Selenium and Immune Cell Functions. I. Effect on Lymphocyte Proliferation and Production of Interleukin 1 and Interleukin 2

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Abstract. **The dietary intake of selenium (Se) has been shown to influence the development and expression of various biologic processes. This study examined the immunologic competence of lymphocytes from C57BL/6J mice maintained for 8 weeks on Se-deficient (0.02 ppm Se), normal (0.20 ppm Se, as sodium selenite), or Se-supplemented (2.00 ppm Se) Torula yeast-based diets. The ability of the cells to recognize alloantigens, to proliferate in response to stimuli, and to produce interleukin 2 (IL-2) was determined. Se deficiency significantly inhibited the ability of the lymphocytes to proliferate in response to allogeneic stimulation in the mixed lymphocyte reaction or to mitogen stimulation by phytohemagglutinin, whereas Se supplementation significantly enhanced both responses. In contrast, the amounts of IL-2 and interleukin 1 (IL-1) produced by lymphocytes and macrophages, respectively, removed from Se-deficient or Se-supplemented animals did not differ significantly from the amounts of IL-2 and IL-1 produced by cells removed from animals maintained on the control diet. These results suggest that the mechanism(s) responsible for the observed effects of Se on lymphocyte proliferation are independent of the levels of IL-2 or IL-1.** [P.S.E.B.M. 1990, Vol 193]

Selenium (Se) appears to affect significantly the function of all components of the immune system (1–3). In animals maintained on Se-modified diets, a deficiency of Se appears to result in immunosuppression, whereas supplementation with low doses of Se appears to result in augmentation and/or restoration of immunologic functions. Although there have been relatively few studies in this area, the apparent effects of Se on immunologic functions indicate that this trace element may be an immunologic response modifier of significant clinical importance. Of particular interest is the indication that modulation of Se levels in the diet may augment or suppress the immune response of a host toward malignant cells (4–6).

The interaction of lymphocytes with foreign soluble or cellular antigens or mitogens in the presence of accessory cells results in the production and release of various mediators of immunity and in the subsequent proliferation and differentiation of the lymphocytes into mature immunocompetent cells (7, 8). This process represents the basis for the expression of all major immunologic functions. An agent that can modify any aspect of this sequential process could, therefore, have a significant effect on the immunologic competence of a host. This study determined whether modulation of the levels of Se in the diet affects the expression of immune functions by influencing the ability of lymphocytes to interact with antigen or mitogen, to produce lymphokine, and to proliferate.

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Materials and Methods

Animals and Diet. Six-week-old male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were 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. The composition of the basal diet (without added Se) and the level of Se in the normal diet were selected on the basis of the ability of the diets to produce significant differences in the Se status of the animals, i.e., deficient versus normal levels (9).

The use of 2 ppm Se as a supplement was based on a report by Medina and Lane (10) that indicated an

optimal protective effect against cancer at this level. The diets were prepared commercially (Teklad, WI), and the composition of the basal diet (without added Se) is shown in Table I. The protein source in the diet was Torula yeast, and the Se content of the yeast in the basal diet was 0.02 ppm, as determined fluorometrically (Hazelton Labs., WI). The same lot of yeast was used in the preparation of all diets. The normal and supplemented diets were prepared by adding to the basal diet the needed amount of sodium selenite to obtain 0.20 and 2.00 ppm Se, respectively. Each animal was provided with 5 g/day of the respective diet and water *ad libitum*. There were no significant differences in the body weights of mice maintained for 8 weeks on the three diets. The Se levels in the pooled serum collected at the end of the 8-week period from the control and experimental groups were fluorometrically determined by National Medical Services, Inc., Willowgrove, PA. The sensitivity of the assay was 0.5 μg/dl 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 27, 2, and 48 μg/dl, 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.

The DBA/2 and C_3H/HeJ mice used in the mixed lymphocyte reaction and IL-1 studies were maintained on the normal diet. All mice were housed in temperature-, humidity-, and light-cycle controlled rooms.

Culture Media. All cells were cultured in a basic medium of RPMI 1640 supplemented with 25 mM 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.

