buffer were added to each tube, the tubes were centrifuged at 9000 rpm for 60 sec in a microcentrifuge, and the amount of free radiolabeled ligand in the aqueous phase was determined. The oil mixture was discarded, and the amount of cell-bound radioisotope in the cell pellet was determined. Specific binding was obtained 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 high, intermediate, and low affinity binding sites/cell and the dissociation constants (K_d). For each sample, the number of p55 subunits was calculated as the sum of the high and low affinity sites/cell and the number of p70/75 subunits as the sum of the high and intermediate sites/cell. The total sites/cell were calculated for each experiment as the sum of the p55 and p70/75 sites.

Immunoassay for Il-2R p55. The relative density of p55 subunits/cell was estimated using the 7D4 monoclonal antibody specific for the p55 subunit of mouse Il-2R (13, 18). Washed lymphoblasts (5×10^5 cells in 50 μl of binding buffer) were incubated in Eppendorf tubes with 100 μl of 7D4 for 30 min at 4°C and then washed once with 500 μl of ice-cold binding buffer. Negative controls were incubated with 100 μl of a 1/100 dilution of rat IgM (2.5 mg/ml; Chemicon, Tenecula, CA) in place of the 7D4 antibody. The cells were resuspended in 50 μl of binding buffer, incubated with 100 μl of MAR 18.5 for 30 min at 4°C, and washed once with 500 μl of ice-cold binding buffer. The washed cells were resuspended in 50 μl of binding buffer and 100 μl of ^{125}I-Protein A (1/80 dilution; New England Nuclear, sp act 118 μCi/μg) and incubated for 45 min at 4°C. Protein A has a binding affinity of >99% for IgG$_2$ class antibodies (19). The cell suspension was layered over 150 μl of the dibutyl/dinonyl phthalate oil mixture and centrifuged at 9000 rpm for 60 sec. The aqueous phase and the oil mixture were discarded and the amount of cell-bound radioisotope was determined. Specific binding was calculated by subtracting the cpm of ^{125}I-Protein A bound to cells in the negative control from the experimental, and the difference was converted to the number of molecules of ^{125}I-Protein A bound/cell, based on a predetermined counting efficiency of 38%.

Kinetics of IL-2 Internalization. The assay was conducted as described by Robb and Green (20), with some modifications. Briefly, washed lymphoblasts (25–30×10^6) were suspended in 2 ml of binding buffer containing 100 μM chloroquine (Sigma) and incubated at 37°C for 5 min. The cells were washed once and 24×10^6 cells were incubated in 2.4 ml of a 100 pM solution of ^{125}I-IL-2 prepared in binding buffer for 40 min at 4°C with constant shaking. The cells were washed once with 4 ml of ice-cold binding buffer to remove unbound IL-2 and resuspended in 2.4 ml of prewarmed binding buffer containing 100 μM chloroquine, and 200-μl aliquots containing 2×10^6 cells were removed immediately and at various intervals during a 60-min incubation period at 37°C with constant shaking. The aliquots were diluted with 400 μl of binding buffer and centrifuged at 9000 rpm, the entire supernatant was removed, and the amount of free radiolabeled ligand was determined to ensure that no significant dissociation of IL-2 from the cells had occurred. The cell pellet was resuspended in 300 μl of a 10-mM citrate buffer (pH 4.0) containing 0.14 M NaCl and 50 μg/ml of bovine serum albumin, and after 30 sec at room temperature, the cells were layered on 150 μl of dibutyl/dinonyl phthalate and centrifuged at 9000 rpm for 1 min. The amount of acid-sensitive, surface-bound radioligand in the aqueous supernatant phase was determined. The oil mixture was discarded, and the amount of radioactivity in the cell pellet was assessed to determine the amount of IL-2 internalized. The half-time for the internalization of IL-2 was determined from the intersection of the plots of the cpm representing the amount of internalized and surface-bound IL-2/cell versus time.

