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Immune responses in rats supplemented with selenium

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SUMMARY

It is generally accepted that Selenium (Se) is necessary for optimum performance of the immune system. Selenium deficiency results in immune suppression but little is known concerning the effect of excess Se on immune function. Recent evidence suggests that oral Se supplementation may impede oncongenesis, but the mechanism of this action is currently unknown. Conversely, under certain conditions, Se is suspected of promoting neoplasia. The studies described herein delineate the effects of excess Se (0·5, 2·0 or 5·0 p.p.m.) on specific immune functions of Se-adequate rats, namely, antibody synthesis, delayed-type hypersensitivity (DTH), natural killer (NK) cell activity, prostaglandin E2 (PGE2) synthesis, and interleukin 1 (IL-1) activity. Selenium administered to female Sprague-Dawley rats for 10 weeks at 0·5 and 2·0 p.p.m. resulted in significant ($P \leqq 0{\cdot}01$) enhancement of splenic NK activity while the NK response in the 5·0 p.p.m. Se-treated rats was equivalent to the non-Se-treated controls. Conversely, the DTH response was significantly ($P \leqq 0{\cdot}01$) suppressed at all three dosages while antibody synthesis and prostaglandin E2 activity were significantly ($P \leqq 0{\cdot}05$) reduced compared to the controls at the highest dosage of Se. IL-1 activity was unaffected by Se exposure. These data could partially explain the contradictory oncogenic characteristics of Se. For instance, tumours that are NK sensitive could be prevented and/or responsive to Se therapy, while NK insensitive neoplasms could be enhanced by Se supplementation due to the impaired function of both humoral and cell-mediated immunity.

Keywords selenium immunity cancer natural killer cells

INTRODUCTION

Selenium (Se) has been recognized for years as an essential trace element for animals (National Research Council, 1980), but not until recently has it been recognized as an essential element for man. Se is an important deterrent of lipid peroxidation, competes with sulphur in biochemical pathways, and is incorporated into the sulphur-containing essential amino acids, cysteine and methionine (Burk, 1978; Underwood, 1977). Se is a component of several enzymes which regulate normal body processes, one of which is glutathione peroxidase (GSH-Px) (Flohe, Gunzler & Schock, 1973). GSH-Px protects cellular membranes and lipid-containing organelles from peroxidative damage by inhibition and destruction of endogenous peroxides, acting in conjunction with Vitamin E to maintain integrity of these membranes (Scott, 1979).

The functioning immune system requires adequate levels of Se for optimum performance. Se deficiency generally impairs immune function (Koller, 1980), but little is known as to the impact of excess Se on immune responses. The objectives of these investigations were to ascertain the effects of

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Se supplementation on various components of the immune system: namely, antibody synthesis, delayed-type hypersensitivity, natural killer (NK) cell cytotoxicity, prostaglandin E2 synthesis, and interleukin 1 (IL-1) activity of macrophages. Known immunosuppressants (cyclophosphamide and dexamethasone) were included to compare the degree of immunomodulation imparted by Se and to serve as a positive control for the immune assays.

MATERIALS AND METHODS

Selenium treatment. Female, weanling Sprague-Dawley rats purchased from Washington State University, Pullman, Washington, USA, were housed four per cage in stainless-steel hanging wire cages, fed a commercial rodent chow, and provided deionized drinking water ad libitum. Room temperature was set at 20°C and the lighting was regulated for alternate 12 h light and dark cycles. Following a 1-week acclimatization period, the rats were divided into groups of 12 each and treated with either 0·5, 2·0 or 5·0 p.p.m. Se as sodium selenite (ICN Nutritional Biochemicals, Cleveland, Ohio, USA) in the drinking water. The control rats were left untreated. Non-treated positive control rats immunosuppressed with subcutaneous or intraperitoneal injections respectively of cyclophosphamide (75 mg/kg) (Sigma, St. Louis, Missouri, USA or dexamethasone (0·4 mg/kg) (Med-Tech., Elwood, Kansas, USA), were included in each experiment.

ELISA assay for humoral antibody. The antibody response to the antigen keyhole limpet haemocyanin (KLH) was analysed by an ELISA (enzyme-linked immunosorbent assay) procedure as modified by Exon *et al.* (1984) on a Gilford PR50 autoanalyser. Briefly, serum samples were collected from KLH-injected rats by cardiac puncture and stored at −20°C until analysed. ELISA microcuvette trays were coated with KLH and serum samples were added at various dilutions. After incubating and washing the samples, goat anti-rat IgG (conjugated to alkaline phosphatase) was added to each well. Following a second incubation, a substrate for alkaline phosphatase was added to each well and the colour reaction was quantitated spectrophotometrically. The values are reported at various dilutions of serum as absorbance at 405 nm. Background absorbance was considered to be 0·30.