Mixed Lymphocyte Reaction (MLR). Spleen cells from control and experimental animals, as well as from DBA/2 mice, were prepared for the MLR as described previously (11, 12). Briefly, minced spleens were passed through a wire mesh, the red blood cells removed by osmotic shock with distilled water, and the washed spleen cells suspended in the basic medium supplemented to 5% with heat-inactivated fetal bovine serum (FBS; GIBCO; Se content: 1–2 ppm, determined by National Medical Services, Inc.) at a cell density of 5×10^6 lymphocytes/ml. No 2-mercaptoethanol was added to the culture medium, as its immunoenhancing effect was not desirable in these studies.

For the one-way MLR, 4×10^5 control or experimental C57BL/6J lymphocytes were co-cultured with an equal number of DBA/2 spleen lymphocytes that were pretreated with 50 μg/ml mitomycin C (Boehringer Manheim, Indianapolis, IN) for 30 min. The cells were plated (eight replicates per cell combination per animal) in the supplemented RPMI 1640 medium in a total volume of 200 μl/well in 96-well flat bottom culture plates (Linbro 76-003-05) and incubated for 96 hr. At the end of this period, 1 μCi of [*methyl*-^{3}H] thymidine (sp act, 6.7 Ci/mmol; New England Nuclear, Boston, MA) in 50 μl of culture medium was added to each well and the plate was incubated for an additional 24 hr. The cells were harvested with an automatic cell harvester (Skatron, Sterling, VA), lysed in distilled water, and the nuclei collected on filters. The amounts of [*methyl*-^{3}H]thymidine incorporation, in cpm, were determined in a liquid scintillation counter, and the average cpm for the eight replicate determinations, per cell combination, for each control or experimental animal were determined. The mean ± SE ($\bar{X}$ ± SE) from nine Se-deficient, eight Se-supplemented, and seven normal animals were compared statistically with Student's *t* test.

Mitogen Stimulation Assay. Spleen cells from control and experimental animals were prepared as for the MLR and suspended in the same medium supplemented with 10 μg/ml phytohemagglutinin-P (Sigma, St. Louis, MO). The cells were plated in 96-well flat-bottom plates at 2×10^5 lymphocytes/well in a total volume of 200 μl and incubated for 48 hr. At the end of the incubation period, the cultures were pulsed with 1 μCi of [*methyl*-^{3}H]thymidine in 50 μl of culture medium for an additional 24 hr, the cells were harvested, and the amount of incorporation was determined as for the MLR. The $\bar{X}$ cpm ± SE from five replicates each from eight Se-deficient, nine Se-supplemented, and three normal animals were compared statistically with Student's *t* test.

Interleukin 2 Production and Assay. Spleen cells from control and experimental animals were prepared as for the MLR and suspended in the same medium supplemented with 3 μg/ml concanavalin A (Con A; Sigma). The cells were adjusted to 5×10^6 lymphocytes/ml, and 2×10^7 lymphocytes were cultured in 25-mm^2 culture flasks (Corning 25100) in 4 ml of medium for

Table I. Composition of Basal Diet

Ingredient	g/kg
Torula yeast	300.00
DL-methionine	3.00
Corn oil	50.00
Mineral mix, selenium deficient[a]	30.87
Calcium carbonate	12.00
Sucrose	592.13
Vitamin mix, AIN-76A (38)	10.00
Choline bitartrate	2.00
Sucrose-selenium mixture[b]	0.00

[a] AIN-76 mineral mix (37) without selenium.
[b] The amount of sucrose-selenium mix (0.045% Na_2SeO_2) added to obtain the normal (0.20 ppm) and Se-supplemented (2.00 ppm) diets was based on the selenium content of the Torula yeast (0.02 ppm), i.e., 591.2323 g/kg sucrose plus 0.899 g/kg sucrose-selenium mix and 582.2433 g/kg sucrose plus 9.888 g/kg sucrose-selenium mix, respectively.

24 hr. The cells from the control animals were pooled prior to culture, whereas cells from each of the experimental animals were cultured separately. At the end of the incubation, supernatants were collected, the Con A was absorbed with 10 mg/ml Sephadex G-10 (Sigma), and the supernatants were filtered (0.45 μm; Millipore, Bedford, MA) and frozen at −20°C until assayed.