To determine the maximal internalization of IL-2, triplicate samples of washed lymphoblasts (5×10^5) were suspended in 0.5 ml of binding buffer containing 100 μM chloroquine and incubated at 37°C for 5 min. The cells were washed once and incubated in 0.15 ml of a 200-pM solution of ^{125}I-IL-2, prepared in binding buffer, for 40 min at 4°C with constant shaking. The cells were washed once with 0.5 ml of ice-cold binding buffer, resuspended in 0.5 ml of prewarmed binding buffer containing 100 μM chloroquine, and incubated at 37°C for 60 min, with constant shaking, to allow maximal internalization of IL-2. The amounts of internalized (acid-insensitive) and surface-bound (acid-sensitive) IL-2 were determined as above. The amount of total IL-2 bound/cell was expressed as the sum of the surface-bound and internalized IL-2.

Statistical Analyses. Results are presented as the mean ± SE for each control and experimental group. The dissociation constants (K_d) are presented as the geometric mean ± SE (21). Differences between the group means were determined using the Student's two-tailed t test, and P values ≤ 0.05 were considered significantly different.

Results

Expression of IL-2R after Dietary or *In Vitro* Exposure to Se. Dietary or *in vitro* supplementation with Se exerted a significant effect on the expression of the IL-2R subunits on stimulated lymphocytes. Scatchard plots of ^{125}I-IL-2 binding data (2 nM–6 pM) on 48-hr lymphoblasts from Se-normal and Se-supplemented animals indicated that Se significantly enhanced the expression of both high affinity (49.2% increase; $P < 0.001$) and low affinity (54.9% increase; $P < 0.02$) IL-2 binding sites (Table I). The number of intermediate affinity binding sites exceeded the number of high affinity IL-2R on cells from both groups of animals equally, i.e., by a factor of 3.2 and 2.9 for cells from Se-normal and Se-supplemented animals, respectively, which was in close agreement with values reported for activated human and murine lymphocytes, i.e., factor of 2.8–5 (22, 23).

The calculated numbers of p55 and p70/75 and the total number of IL-2 binding sites expressed on the cell surface indicated a 51.1% increase ($P < 0.005$) in the expression of the total number of IL-2 binding sites on cells from Se-supplemented animals that was associated with a 52.9% increase ($P < 0.02$) in the expression of the p55 sites, and a 31.8% increase in the expression of the p70/75 sites ($P < 0.05$) (Fig. 1). The expression of the p55 sites exceeded that of the high

Table I. Effect of Se on the Expression of Il_2 Binding Sites on 48-hr Con A-Stimulated Spleen Lymphoblasts from C57BL/6J Mice

Selenium content	Il_2 binding sites/cell ± SE[a]		
	High affinity	Intermediate affinity	Low affinity
Diet			
Normal (0.20 ppm)	2,169 ± 95	7,872 ± 898	34,648 ± 2,923
	4.3×10^{-11}	2.6×10^{-10}	1.8×10^{-9}
Supplemented (2.00 ppm)	3,236 ± 216	9,500 ± 730	53,687 ± 6,160
	6.9×10^{-11}	3.0×10^{-10}	3.3×10^{-9}
Percentage of change	+49.2	+20.7	+54.9
P	<0.001	<0.20	<0.02
In vitro			
Control (no Se)	1,229 ± 128	4,333 ± 629	19,468 ± 1,723
	1.3×10^{-11}	1.6×10^{-10}	2.1×10^{-9}
1×10^{-7} *M* Se	1,873 ± 95	5,747 ± 449	28,585 ± 1,896
	3.4×10^{-11}	2.2×10^{-10}	1.9×10^{-9}
Percentage of change	+52.4	+32.6	+46.8
P	<0.005	<0.10	<0.01

[a] Data were calculated from Scatchard plots of $^{125}\text{I-Il}_2$ binding data (2 n*M*–6 p*M*).

