Selenium and Immune Cell Functions. II. Effect on Lymphocyte-Mediated Cytotoxicity

(43015)

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Abstract. **Selenium (Se) is an essential nutritional factor with a chemopreventive potential. This study examined the ability of C57BL/6J mice, maintained for 8 weeks on Se-deficient (0.02 ppm Se), normal (0.20 ppm Se), or Se-supplemented (2.00 ppm Se) Torula yeast-based diets, to generate cytotoxic lymphocytes (CTL) and to destroy tumor cells. CTL were generated *in vivo* by intraperitoneal immunization with P815 cells and *in vitro* by allogeneic stimulation of cells from animals maintained on a normal diet in media supplemented with 1×10^{-9} to 1×10^{-6} *M* Se (as selenite). Lymphocytes from animals maintained on the Se-supplemented diet had a greater ability to destroy tumor cells than lymphocytes from animals maintained on the normal diet, whereas Se deficiency reduced the cytotoxicity. The effects on cytotoxicity were accompanied by parallel changes in the levels of lymphotoxin produced. The greatest enhancement of tumor cytodestruction occurred with supplementation of 1×10^{-7} *M* Se, whereas with 1×10^{-6} *M* there was inhibition of the cytotoxic responses. The stimulatory effect of Se occurred during the phase of CTL generation rather than during the lytic phase of cytotoxicity. These results indicated that Se supplementation enhances CTL generation and the ability of a host to destroy malignant cells, whereas Se deficiency has the opposite effect.** [P.S.E.B.M. 1990, Vol 193]

The available data on the ability of selenium (Se) to prevent the development of malignant neoplasms indicate that Se is an important chemopreventive agent (1). The majority of the reported studies have demonstrated that Se supplementation inhibits tumorigenesis in a number of mammalian species and that the protective effect extends to chemical, radiation, and oncogenic virus-induced transformation. Other studies have indicated that a nutritional deficiency of Se can result in a significant enhancement of tumor development in a number of model systems and, as based on epidemiologic data, also in humans (2).

The mechanism(s) involved in the chemopreventive effect of Se is still unknown. Among possible mechanisms, Se may be involved in the regulation of DNA synthesis (3), act as an antioxidant (4), regulate cell function by maintaining or increasing the level of total cellular glutathione (5), or it may enhance the functions of immunocompetent cells (6). The purpose of this study was to determine whether modulation of the levels of Se in the diet can result in significant changes in the ability of a host to generate cytotoxic lymphocytes and to destroy tumor cells.

Received May 25, 1989. [P.S.E.B.M. 1990, Vol 193]
Accepted October 24, 1989.

0037-9727/90/1932-0143$2.00/0

Materials and Methods

Animals and Diet. Male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME), 6 weeks of age, were housed in a temperature- and humidity-controlled animal facility, with a 12-hr/12-hr light/dark cycle, and given water *ad libitum*. All animals were fed at midday, and all experimental animals were sacrificed between 9 AM and 12 noon to maintain uniformity and avoid circadian variations.

The animals were maintained on the Se-deficient (0.02 ppm Se), 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 the full composition of the diets appears in the accompanying paper. The protein source in the diet was Torula yeast and the Se content of the yeast was 0.02 ppm, as determined fluorometrically (Hazelton Labs, Madison, WI). The normal and supplemented diets were prepared by adding to the basal diet the needed amount of sodium selenite to obtain

0.20 ppm and 2.00 ppm Se, 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 significant differences in the body weight of animals maintained for 8 weeks on the three diets. Blood samples were taken by cardiac puncture, under light ether anesthesia, and, for each diet group, equal volumes of blood from each animal were pooled. Serum was separated, filter sterilized (0.45 μm; Millipore, Bedford, MA), and serum Se concentrations were fluorometrically determined by National Medical Services, Inc., Willowgrove, PA. The sensitivity of the assay was 6.85×10^{-8} *M* Se and all assays were run against quality control standards (standard concentration 9.4 μg/dl; SD ± 0.95 μg/dl). The Se concentrations of the pooled serum samples from 10 each, Se-normal, Se-deficient, and Se-supplemented mice were 3.6×10^{-6} *M*, 2.7×10^{-7} *M*, and 6.6×10^{-6} *M*, respectively. Although the final Se concentration in the Se-modified diets was not assessed, the significant differences in the Se levels in the serum from animals maintained on the three diets attest to the Se content of the diets.