IL-2 activity in the supernatants was assayed as described by Gillis *et al.* (13). IL-2-dependent CTLL cells, provided by Dr. K. Welte (Memorial Sloan-Kettering Institute, New York, NY), were suspended in RPMI 1640 supplemented with 2.8×10^{-5} *M* 2-mercaptoethanol and to 10% FBS. CTLL cells (4×10^3) were cultured in 200 μl in 96-well flat-bottom plates with serial dilutions (1/4–1/128) of the IL-2-containing supernatants or with 1 half-maximal unit of human IL-2 (Electro-Nucleonics, Silver Spring, MD), which served as a calibration standard. The plates were incubated for 18 hr, 0.5 μCi of [*methyl*-^{3}H]thymidine (sp act, 6.7 Ci/mmol) in 50 μl of culture medium was added to each well, and the cultures were incubated for an additional 4 hr. The cells were harvested, and the amount of radiotracer incorporated in three wells per dilution of IL-2 was determined as described for the MLR. For each sample, the $\bar{X}$ cpm for the triplicates were plotted against the ln of the dilution and a linear regression analysis was performed. The cpm value of the standard was extended to intercept the curve, and the reciprocal of the dilution (anti-log of ln dilution) was determined as half-maximal units/200-μl sample and adjusted to half-maximal units/ml. The $\bar{X}$ units IL-2 ± SE produced in supernatants from lymphocyte cultures from 17 Se-deficient and 20 Se-supplemented animals were compared statistically (Student's *t* test and one-way analysis of variance) with the amount of IL-2 in the supernatant from cultures of pooled lymphocytes from nine animals maintained on the normal diet.

Interleukin 1 Production and Assay. Resident (nonimmune) peritoneal macrophages and activated (immune) peritoneal macrophages, elicited by intraperitoneal immunization with 5×10^6 P815 mastocytoma cells 10 days earlier, were collected from control and experimental animals by peritoneal lavage as described previously (14). The peritoneal cells were washed and suspended in basic medium supplemented to 10% FBS. The number of macrophages in these preparations were determined on Giemsa-stained smears, based on morphologic criteria for identification of macrophages. These differential counts correlated with counts made on peritoneal cell preparations from the same animal incubated in the presence of 100 mg/ml of carbon particles and stained with Giemsa. Monolayers were prepared by seeding 1.5×10^6 macrophages in 1 ml of medium into each well of 24-well culture plates (Linbro 76-033-05); the plates were incubated for 2 hr to allow the macrophages to adhere. The monolayers were washed three times with warm medium by vigorously decanting the fluid from the plate and blotting on sterile absorbent paper after the final wash. The procedure yielded monolayers free of nonadherent cells.

IL-1 production was stimulated by incubating the washed macrophage monolayers in 1 ml of medium containing 20 μg of lipopolysaccharide W from *Escherichia coli* D 128:B12 (Difco Laboratories, Detroit, MI) for 48 hr. Control cultures were prepared without lipopolysaccharide. The culture supernatants were filtered (0.45 μm; Millipore) and frozen at −20°C until assayed.

The mouse thymocyte assay for IL-1, which measures the ability of IL-1 to stimulate proliferation of C_3H/HeJ mouse thymocytes in the presence of a suboptimal concentration of phytohemagglutinin (PHA), was performed as described by Schultz and Alton (15). Briefly, thymocytes from 5-week-old female mice were suspended in basic medium supplemented with 5×10^{-5} *M* 2-mercaptoethanol and to 5% FBS. The cells were seeded at 1.5×10^6 cells/well in 96-well flat-bottom culture plates in a total volume of 200 μl of assay medium containing serial dilutions (1/4–1/64) of IL-1 and 1 μg/ml phytohemagglutinin-P (Sigma). The plates were incubated for 72 hr. Four hours before harvest, 1 μCi of [*methyl*-^{3}H]thymidine (sp act, 6.7 Ci/mmol) in 50 μl of culture medium was added to each well. The cells were harvested, and the amounts of radiotracer incorporated in three wells per dilution were determined as described for the MLR.

A single preparation of murine IL-1 was prepared in our laboratory, as described above, and served as the standard for all assays. This preparation was assigned an arbitrary concentration of 100 units/ml. Serial dilutions (1/4–1/64) of the standard were tested concurrently with the test samples for each assay. The mean cpm of radiotracer incorporation was determined for all dilutions of standard and experimental samples. The cpm of the dilutions of the standard were plotted against the ln of the dilution factor, and a regression analysis was performed on the linear portion of the curve. The cpm value of the standard curve that intercepted the value of ln 10 (dilution factor of 10) was used as the standard activity of 10 units/0.1 ml for the individual assays. The mean cpm for the various dilutions of the experimental samples for each assay were determined, and each was plotted against the ln of the dilution factor to determine the linear portion of the curve. A regression analysis was performed on the linear portion of the plot, and the dilution factor at which each curve intercepted the cpm of the standard activity for that assay was calculated. This dilution factor was multiplied by 10 to yield the number of units/ml.