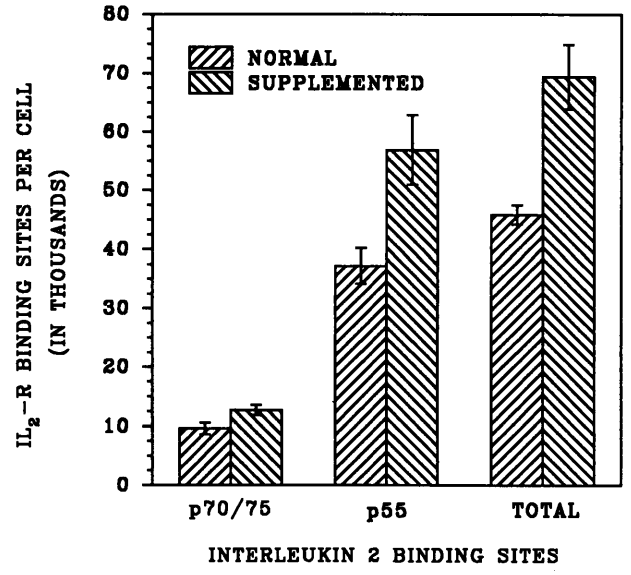

Figure 1. Calculated numbers of IL-2 binding sites/cell for spleen lymphoblasts derived from C57BL/6J mice maintained on Se-normal (0.20 ppm Se) or Se-supplemented (2.00 ppm Se) diet for 8 weeks. The numbers were calculated using data from Scatchard plot analyses of data derived from cells stimulated with 5 μg/ml of Con A for 48 hr (Table I). Data are presented as the mean number of IL-2 binding sites/cell ± SE.

affinity IL-2R by a factor of 17.1 and 17.5 for cells from Se-normal and Se-supplemented animals, respectively, and the high affinity IL-2R represented 4.7% and 4.3% of the total number of sites, respectively (Table I and Fig. 1). These results were in agreement with the reported distribution of the total IL-2 binding sites and high affinity IL-2R on fully activated lymphocytes (i.e., activated T cells have 10–20 times more p55 sites than high affinity sites, and high affinity IL-2R usually comprise less than 5% of total cell surface sites [24, 25]) and indicated that Se augments the expression of the high affinity IL-2R at ratios comparable to those seen on cells from Se-normal animals.

The results from parallel studies on cells supplemented with 1×10^{-7} *M* Se *in vitro* showed identical relationships (Table I and Fig. 2) and supported the results from the studies involving dietary supplementation with Se.

Immunoassay for IL-2R p55. To confirm the stimulatory effect of Se on the expression of the p55 subunit of the IL-2R, the relative density of p55 sites/cell was estimated by exposing spleen lymphocytes stimulated with Con A for 48 hr from each dietary group of animals to the 7D4 monoclonal antibody. The number of p55 sites on cells from the Se-supplemented group was 46.8% greater ($P < 0.02$) than on the Se-normal group (Fig. 3) and confirmed the results from the $^{125}\text{I-IL-2}$ binding assay.

Kinetics of IL-2 Internalization. Analysis of the kinetics of receptor-mediated internalization of IL-2 indicated that maximal internalization was attained after 30 min by cells from both dietary groups (Fig. 4 shows results from a typical experiment). The calculated $t_{1/2}$ of receptor-mediated internalization of IL-2 was 15.0 ± 0.9 min and 14.0 ± 1.2 min ($P = 0.50$) for cells from Se-normal and Se-supplemented animals, respectively, and indicated no difference in the rate of IL-2 internalization between the two groups. The ratios of the amounts of internalized IL-2 to that of the total