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 tumor 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 penicillin, 100 μg/ml streptomycin, and 2 m*M* glutamine (GIBCO, Grand Island, NY), and additional supplementation as specified for each assay. All cultures were incubated at 37°C with 5% CO_2.

***In Vivo* Generation of Cytotoxic Lymphocytes (CTL).** Eleven days before the end of the 8-week feeding period, groups of 10 Se-normal, Se-supplemented, and Se-deficient mice were immunized by intraperitoneal injection with 5×10^6 freshly isolated P815 mastocytoma cells, maintained by serial passage in DBA/2 mice. On the day of assay, peritoneal exudate cells were obtained by lavage with 10 ml of the basic medium supplemented with 1 unit/ml sodium heparin, and to 5% with fetal bovine serum (FBS; GIBCO). The cells were washed once in the basic culture medium supplemented with 0.1 m*M* nonessential amino acids (GIBCO) and to 10% with FBS, resuspended in the same medium, and cultured for 2 hr. The nonadherent cells (lymphocytes) were collected and counted for use in the cytotoxicity assay.

***In Vitro* Generation of CTL.** CTL were generated *in vitro* by allogeneic stimulation of spleen lymphocytes removed from animals maintained on standard laboratory diet. The spleen cells were removed as described previously (7), washed once in basic medium supplemented to 5% with FBS, and counted. Lymphocytes (4×10^6) were cocultured in 2 ml of the basic medium supplemented with 0.1 m*M* nonessential amino acid, 5×10^{-5} *M* 2-mercaptoethanol, 1×10^{-9} to 1×10^{-6} *M* sodium selenite (Sigma, St. Louis, MO), and to 5% with FBS, with 8×10^4 P815 cells that had been treated with mitomycin C (100 μg/ml, 60 min at 37°C; Boehringer Manheim, Indianapolis, IN). The cells were plated in 24-well culture plates (Linbro 76-033-05) and cultured for 5 days. The lot of FBS used for these studies was specifically selected for its low Se content, i.e., 0.9 μg/dl (National Medical Services, Inc.), which is equal to 6.33×10^{-9} *M* Se in the final culture medium. At the end of the incubation period, the cells were harvested, washed once, counted, and used for the cytotoxicity assay.

Cytotoxicity Assay. Lymphocyte-mediated tumor cytotoxicity was assayed using a 4-hr ^{51}Cr release assay. Activated lymphocytes were cocultured with 2×10^4 P815 cells labeled with ^{51}Cr (400 mCi/mg; New England Nuclear, Boston, MA), at effector cell to target cell ratios of 1.25:1–10:1 in 96-well round-bottom culture plates (Linbro 76-013-05). The cells were cultured in a total volume of 200 μl/well of the basic culture medium supplemented to 5% with FBS for 4 hr. 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, in 100 μl of medium, 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 follows:

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

where the cpm values represent the means of four replicates/sample. The total number of peritoneal exudate cell lymphocytes required to kill 2×10^4 target P815 cells was determined by regression analysis of the mean percentage of cytotoxicity at the various effector to target cell ratios for each individual animal at the point at which the regression line intercepted 63% specific lysis, which is a measure of the cytotoxic cell frequency in a given population of lymphocytes (8). The cytotoxic cell activity, *A* (which represents the relative efficiency of CTL to kill a specific target tumor cell), for each control and experimental animal was calculated as:

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

where *Y* is the % specific lysis, *x* is the number of lymphocytes/well at the 10:1 effector to target cell ratio, and *k* is the cytotoxic cell frequency (reciprocal of the number of lymphocytes required to kill 2×10^4 target cells) (8).