Delayed-type hypersensitivity (DTH) assay. DTH reactivity was used to analyse *in vivo* cell-mediated immunity in rats using a method described by Henningsen *et al.* (1984). Briefly, bovine serum albumin (BSA) was mixed 1 : 10 with Evans blue dye (No. 3873, Eastman Kodak, Rochester, New York, USA) in physiological saline, and 100 μg BSA in a total volume of 100 μl was injected s.c. at the base of the tail. Seven days later, the left footpad was challenged with a s.c. injection of 75 μl of a 2% heat-aggregated BSA suspension. The right footpad was sham-injected with sterile physiological saline. 24 h later, footpad swelling in both hind feet was measured using pressure calipers. The thickness of the saline-injected footpad was subtracted from the BSA-injected footpad to determine the DTH reaction. The data were reported as millimetres of swelling.

Natural killer cell assay. This method was recently described in detail by Talcott, Exon & Koller (1984). Briefly, single cell suspensions were prepared from rat spleens in RPMI medium containing antibiotics, and the RBCs were lysed by hypotonic shock. Adherent cells were removed by incubating the cells on nylon wool columns followed by 1-h incubation in tissue culture flasks. YAC-1 tumour cells were labelled with ^{51}Cr and used as NK target cells. The target cells were added to 96-well round-bottomed microtest plates containing different concentrations of NK effector cells. After a 4 h incubation period, the cell-free supernatant was collected from each well and counted on a gamma counter. Specific ^{51}Cr release from lysed target cells was calculated by the formula:

$$\frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release by 2\% SDS} - \text{spontaneous release}} \times 100\% = \% \text{ cytotoxicity}$$

Prostaglandin E (PGE) analysis. Resident peritoneal cells (RPC) were collected by peritoneal lavage with Hank's balanced salt solution (HBSS). The macrophage-laden RPCs were plated at 2×10^6 cells per well in a 24-well cluster plate. After 1–2 h, wells were rinsed three times to remove non-adherent cells. 1 ml culture media (Dulbecco's MEM, 5% fetal bovine serum, 100 u penicillin/

ml, and 100 μg streptomycin/ml) was added to each well. Four cultures were assayed for each rat. Two were control cultures with no additive, and two were cultures with 0·2 μg/ml LPS (lipopolysaccharide, *Escherichia coli*, 055:B5). Media controls were run for each assay. The cluster plates were incubated at 37°C, 10% CO_2 for 20 h, at which time supernatants were removed and centrifuged. The cell-free supernatants were assayed for prostaglandin E2 (PGE) using a Clinical Assays (Cambridge, MA 02139) radioimmunoassay kit (CA-501). The assay was sensitive to approximately 0·03 ng/1 ml media. Samples were counted on a liquid scintillation counter. Unknowns were compared to the standard curve and PGE concentrations were reported as ng/ml. These values were corrected for the amount of protein contributed by cells in the two control cultures. Final PGE levels were reported as ng/100 μg protein contributed by adherent resident peritoneal cells.

Interleukin 1 (IL-1) assay. The activity of IL-1 produced *in vitro* by macrophages was quantitated directly by the ability of IL-1 to induce proliferation of lectin-treated thymocytes. Resident peritoneal cells (RPC) were collected in HBSS by peritoneal lavage from rats. The macrophage-enriched RPC were washed once and resuspended to 4×10^6 cells/ml in M199–10% fetal calf serum. 1 ml aliquots of RPC were cultured in multi-well plates for 2 h, after which non-adherent cells were removed by washing. The adherent cells were incubated 24 h in 1 ml culture medium containing 20 μg/ml *E. coli* lipopolysaccharide (LPS). Supernatants containing IL-1 were collected and quantitated for IL-1 activity by measuring the supernatants activity to induce proliferation of cultured thymocytes. Non-LPS-stimulated cultures were included for background controls. Thymocytes collected from 3-week-old C3H/HeJ mice were processed and diluted in culture media to $1{\cdot}5 \times 10^6$ cells/0·1 ml. Phytohaemagglutinin (PHA) and 2-mercaptoethanol were

Table 1. Antibody production, delayed-type hypersensitivity response, and Natural Killer cell cytoxicity of rats exposed to selenium