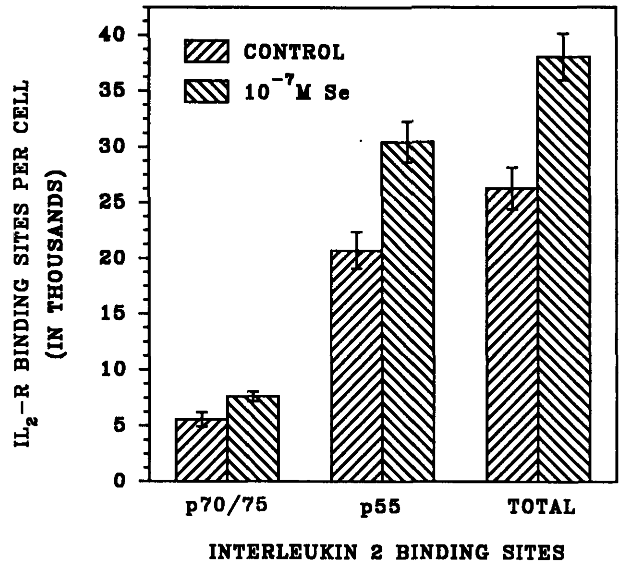

Figure 2. Calculated numbers of IL-2 binding sites/cell for spleen lymphocytes derived from C57BL/6J mice maintained on normal laboratory diet after stimulation with 5 μg/ml of Con A for 48 hr in the presence (1×10^{-7} *M*) or absence of exogenous Se. The numbers were calculated using Scatchard plot analyses data (Table I). Data are presented as the mean number of IL-2R binding sites/cell ± SE; 45.0% increase in number of total sites ($P < 0.05$); 47.1% increase in total p55 sites ($P < 0.005$); 37% increase in total p70/75 sites ($P < 0.05$).

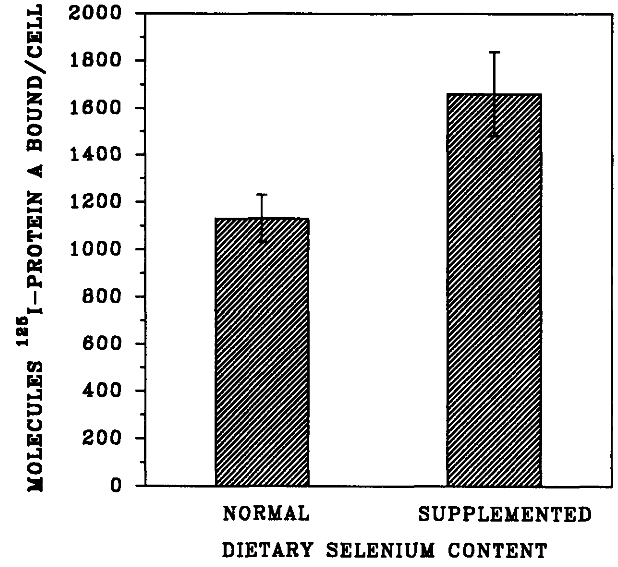

Figure 3. Estimates of the relative density of p55 subunits/cell for spleen lymphoblasts derived from C57BL/6J mice maintained on Se-normal (0.20 ppm Se) or Se-supplemented diet (2.00 ppm Se) for 8 weeks, using the 7D4 monoclonal antibody specific for the p55 site. Cells treated with 7D4 were exposed to MAR 18.5 and ^{125}I-Protein A. Data are presented as the mean number of molecules of ^{125}I-Protein A bound/cell ± SE.

cell-bound and internalized IL-2 were the same for both groups, i.e., 0.71, and indicated no differences in the function of the IL-2R (Fig. 5). However, the amount of IL-2 internalized by cells from Se-supplemented animals was significantly higher (56.2% greater; $P < 0.02$),