For immunized animals, the $\bar{X}$ units IL-1 ± SE produced by activated macrophages and the $\bar{X}$ cpm [methyl-^{3}H]thymidine incorporation stimulated in thymocytes by the IL-1 preparations (1/8 dilution) were compared statistically (Student's *t* test). For nonimmunized animals, the $\bar{X}$ cpm radiotracer incorporation

stimulated by IL-1 (1/8 dilution) produced by resident macrophages were compared statistically with Student's *t* test and analysis of variance.

Results

MLR. Splenocytes from C57BL/6J mice that were maintained for 8 weeks on the Se-supplemented diet and allogeneically stimulated with mitomycin C-treated DBA/2 spleen cells showed a significant increase in their ability to proliferate. The mean [*methyl*-^{3}H]thymidine incorporation value for these cells was 114.0% higher ($P < 0.0005$) than that of cells from animals maintained on the control diet (Fig. 1). Conversely, cells from animals maintained on the Se-deficient diet showed a 30% decrease ($P < 0.05$) in their ability to mount an antigen-induced blastogenic response. The background [^{3}H]thymidine incorporation values for all groups of animals were the same and the intrasample variation within replicates averaged 10.8%. The correlation between [*methyl*-^{3}H]thymidine incorporation and cell proliferation is supported by data presented in the accompanying article that indicated quantitative changes in the T cell population following allogeneic stimulation both *in vivo* and *in vitro*.

Mitogen Stimulation Assay. Stimulation with phytohemagglutinin P of spleen cells from animals maintained on the Se-supplemented diet resulted in a significant increase in the ability of lymphocytes to undergo blastogenesis. The mean [*methyl*-^{3}H]thymidine incorporation value for these cells was 110.6% higher ($P < 0.0005$) than the mean incorporation value for cells from control animals (Fig. 2). In contrast, cells from animals maintained on the Se-deficient diet showed a 49.5% decrease ($P < 0.0025$) in their ability to proliferate in response to mitogen stimulation. The background [^{3}H]thymidine incorporation values for all groups of animals were the same and the intrasample variation within replicates averaged 5.0%.

IL-2 Production. Alterations in the dietary Se levels did not result in a statistically significant change in the ability of spleen cells to produce IL-2 *in vitro*. The units of IL-2 produced in cultures of cells from animals maintained on a Se-supplemented or Se-deficient diet did not differ significantly ($P > 0.95$) from the amount produced by cells from animals maintained on the control (normal) diet (Fig. 3).

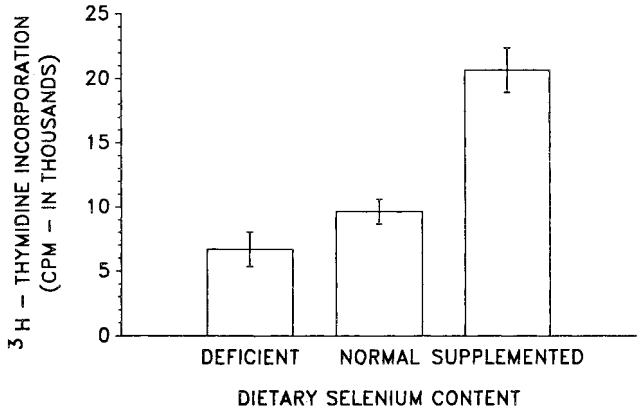

Figure 1. Effect of Se supplementation and deficiency on the mixed lymphocyte response of spleen cells from C57BL/6J mice. Spleen lymphocytes were stimulated with mitomycin C-treated spleen cells from DBA/2 mice, and incorporation of the radiotracer was evaluated after 5 days in culture.

