IMMUNOTOXICITY OF CHRONIC SELENIUM EXPOSURE[1]

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Abstract: The immunotoxicity of chronic selenium exposure was examined. *In vitro* studies with cultured bovine lymphocytes demonstrated the relative immunotoxicity of three forms of selenium with toxicity due to selenite greater than that of selenocystine, which was greater than that of selenomethionine. By examining different concentrations of the three forms in lymphocyte cultures, the thresholds of inhibition for each were determined. Oral exposure of mice to selenomethionine, selenocystine and selenite demonstrated the greater immunotoxicity of selenite by inhibition of primary antibody responses to protein antigen. Although selenomethionine resulted in appreciable liver selenium accumulation, it inhibited antibody responses the least. Oral exposure to selenomethionine and selenite also inhibited antibody responses in steers and in a dose dependent manner. *In vitro* assessment of immune cell function of these steers revealed limited immune impairment. However, the addition of selenite to cultures at subthreshold levels to more closely simulate the *in vivo* environment, caused impairment of lymphoproliferative responses. Feeding pronghorn antelope high selenium forage also caused impairment of antibody responses. These results demonstrate the immunotoxicity of selenium at concentrations potentially available in a selenium-rich environment.

Additional Key Words: Immunity; Immune Suppression; Selenite; Selenomethionine

Introduction

A substantial part of the Western United States contains selenium-rich soil derived from Cretaceous shales. In these soils, selenium is often found in close association with coal and uranium ore. (National Research Council, 1983). Surface mining may expose selenium-rich strata, and the subsequent accumulation of soluble selenium compounds by plants may serve as a source of toxic selenium concentrations for grazing animals (National Research Council, 1983). Reports of suspected chronic selenosis date back to the 1930's (Beath et al.,1935). However, it has been suggested that repetitive citation of early studies may be responsible for overstating the occurrence of chronic selenosis (Raisbeck, et al.,1993).

Because of its extreme sensitivity, the immune system may be an important target for potential toxicity. Toxic insults to the immune system can often be detected very early and injury to the immune system may persist longer than other types of injury (Spallholz et al.,1990). The immune system can thus be pictured as a sentinel organ system for detection of toxicity.

To better assess chronic selenosis, the influence of different forms of selenium on function of lymphocytes in cell culture was examined as were immune parameters for laboratory mice and steers fed controlled amounts of selenium in organic or inorganic forms. Immune parameters of captive antelope fed high selenium forage were monitored as well.

Materials and Methods

In vitro assays of lymphocyte function

Whole blood was collected from a healthy donor steer in heparin vacutainer tubes by jugular venipuncture. Mononuclear cells were harvested as described in Belden et al., 1981. Briefly, blood was diluted 1:2 with Hank's balanced salt solution and underlaid with Ficoll/Hypaque. Mononuclear cells were collected after centrifugation at 1000 xg. Cells were rinsed by further centrifugation until free of platelets, counted, and diluted to 4×10^6 cells/ml.

Blastogenesis assays were performed in flat-bottomed microtiter plates using concanavalin A at 2.5 ug/ml and phytohemagglutinin P at a 1:1800 dilution. Plates were incubated for 48 hr, 0.5 uCi tritiated thymidine was added, and plates were then incubated an additional 18 hr. Mitogen stimulation was detected by harvesting the cells onto glass fiber filters followed by scintillation counting. Results are expressed as the difference between stimulated counts and background, unstimulated counts.

The function of bovine B cells was monitored with an enzyme-linked antiglobulin spot (elispot) assay which measures the ability of individual cells to produce antibody. Microtiter plates were sensitized with rabbit anti-bovine immunoglobulin. Peripheral blood lymphocytes were cultured for 96 hr with pokeweed mitogen to stimulate immunoglobulin synthesis. Cells were diluted and centrifuged onto antiglobulin sensitized plates. Trapped immunglobulin produced by individual cells was then detected with an enzyme-linked antibody. The assay results in spot development representing individual cells.