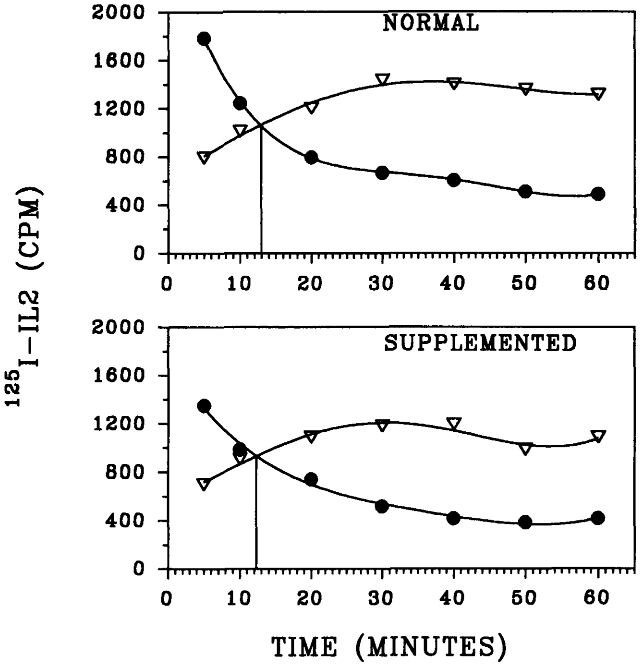

Figure 4. Kinetics of ^{125}I-IL-2 internalization by 48-hr Con A-stimulated spleen lymphocytes from C57BL/6J mice maintained on Se-normal (0.20 ppm Se) or Se-supplemented diet (2.00 ppm Se) for 8 weeks. The data represent the internalization patterns from a typical experiment. ●, Bound but not internalized IL-2 (pH 4-sensitive fraction); ∇, internalized IL-2 (pH 4-resistant fraction).

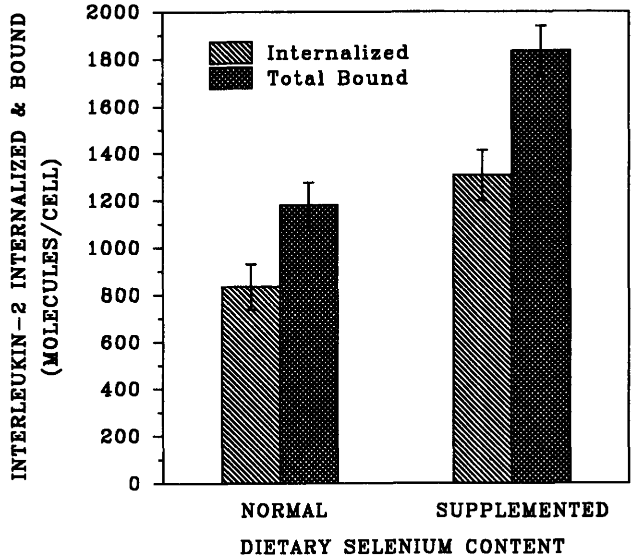

Figure 5. Maximum ^{125}I-IL-2 internalized and total IL-2 bound/cell (surface bound and internalized) for 48-hr Con A-stimulated spleen lymphocytes derived from C57BL/6J mice maintained on Se-normal (0.20 ppm Se) or Se-supplemented (2.00 ppm Se) diet for 8 weeks. Cells exposed to ^{125}I-IL-2 for 40 min at 4°C were incubated at 37°C for 60 min to allow maximal internalization. Data are presented as the mean number of ^{125}I-IL-2 molecules/cell internalized and total bound ± SE.

which probably reflected the higher number of high affinity receptors on their surfaces.

Discussion

The results from these studies demonstrated that dietary (2 ppm) or *in vitro* (1×10^{-7} *M*) supplementation with Se results in a significant upregulation of the expression of both the p55 and p70/75 subunits of the IL-2R on the surface of activated mouse lymphocytes. The upregulated expression of the subunits resulted in the formation of significantly higher numbers of high affinity IL-2R/cell, while preserving the normal ratio of high affinity to total IL-2 binding sites/cell. The high affinity IL-2R on cells from Se-supplemented animals functioned normally in terms of ligand binding and the kinetics of the internalization of IL-2, but their greater numbers/cell resulted in the internalization of significantly larger amounts of IL-2/cell. As supplementation with Se results in an earlier expression and in greater numbers of high affinity IL-2R (a critical threshold of triggered receptors must accumulate before a cell enters the S phase of the cell cycle [11]), the higher amounts of IL-2 internalized by the Se-supplemented cells may support their earlier and faster expansion in the presence of continuous immunologic stimulation and a constant supply of IL-2. Our previous studies (8–10), which indicated that supplementation with Se results in an enhanced proliferation and clonal expansion of cytotoxic lymphocytes, support this conclusion.