Effect of Exogenous Selenium on ^{51}Cr Release by P815 Cells. The effect of Se on nonspecific release of ^{51}Cr by P815 cells was tested by incubating 2×10^4

^{51}Cr-labeled P815 cells, in the basic culture medium supplemented with 1×10^{-9} to 1×10^{-6} M sodium selenite and to 5% with FBS, as in the 4-hr ^{51}Cr release assay. The percentage of specific ^{51}Cr release was calculated as in the cytotoxicity assay.

Effect of Exogenous Selenium on Lysis of P815 Cells by CTL. The effect of exogenous Se on the lysis of P815 target cells by CTL generated *in vitro* was determined with spleen cells from 14-week-old standard-fed mice. The CTL were generated as described above but without exogenous Se in the medium. The lymphocytes were harvested, washed once, and the cytotoxic lymphocyte activity was measured in the 4-hr ^{51}Cr release assay in the presence of 1×10^{-9} to 1×10^{-6} M of added sodium selenite.

Lymphotoxin Production. CTL were generated *in vivo* in animals maintained on the control or experimental diets (10 animals/diet) and collected as described above. Peritoneal lymphocytes (5×10^6) were cocultured in 25-mm^2 culture flasks (Corning 25100) with 2×10^5 mitomycin C-treated P815 cells in 5 ml of the basic culture medium supplemented to 5% with FBS. The cells were incubated for 4 hr, the culture supernatants were collected, filtered (0.45 μm; Millipore), and immediately assayed for lymphotoxin content. L929 cells/well (2.5×10^5), in 0.2 ml of the basic culture medium supplemented with 10 μg/ml actinomycin-D (Sigma) and to 3% with FBS, were plated in 96-well flat-bottom culture plates (Linbro 76-003-05) with serial dilutions (1/4 to 1/512; six replicates/dilution) of lymphotoxin containing medium or medium alone, and incubated for 21 hr. The remaining live cells in each well were fixed, stained with 0.5% crystal violet, and washed three times in tap water. The dye was eluted with 95% ethanol and the optical density of the material in each well was measured (Titertek Uniskan II; Flow Labs, McLean, VA) at 570 nm. One unit of lymphotoxin was defined as the amount of supernatant that caused lysis of 50% of the cells/well.

Statistical Analysis. The results are presented as the arithmetic $\bar{X} \pm$ SE for each control and experimental group. Differences among the $\bar{X}$ of groups were determined using Student's two-tailed t test and P values ≤ 0.05 were considered to be significantly different.

Results

Effect of Dietary Selenium on *In Vivo* Generation of CTL. Se supplementation significantly enhanced (by 22.3%; $P < 0.05$) and Se deficiency significantly reduced (by 16.8%; $P < 0.05$) tumor cell cytotoxicity mediated by alloantigen-induced *in vivo*-generated lymphocytes (Fig. 1). The response curves for all lymphocyte dilutions (i.e., effector to target cell ratios) were linear for the three dietary levels of Se (Fig. 2), and the percentage of cytotoxicity was significantly different at all effector to target cell ratios tested.

The observed changes in cytotoxicity were apparently related to an increase or decrease in the cytotoxic

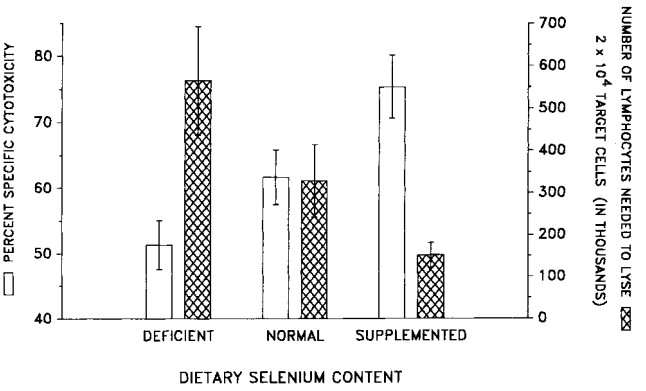

Figure 1. Effect of Se supplementation and deficiency on tumor cytodestruction by peritoneal cytotoxic lymphocytes generated by intraperitoneal immunization with P815 mastocytoma cells 11 days before the assay. Data are presented as the $\bar{X} \pm$ SE of the percentage of specific lysis of P815 cells at a 10:1 effector to target cell ratio (open bars), and as $\bar{X} \pm$ SE of the number of peritoneal lymphocytes required to lyse 2×10^4 P815 cells (closed bars).