Treatment†	*n*	Antibody‡ mean absorption at 405 nm per dilution (1:6,000)	Delayed-type hypersensitivity§ mean mm footpad swelling	Natural Killer cell¶ % cytotoxicity effector:target cell ratio (100:1)
Control	12	1·31 ± 0·09††	1·13 ± 0·16	27·7 ± 1·9
Selenium				
0·5 p.p.m.	12	1·17 ± 0·07	0·68 ± 0·01**	40·0 ± 1·0**
2·0 p.p.m.	12	1·26 ± 0·08	0·51 ± 0·06***	34·2 ± 1·8**
5·0 p.p.m.	12	1·09 ± 0·07*	0·73 ± 0·14**	29·5 ± 2·1
Cyclophosphamide (CY)				
75 mg/kg	6	0·44 ± 0·01****		8·4 ± 1·9***
Dexamethosone (Dx)				
0·4 mg/kg	6		0·46 ± 0·03***	

* $P \leq 0{\cdot}05$ by ANOVA, least squares means comparison.

** $P \leq 0{\cdot}01$ by ANOVA, least squares means comparison.

*** $P \leq 0{\cdot}001$ by ANOVA, least squares means comparison.

**** $P \leq 0{\cdot}0001$ by ANOVA, least squares means comparison.

† Weanling, female Sprague-Dawley rats received 0·5, 2·0 or 5·0 p.p.m. Se in the drinking water for 10 weeks. CY was administered as a single i.p. injection 6 days prior to collection of serum (antibody) and cells (NK). Dx was injected twice s.c. at 60 and 18 h prior to footpad injection of BSA.

‡ Rats were injected s.c. with 1 mg KLH 6 and 14 days prior to serum collection. IgG antibody synthesis specific for KLH was measured by ELISA.

§ Rats were injected with 100 mg BSA into the base of the tail 8 days prior to footpad measurement, and challenged with 75 mg BSA into the footpad 1 day before footpad measurement.

¶ Percent cytotoxicity of splenic NK cells against YAC-1 tumour cells.

†† ± s.e.m.

added to the thymocyte cultures along with the appropriate dilutions of IL-1-containing supernatants. The cell cultures were incubated in flat-bottomed microplates for 3 days at 37°C in 5% CO_2. The cultures were pulsed with 0·5 μCi/well of ^{3}H-thymidine and harvested 4 h later onto glass-fibre filters. Radiolabelled thymidine uptake was determined by scintillation counting. Results are expressed as mean counts/min.

Pathology and statistics. Complete necropsies were performed on each rat. Body, spleen, thymus, and liver weights were recorded. Tissue samples were collected from all internal organs for histopathological examinations. Samples were fixed in 10% buffered formalin, and were sectioned and stained with haematoxylin and eosin. Statistical analyses were performed by analysis of variance (ANOVA) and least squares means comparison.

RESULTS

Humoral antibody (IgG) production directed against the antigen KLH was reduced in all Se-treated rats compared to the controls, but this effect was only statistically significant ($P \leqq 0{\cdot}05$) at the highest dose (Table 1). A single injection of cyclophosphamide (CY) significantly ($P \leqq 0{\cdot}0001$) impaired antibody synthesis.

The delayed-type hypersensitivity response was significantly reduced ($P \leqq 0{\cdot}01$) in all Se-treated rats (Table 1). Rats injected with dexamethasone (DX) had a significantly ($P \leqq 0{\cdot}001$) impaired DTH response.

Rats exposed to 0·5 or 2·0 p.p.m. Se in drinking water for 10 weeks exhibited significantly ($P \leqq 0{\cdot}01$) enhanced NK cytotoxicity against YAC-1 tumour cells (Table 1). The NK activity in rats treated with 5·0 p.p.m. Se was equivalent to the non-selenium treated controls. A single injection of CY markedly ($P \leqq 0{\cdot}001$) impaired the cytotoxic activity of splenic NK cells.

Prostaglandin synthesis was significantly ($P \leqq 0{\cdot}05$) decreased in the 5·0 p.p.m. selenium treated rats compared to the non-Se controls (Table 2). CY markedly ($P \leqq 0{\cdot}01$) enhanced PGE2 activity.

Selenium treatment did not affect resident peritoneal cells' ability to produce IL-1 (Table 2). Cyclophosphamide treatment resulted in significant ($P \leqq 0{\cdot}05$) enhancement of IL-1 activity.