The regulation of high affinity IL-2R expression is achieved by modulation of the expression of both the p55 and p70/75 subunits of IL-2R. The p55 subunits of the IL-2R are usually not present on resting T cells, but after antigen/mitogen stimulation, activated cells express 10- to 100-fold more p55 IL-2 binding sites than high affinity IL-2R (1, 24). In contrast, the p70/75 chain, which is constitutively expressed on unstimulated T cells, is upregulated only 3- to 4-fold after stimulation (26, 22). Thus, the total number of high affinity IL-2R expressed after T cell activation is directly related to the availability of both the p55 and p70/75 subunits. The earlier and greater accumulation of p55/p75 receptors on the surface of activated lymphocytes in the presence of Se is, therefore, the probable result of a more rapid, but coordinated, production of greater amounts of each protein. Among a number of possible mechanisms, this could result from an earlier and augmented expression of the genes coding for IL-2R α and β or through posttranscriptional modulation of protein production and/or mRNA stabilization.

Most primary activation of genes in eukaryotic cells is accomplished within 15–20 min after initial stimulation (1). After stimulation of T lymphocytes, the mRNA that codes for IL-2Rα peaks at 8–12 hr after activation (3), and the cellular expression of the corresponding protein reaches a peak 24 hr after stimulation. However, the surface expression of the protein continues to increase beyond the peak of intracellular protein expression (27, 28). The mRNA that codes for IL-2Rβ peaks 6 hr after stimulation (29), but the corresponding expression of the protein at the surface peaks 3–4 days after stimulation (22). Our previous studies (10, 11) have shown that Se, in the absence of mitogen stimulation, has no effect on the expression of high affinity IL-2R and that the presence of Se in the cell environment at the time of stimulation, or during the first 8 hr after activation, is not required for the augmentation of IL-2R expression. Therefore, it appears unlikely that Se augments the surface expression of both the p55 and p70/75 chains through a direct effect on the activation of the IL-2R α and β genes and/or the rate of their transcription.

Inasmuch as Se exerts its effect on the expression of IL-2R 8–24 hr after activation (10, 11), i.e., during the period of active protein synthesis of receptor subunits (27, 28), it appears more likely that Se affects posttranscriptionally protein production and/or mRNA stabilization. The translation of the IL-2Rα mRNA chain appears to be downregulated through a "leaky scanning" mechanism that results in reduced translational efficiency as a consequence of the presence of two AUG start codons separated by a UGA stop codon, located 5′ to the IL-2R initiation site; removal of the repeat segment results in a 5- to 6-fold increase in the expression of IL-2R on the cell surface without changes in the IL-2R mRNA levels (30). The IL-2R mRNA appears to be inefficiently translated at all times; stimulation of primed T cells with Con A results in elevated levels of IL-2R transcripts but inefficient translation of the IL-2R mRNA. It would be of interest to know whether the presence of Se in the environment increases the efficiency of IL-2R mRNA translation and, thus, whether it can augment the expression of IL-2R on the cell surface. Se has been shown to regulate posttranscriptionally the production of several enzymes at the level of mRNA translation, e.g., glutathione peroxidase and iodothyronine deiodinase (31–33), and, in some instances, at the level of mRNA degradation (34). Although there is no direct evidence of the role of Se in the regulation of the expression of IL-2R α and β by the above mechanisms, our results provide the basis for further investigation in this area. Elucidation of the mechanism(s) involved in the augmentation of IL-2R expression on activated lymphocytes could provide the rationale for the efficacy of Se in modulating IL-2-dependent immune responses.

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