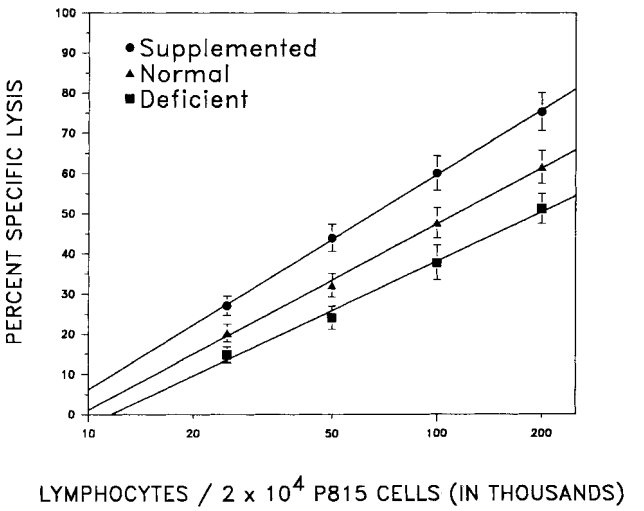

Figure 2. Effect of dietary Se supplementation and deficiency on tumor cytodestruction by cytotoxic lymphocytes generated *in vivo*. Data are presented as the $\bar{X} \pm$ SE of the percentage of specific lysis of 2×10^4 P815 mastocytoma cells by 2×10^5, 1×10^5, 5×10^4, and 2.5×10^4 peritoneal lymphocytes, representing effector to target cell ratios of 10:1, 5:1, 2.5:1, and 1.25:1. The least-squares linear regression is given for each experimental and control diet.

cell frequency (i.e., relative numbers of activated cytotoxic lymphocytes within the peritoneal exudate pools). The number of peritoneal exudate lymphocytes required to kill a fixed number of target tumor cells (2×10^4) was significantly increased in Se-deficient (by 72.0%; $P < 0.01$) and significantly decreased in Se-supplemented (by 53.9%; $P < 0.05$) mice (Fig. 1). The cytotoxic cell activity, however, was not affected as mice on all three diets generated cytotoxic lymphocytes with A values in the range of 0.945–1.02.

Effect of Selenium Supplementation on *In Vitro* Generation of CTL. The concentration of Se in the culture medium contributed by the FBS was 6.33×10^{-9} M. Supplementation of the medium with the lowest level (1×10^{-9} M) of Se tested, which was below the endogenous concentration of Se in the culture

medium, had no apparent effect ($P > 0.9995$) on the percentage of specific lysis of target cells (Fig. 3). However, supplementation of the medium with 5×10^{-9} to 5×10^{-7} M Se resulted in significant increases ($P < 0.05$–$P < 0.0025$) in the percentage of cytotoxicity that were from 7.2% to 19.2% above baseline controls. When the Se supplementation dose was plotted semilogarithmically against the percentage of specific tumor cell lysis, the curve was linear in the range of 1×10^{-9} to 1×10^{-7} M, and then plateaued at 5×10^{-7} M. However, the percentage of cytotoxicity decreased to 32.41 ± 10.81 ($P < 0.05$) at 1×10^{-6} M Se, and the number of viable lymphocytes recovered after 5 days of culture in the presence of 1×10^{-6} M Se was decreased to 54.3% of control.

In vitro supplementation with 5×10^{-9} to 5×10^{-7} M Se during CTL generation in allogeneically stimulated cultures resulted in a significant reduction ($P < 0.05$) in the number of lymphocytes required to destroy a fixed number of target cells (Fig. 4). The increase in cytotoxic cell frequency was not accompanied by an alteration in cytotoxic cell activity, since all concentrations of Se (1×10^{-9} to 5×10^{-7} M) tested resulted in A values in the range of 0.86–0.91.