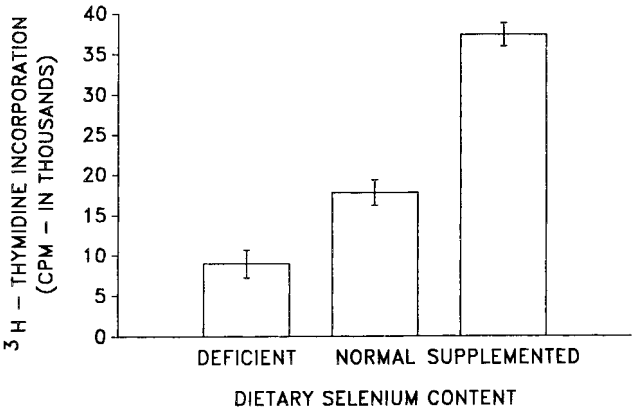

Figure 2. Effect of Se supplementation and deficiency on the ability of C57BL/6J spleen cells to proliferate in response to phytohemagglutinin. The incorporation of the radiotracer was evaluated after 3 days in culture.

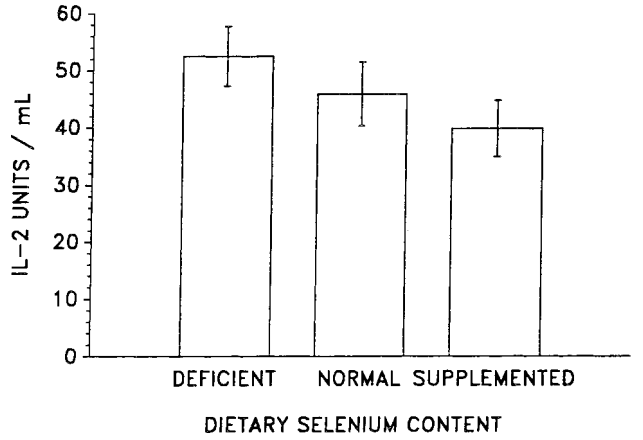

Figure 3. Effect of Se supplementation and deficiency on the ability of C57BL/6J spleen lymphocytes to produce IL-2 in response to stimulation for 24 hr with Con A. IL-2 production was measured using the CTLL proliferation assay and data are expressed as $\bar{X}$ units IL-2/ml ± SE.

IL-1 Production. There were no statistically significant differences in the incorporation of [*methyl*-^{3}H] thymidine ($P > 0.20$–0.90) by C_3H/HeJ thymocytes stimulated with IL-1 produced by immune (activated) or nonimmune (resident) macrophages from animals maintained on the Se-supplemented, Se-deficient, or normal diets (Table II). The units of IL-1 produced by immune macrophages from animals maintained on the three diets did not differ significantly from each other ($P > 0.30$–0.70, Table II).

Discussion

The first activation signal for T lymphocyte proliferation is provided by the interaction of a specific

Table II. Effect of Selenium Supplementation and Deficiency on the Ability of C57BL/6J Peritoneal Macrophages to Produce IL-1

	Dietary selenium content		
	Deficient (0.02 ppm)	Normal (0.20 ppm)	Supplemented (2.00 ppm)
[^{3}H]Thymidine incorporation[a] (non-immune mice[b])	5,957[c] ± 663 (6)	6,136 ± 1,287 (7)	7,643 ± 1,370 (7)
[^{3}H]Thymidine incorporation (immune mice[d])	16,488 ± 2,938 (10)	24,057 ± 3,536 (11)	18,665 ± 3,957 (11)
IL-1 units/ml (immune mice[d])	19.76 ± 2.10 (10)	20.87 ± 2.64 (11)	23.94 ± 3.26 (11)

[a] IL-1 was produced in response to stimulation with lipopolysaccharide for 48 hr and culture supernatants containing IL-1 (1/8 dilution) were tested for their ability to stimulate proliferation ([^{3}H]thymidine incorporation) of C3H/HeJ thymocytes.
[b] IL-1 produced by resident (nonimmune) peritoneal macrophages.
[c] $\bar{X}$ cpm ± SE (number of animals tested).
[d] IL-1 produced by activated (immune) peritoneal macrophages from mice immunized with P815 cells by intraperitoneal injection 10 days before the assay.

antigen, which is presented to lymphocytes in association with the major histocompatibility complex Class I or Class II molecules on the surface of macrophages, with the T cell receptor on the surface of resting lymphocytes (16). The same activation signal can also be initiated by mitogenic plant lectins (e.g., Con A or PHA), antibodies to the T cell receptor accessory proteins (T_3), or phorbol esters (17, 18). Maximum stimulation, however, requires the presence of the co-stimulatory monocyte-derived peptide, IL-1 (19–21). In response to the stimulation by antigen (mitogen) and IL-1, a specific subpopulation of T cells begins to synthesize and secrete IL-2, while other specific subsets synthesize and express, at their surface, receptors for IL-2 (IL-2R) (22–24). The interaction of IL-2 with high-affinity IL-2R is believed to provide a unique second signal to the IL-2R-positive cells to begin DNA synthesis and proliferate (24, 25). Although the biochemical events initiated by the binding of antigen or mitogen to T lymphocytes are the same, the signal resulting in cell proliferation may not use the same intracellular messengers (26).