Table 2. Number of resident peritoneal cells (RPC), prostaglandin E2 synthesis, and interleukin 1 production by adherent RPC from rats exposed to selenium

Treatment†	*n*	Mean RPC/rat‡ ($\times 10^7$)	PGE secretion (ng/100 μg protein)	Mean count min/1·5 $\times 10^6$ adherent RPC dilution (1:8)
Control	12	2·0	28·0 ± 5·7§	4365·0 ± 462·8
Selenium				
0·5 p.p.m.	12	1·7	16·9 ± 5·4	5938·5 ± 785·8
2·0 p.p.m.	12	1·9	31·5 ± 5·7	5213·2 ± 646·5
5·0 p.p.m.	12	1·9	10·1 ± 5·4*	4756·8 ± 476·0
Cyclophosphamide (CY)				
75 mg/kg	6	0·9**	95·8 ± 7·7**	6805·0 ± 488·9

* $P \leq 0{\cdot}05$ by ANOVA, least squares means comparison.

** $P \leq 0{\cdot}01$ by ANOVA, least squares means comparison.

† Weanling, female Sprague-Dawley rats received 0·5, 2·0, or 5·0 p.p.m. Se in the drinking water for 10 weeks. CY was administered as a single i.p. injection 6 days prior to collection of cells.

‡ RPC were harvested by peritoneal lavage and enriched for macrophages. Adherent RPC were cultured with LPS. The cell-free supernatants were measured for PGE by radioimmunoassay, while IL-1 activity was indirectly measured by the induction of proliferation of cultured thymocytes.

§ ± s.e.m.

Table 3. Body and organ weights of rats exposed to Se for 10 weeks

Treatment†	*n*	Body weight (g) (±s.e.m.)	Mean percentage body weight‡ Spleen	Thymus	Liver
Control	12	240±5	0·27±0·01§	0·22±0·01	3·9±0·08
Selenium					
0·5 p.p.m.	12	239±4	0·26±0·01	0·22±0·01	4·1±0·07*
2·0 p.p.m.	12	250±5	0·26±0·01	0·20±0·02	4·3±0·07**
5·0 p.p.m.	12	241±5	0·25±0·01	0·25±0·01*	4·4±0·10**
Cyclophosphamide (CY)					
75 mg/kg	6	231±8	0·16±0·01**	0·16±0·02***	4·4±0·10**

$P \leq 0{\cdot}05$ by ANOVA, least squares means comparison.
$P \leq 0{\cdot}001$ by ANOVA, least squares means comparison.
$P \leq 0{\cdot}01$ by ANOVA, least squares means comparison.

† Weanling, female Sprague-Dawley rats received 0·5, 2·0 or 5·0 p.p.m. Se in the drinking water for 10 weeks. CY was administered as a single i.p. injection 6 days prior to termination of the experiment.

‡ After 10 weeks of chemical exposure, rats were killed, body and organ weights were recorded and reported as mean percentage body weight by treatment group.

§ ±s.e.m.

Selenium treatment resulted in a significant ($P \leq 0{\cdot}05$) dose-related increase in liver weights and an increase in thymus weights at 5 p.p.m. (Table 3). Cyclophosphamide significantly ($P \leq 0{\cdot}01$) decreased both spleen and thymus weights and increased liver weights. All rats appeared grossly normal throughout the exposure period. No significant gross lesions were observed at necropsy. Microscopic examination of the liver did not reveal any remarkable hepatocellular damage except for mild hepatocyte hypertrophy. The rats treated with selenium had mild congestion of renal cortical veins. No other significant lesions were observed in other organs.

DISCUSSION

The functioning immune system requires adequate levels of Se for optimum performance. Animals deficient in Se have been shown to express lower antibody titres in response to vaccination (Sheffy & Schultz, 1979), while excess Se potentiated the protective effect of vaccination (Serfass & Ganther, 1976). Likewise, Se deficiency has resulted in an impaired mitogenic stimulation of lymphocytes in both dogs. (Sheffy & Schultz, 1979) and mice (Parnham, Winkelmann & Leyck, 1983). Neutrophils, peritoneal macrophages, and pulmonary alveolar macrophages from Se-deficient animals have been shown to contain lower than normal amounts of GSH-Px activity and decreased microbicidal activity (Boyne & Arthur, 1979; Gyang *et al.*, 1984; Serfass & Ganther, 1975, 1976). Excess Se has been reported to enhance antibody synthesis, thereby amplifying the immune system in response to antigenic stimulation (Koller, Kerkvliet & Exon, 1979).