Effect of Dietary Selenium Levels on Lymphotoxin Production. Dietary supplementation with Se significantly enhanced ($P < 0.005$) the *in vitro* production of lymphotoxin to a level that was approximately double (121% increase) that in mice maintained on the normal diet (Fig. 5). Dietary deficiency in Se significantly decreased lymphotoxin production (by 35.7%; $P < 0.05$).

Effect of Exogenous Selenium on Tumor Cell Lysis by Preactivated CTL. To test the effect of Se on the lytic phase of tumor cytodestruction, various amounts of Se were added to the culture media at the beginning of the 4-hr ^{51}Cr release assay. No significant alterations in the percentage of specific lysis were evident at any of the Se concentrations tested (1×10^{-9} to 5×10^{-7} M), when compared with control cultures that contained only endogenous (FBS contributed; 6.33×10^{-9} M) levels of Se (Fig. 6).

Effect of Exogenous Selenium on Release of

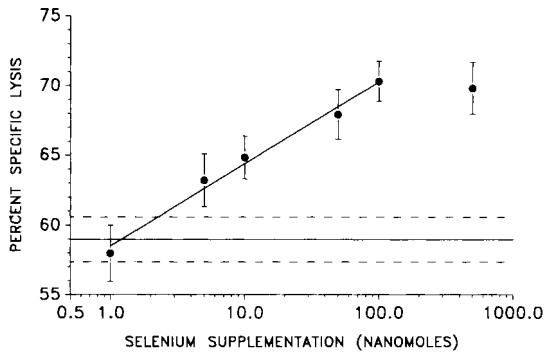

Figure 3. Effect of selenium supplementation on tumor cytodestruction by *in vitro*-generated splenic cytotoxic lymphocytes. Data are given as the $\bar{X} \pm$ SE of the percentage of specific lysis of P815 mastocytoma cells at an effector to target cell ratio of 10:1. The linear regression line for 1×10^{-9} to 1×10^{-7} M of added Se is given. The horizontal lines represent the $\bar{X}$ (solid line) $\pm$ SE (dashed lines) for nonsupplemented control lymphocytes. Supplementation with 1×10^{-6} M Se (not shown) resulted in 32.41 ± 10.81% specific lysis.

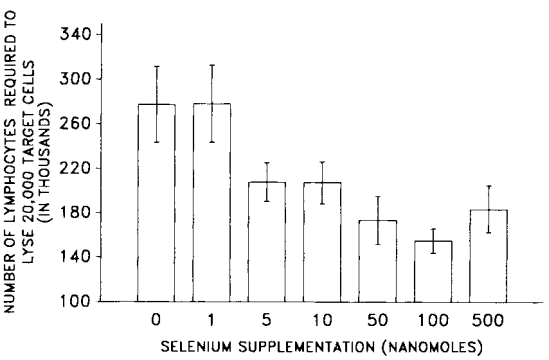

Figure 4. The effect of 1×10^{-9} to 5×10^{-7} M exogenous Se on the *in vitro* generation of splenic cytotoxic lymphocytes by allogeneic stimulation with mitomycin C-treated P815 mastocytoma cells for 5 days. The data are presented as the $\bar{X} \pm$ SE of the number of lymphocytes required to lyse 2×10^4 target cells.

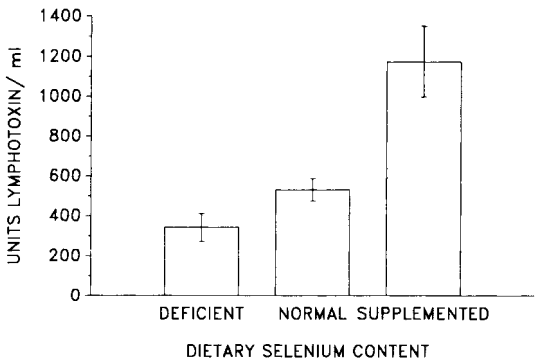

Figure 5. Effect of dietary Se supplementation on the production of lymphotoxin by peritoneal exudate lymphocytes generated after intraperitoneal immunization with P815 mastocytoma cells 11 days before the assay. Lymphocytes were cocultured with mitomycin C-treated P815 cells for 4 hr, and the culture supernatants were assayed for cytotoxicity on L929 cells. Data are given as the $\bar{X} \pm$ SE units of lymphotoxin/ml.