The results of the present studies showed that spleen lymphocytes from mice maintained on the Se-supplemented diet have a significantly enhanced ability to proliferate when stimulated by antigen (MLR) or mitogen, whereas splenocytes from animals maintained on the Se-deficient diet have a significantly decreased ability to mount an antigen- or mitogen-induced blastogenic response. These results confirmed previous reports on the altered ability of lymphocytes from a number of animal species to respond to mitogen stimulation following exposure to a Se-deficient or Se-supplemented diet (27–30). However, the proliferative responses observed in this study appeared to be independent of the endogenous levels of IL-2 or IL-1, as the amounts of these cytokines produced in cultures of spleen cells or resident or immune macrophages, respectively, from animals maintained on all three Se-modified diets did not differ significantly.

As lymphocyte proliferation in response to antigen stimulation (e.g., MLR) requires the proper functioning of accessory cells (i.e., macrophages) in terms of, both, antigen presentation and IL-1 production, it is possible that the observed decreases or increases in cell proliferation in relation to the Se status of the animal could have resulted from a modulation in the ability of the accessory cells to present antigen. However, this possibility seems unlikely because the stimulation of T cell subsets to produce IL-2 in response to Con A requires intimate contact with accessory cells and interactions with Class I major histocompatibility complex antigens (31), and the amounts of IL-2 produced in Con A-stimulated cultures of spleen cells from animals maintained on all three diets did not differ significantly. Furthermore, studies on polyclonal activation of T lymphocytes by lectins have shown that human T lymphocytes stimulated by PHA can proliferate without the requirement for IL-2 or accessory cells (32). On this basis, it can be inferred that the observed decreases or increases in the ability of T lymphocytes to proliferate, as a function of changes in the Se level in the diet, does not appear to be related to the ability of accessory cells (macrophages) to produce IL-1 or to present antigen.

Inasmuch as the levels of IL-2 detected in 24-hr culture supernatants of Con A-stimulated spleen cells from animals maintained on all three diets did not differ significantly, Se may have exerted its effect through modulation of IL-2R expression. Se may be required for the expression of IL-2R and/or it may increase the number of IL-2R on individual cells as well as the number of cells expressing IL-2R. This possibility is supported by the fact that optimal growth of mouse hematopoietic cells *in vitro* requires the presence of 10^{-7} *M* Se in the medium (33). The ability of Se to modulate IL-2R expression could, thus, explain the altered ability of lymphocytes from animals maintained on Se-deficient or Se-supplemented diets to proliferate.

The experimental protocol in the present studies involved the incubation of spleen cells removed from animals maintained on the three diets for 3 (blastogen-

esis) to 5 (MLR) days in media that were supplemented to 5% with FBS. The Se content of the FBS used was 1–2 ppm. Nevertheless, although the cells were incubated in the presence of Se at concentrations considered to be optimal for growth (33), the lymphocytes exhibited significantly different proliferative responses. Thus, modulation of the Se levels *in vivo* apparently resulted in alterations in the functional properties of the cells that were not readily reversible and were not altered by the subsequent availability of Se *in vitro*. Although this fact does not entirely exclude the possibility that Se may influence IL-2R expression, it suggests that Se may also influence the transmembrane and/or intracellular transmission of signals necessary to initiate proliferation. Se interacts strongly with cellular membranes (34) and it can be incorporated into the polynucleotide structure of tRNA in animals and bacteria (35, 36). It has also been suggested that seleno-tRNA may modulate the protein translation efficiency of certain mRNAs (36). Thus, the intracellular Se status, resulting from the turnover rate of Se containing regulatory molecules, may have an effect similar to or greater than that exerted by the Se content of the cellular environment.

Although variations in the nutritional intake of Se apparently affect the function of immunocompetent cells, the mechanisms involved are not clear. Nevertheless, the results of these studies reinforce the potential importance of Se as a biologic response modifier and emphasize the need for information on the mechanism(s) of its action.

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