In our studies, we observed that excess dietary Se significantly ($P \leq 0{\cdot}05$) stimulated natural killer (NK) cell cytotoxicity and concurrently reduced both cell-mediated and humoral immune responses in rats. The delayed-type hypersensitivity response was suppressed in rats fed 0·5, 2·0, or 5·0 p.p.m. Se for 10 weeks while the humoral immune response was significantly lower than the controls at the highest dosage (5·0 p.p.m.). Conversely, the NK activity was enhanced at 0·5 and 2·0 p.p.m. with no effect occurring at 5·0 p.p.m. Se. PGE2 activity was significantly ($P \leq 0{\cdot}05$) suppressed by 5·0 p.p.m. Se. IL-1 production was unaffected by Se treatment.

Several recent studies indicate that Se may indeed be an anticarcinogen. A recent review (Whanger, 1983) discusses the interactions of Se with a variety of carcinogens and illustrates the anticarcinogenic activity of Se. Selenium supplementation has been shown to significantly inhibit

chemical-induced neoplasia (Banner, Tan & Zedeck, 1982; Jacobs, 1983). The development of preneoplastic lesions were shown to be very sensitive to Se-mediated inhibition (Medina & Shepard, 1981). An increased incidence of cancer in humans occurred in geographic regions deficient in Se (Jansson, Seibert & Speer, 1975; Shamberger, 1970). Further, the blood Se levels in humans who developed cancer during a 5-year period were significantly lower than those for matched control subjects (Willett *et al.*, 1983). Upon withdrawal of the Se supplementation, tumours will emerge at the same rate as those in Se-untreated animals (Griffin, 1979).

It has been suggested that Se protects against oncogenesis by inhibiting metabolic activation of carcinogens (Daoud & Griffin, 1978; Marshall *et al*, 1979) but this mechanism remains unconfirmed. Another study (Clement, 1981) concluded that Se can inhibit both the initiation and promotion phases of carcinogenesis and that a continuous intake of Se is necessary to achieve maximum inhibition of tumour genesis.

Immune surveillance of neoplasia requires a variety of cells often working together in an interrelated network which are intricately regulated by numerous immunocytokines. Different types of cancer may be sensitive to a particular immunocyte population(s) such as cytotoxic T cells, while others may be controlled more by macrophages or NK cells. Recent information favours the NK cell as a primary effector of immune surveillance, keeping in mind that not all tumour cells are NK-cell-sensitive. Nevertheless, the NK serves as a model system to assess the development, progression, regression as well as prognosis for many types of tumours. The enhanced NK cytotoxicity observed in these studies following Se exposure could, in part, account for the anticarcinogenic properties of this element. These results therefore suggest that, in some cases, Se may protect against oncogenesis by enhancing the host's NK cytotoxicity levels.

In contrast to the enhancing effects of Se on NK activity, DTH was significantly ($P \leqq 0{\cdot}01$) suppressed at all three doses as was antibody production to KLH at the high dose of Se ($P \leqq 0{\cdot}05$) (Table 1). This data may be relevant to the contradictory reports in the literature of the effects of selenium on carcinogenesis. For instance, enhanced NK cell function in Se-exposed individuals could result in greater resistance to NK-sensitive but not NK-insensitive tumour cells. In fact, growth of NK-resistant tumour cells could be enhanced due to depressed cell-mediated responses (e.g. DTH) or impaired antibody synthesis.

Prostaglandins (PG) are produced by nearly every cell and tissue of the body as oxygenation products of arachidonic acid via the cyclooxygenase pathway and are known to act as local hormones. It is clear that PG can modulate many immune functions and are capable of negative regulation of both cellular and humoral immunity (Davies *et al.*, 1980; Goodwin & Webb, 1980). PGE1 and PGE2 have been shown to inhibit lymphokine production (Varesio, Holden & Taramelli, 1981), depress antibody synthesis (Webb & Nowowiejski, 1977), prevent development of T cell-mediated cytotoxicity (Wolf & Droege, 1982), and suppress mitogen-induced proliferation of lymphocytes (Baker, Fahey & Munck, 1981). PG has also been implicated in the control of macrophage tumour cytotoxicity (Taffet & Russell, 1981).

In the present study, high selenium exposure resulted in suppressed PGE2 synthesis by resident peritoneal cells. Perhaps the regulatory action of PGE2 could account, in part, for some of the altered immune responses observed in these studies.

We postulate that the enhancement of NK activity may be an underlying mechanism of the anticarcinogenic properties of Se. Thus, neoplasms that are NK sensitive could be prevented and/or responsive to Se therapy. On the other hand, those neoplasms that are NK insensitive could actually be augmented since excess Se may abate both humoral and cellular immunity. Further studies are warranted to test this hypothesis.

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