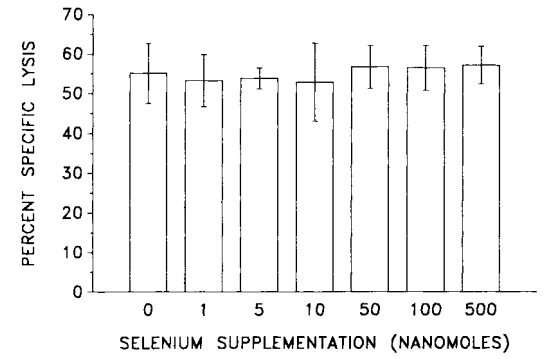

Figure 6. Effect of exogenous selenium on the lytic phase of tumor cytodestruction by *in vitro*-generated splenic cytotoxic lymphocytes. Splenic lymphocytes were cocultured with mitomycin C-treated P815 mastocytoma cells for 5 days. The ^{51}Cr release assay was conducted in the presence of 1×10^{-9} to 5×10^{-7} M exogenous Se. Data are presented as the $\bar{X} \pm$ SE of the percentage of specific lysis of P815 cells at a 10:1 effector to target cell ratio.

^{51}Cr from P815 Cells. To determine whether the observed increases in cytodestruction resulted from an enhanced release of ^{51}Cr by P815 cells in the presence of Se, ^{51}Cr-labeled P815 cells were incubated for 4 hr in the presence of various concentrations of Se. Exogenous Se in the range of 1×10^{-9} to 5×10^{-7} *M* had no demonstrable effect on the nonspecific release of ^{51}Cr from P815 cells (Fig. 7).

Discussion

CTL differentiation in response to alloantigens occurs as a result of interactions between the alloantigen-bearing cells, accessory cells, helper T cells, and CTL precursor cells, CTL-P (9, 10). As a result of these interactions, soluble factors are produced that activate CTL-P and stimulate their proliferation. Among these factors, interleukin 2 (IL-2) is the most studied (11, 12) but other lymphokines may also be required for the induction of cytotoxic responses (13, 14). These factors appear to be necessary for the differentiation of CTL and for the induction of IL-2 receptor (IL-2R) expression. It is postulated that the CTL-P acquire receptors for IL-2 and non-IL-2 factors after the delivery of the activation signal through the T cell receptor complex (17).

The destruction of tumor cells by CTL occurs in two distinct phases (18, 19). In the first phase, conjugates between CTL and target cells are formed that trigger the CTL, and lysis is initiated. In the second phase, the CTL detach from the tumor cells and lysis occurs independently of the presence of CTL. The detached CTL can then attack another target cell.

The results from the present studies showed that peritoneal exudates generated by allogeneic stimulation with tumor cells in mice maintained on the Se-modified diets contained significantly different numbers of CTL. Supplementation with Se resulted in a significant reduction in the number of peritoneal lymphocytes (a pool of lymphocytes which contains CTL) required to destroy a fixed number of tumor cells, whereas deficiency in Se had the opposite effect. As the cytotoxic activity of CTL from animals maintained on all three

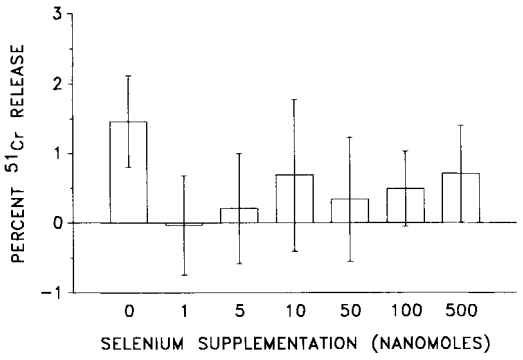

Figure 7. Effect of exogenous selenium on nonspecific ^{51}Cr release by P815 mastocytoma cells. ^{51}Cr-labeled P815 cells were incubated for 4 hr in the presence of 1×10^{-9} to 5×10^{-7} *M* Se. Data are given as the $\bar{X} \pm$ SE of percentage of ^{51}Cr release.

diets remained the same, it appears that Se supplementation may stimulate CTL differentiation while Se deficiency may inhibit the process.

The stimulatory effect of Se on tumor cytodestruction and generation of CTL *in vivo* was confirmed in our *in vitro* cytotoxicity studies. The serum Se level for animals maintained on the normal and Se-supplemented diets was 3.6×10^{-6} and 6.6×10^{-6} *M*, respectively, which falls within the physiologic serum level range reported by others (20, 21). This physiologic range, however, reflects the total Se levels rather than the various organic and inorganic forms of the element (22, 23). *In vitro* studies that have utilized selenite, because of its significant biologic activity, have consistently reported a growth inhibitory effect in a number of cell types at 1 to 5×10^{-6} *M* and a cytotoxic effect at greater concentrations (24–28). On this basis, 1×10^{-9} *M* to 1×10^{-6} *M* Se (as sodium selenite) was chosen for our *in vitro* studies. The greatest amount of cytodestruction and the lowest number of peritoneal exudate lymphocytes required to destroy a fixed number of tumor cells occurred at 1×10^{-7} *M* Se, whereas at 1×10^{-6} *M* Se, there was a significant inhibition of the stimulatory effect. As the growth of hematopoietic and other cell types is optimal at 1×10^{-7} *M* Se (as sodium selenite) (29, 30), it is possible that the observed effect of Se supplementation in our studies may be associated with enhanced generation of CTL. That the effect of Se was exerted during the CTL generation phase rather than in the process of cytodestruction was further indicated by the fact that Se had no effect on the lytic phase of the process, or on the release of ^{51}Cr from P815 cells.

It has been proposed that the cytotoxicity of CTL is expressed through the release of a toxic product termed lymphotoxin (TNF-β) (31). This material is released by lymphocytes activated under various conditions, e.g., when lymphocytes are stimulated with alloantigen, the material is released within 4–6 hr after interaction with the stimulating antigen (32, 33). Moreover, lymphotoxin has properties that are consistent with those of a toxin injected by CTL into their targets (34). In our system, significantly greater amounts of lymphotoxin were produced in cultures of allogeneically stimulated peritoneal exudate lymphocytes from animals maintained on the Se-supplemented diet, as compared with control cultures. The reverse was true for cells from animals maintained on the Se-deficient diet. These results support our conclusion that the same lymphocyte populations, i.e., allogeneically stimulated peritoneal exudate lymphocytes, also contain greater or lower numbers of CTL depending on the amount of Se in the diet.

Recently, it was shown that lymphotoxin produced following allogeneic stimulation significantly enhanced the proliferative responses in the mixed lymphocyte reaction, and the enhanced proliferation appeared to be the result of the ability of the lymphokine to increase

IL-2R expression (35). As the expression of IL-2R is one of the key factors involved in the regulation of CTL-P proliferation, the increased amounts of lymphotoxin produced after allogeneic stimulation in our system can be related, at least in part, to the increased proliferation and differentiation of CTL-P in the same cultures.

The mechanism(s) involved in the enhancement of cell proliferation as the result of supplementation of the diet with low doses of Se, as well as the inhibition of the process as the result of Se deficiency, is unknown. As suggested in the accompanying paper, the observed effects may have resulted from the ability of Se to directly influence the expression of IL-2R, or, indirectly, through an effect on the transmission of transmembrane and/or intracellular signals. Although these studies were not designed to elucidate the mechanism(s) of the action of Se, the results indicate the necessity for further studies, especially as the maintenance of an adequate nutritional intake of Se appears to be a significant factor in the ability of a host to prevent the expression of malignant neoplasms (1).

This work was supported by Grants 86A08 and 86A08-R87B from the American Institute for Cancer Research.

The authors thank Ms. Jeanine Auszmann for technical assistance.

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