Prior work in our lab has resulted in bioassays for the detection of interleukin 2 (Il 2), which is a T-lymphocyte growth factor and is important in cell-mediated immunity (Zelamey and Belden, 1988). Methods have also been developed for the production of interleukin 4-like activity. Interleukin 4 (IL-4) promotes growth and differentiation of B-lymphocytes and is important for humoral or antibody mediated immunity. Because the presence of IL-4 has not yet been confirmed by antibody inhibition, the term "IL-4 like" activity is used. Cells were stimulated with 2.5 ug/ml concanavalin A, supernatants collected, and assessed for their ability to support Il-2 dependent cells, and for their ability to promote immunoglobulin production in the elispot assay.

Total immunoglobulin in serum samples was determined with an antigen trap enzyme-linked immunosorbent assay (ELISA) using rabbit or goat antiglobulin. Serum samples were assayed for specific primary antibody responses using an ELISA procedure with ovalbumin sensitized plates.

In vitro immunotoxicity

Peripheral blood mononuclear cells (PBM) were harvested from a donor steer on normal diet. Cells were placed in culture plates, and the different forms of selenium were added to achieve final selenium concentrations of between 0.007 and 5 ppm in the culture media.

Different concentrations of three forms of selenium: sodium selenite, selenomethionine and selenocystine were examined for their effect on immune function. Blastogensesis assays for T-cell function, elispot assays for B-cell function and Il-2 and Il-4-like cytokine production were performed on the donor steer lymphocytes.

Animal studies

Mice

Sixteen BALB/C mice (5 weeks of age) were assigned by cages (4 mice per cage) to one of 4 groups: control, selenomethionine, selenocystine, or sodium selenite. Mice were fed commercial lab chow reported

to contain approximately 0.5 ppm Se, and housed in plastic cages in approved animal facilities.

All drinking water was prepared to contain 7 ppm of the various forms of selenium in distilled water. Water consumption and weights were monitored weekly. On day 14, mice were immunized with a single subcutaneous injection of 2 mg hen egg albumin (OVA) in potassium alum adjuvant. On day 47, blood for serum was collected from each mouse via eye bleed and the mice were then killed by cervical dislocation. Spleens were collected for immune competency assays and livers were collected for selenium analysis.

Steers

Twenty hereford cross steers (Bos taurus, average weight 194 kg) were housed in cement-floored pens. The animals were provided water *ad libitum* (50 ppb Se) and were acclimated for a period of 45 days on finely chopped, low-selenium (approximately 200 ppb selenium) hay with 1/4 lb cottonseed and 1/2 lb soybean meal supplement.

After acclimation, the steers were divided into control, and high, medium and low selenium dose groups. Dosage groups received 0.8, 0.28 or 0.15 mgSe/kg body weight of sodium selenite or selenomethionine. Selenium doses were calculated on a body weight basis and were prepared fresh each day by addition to 500 g ground corn cob. Controls received the same diet except that distilled water was added to ground corn cob. Steers were supplied each day with the amount to be consumed within 18 hr. Access to the feed was controlled by electronic feed gates.

On day 42 of the 120 day trial, each steer received a single subcutaneous injection of chicken ovalbumin (40 mg, in potassium alum adjuvant).

Antelope

Eight captive-raised antelope (Antilocapra americana) were housed at the Sybille Wildlife Research and Conservation Education Unit (Wyoming Game and Fish Department). The antelope were acclimated for one month on feed with a 60:40 ratio of low-selenium (0.3 to 0.5 ppm)grass hay and alfalfa. They were then fed 60:40 high selenium (23-25 ppm) grass hay from a mine site in central Wyoming mixed with low selenium alfalfa. The antelope were supplemented with 1/2 lb of corn/oat/barley mixture per head per day. Average selenium content of the diet was between 13-16 ppm. The high selenium ration was fed free choice for 167 days.

The antelope were bled at three week intervals. On day 28, each antelope received a single subcutaneous injection of chicken ovalbumin (40 mg, in potassium alum adjuvant).

Data analysis and statistics

Appropriate data were submitted to statistical analysis by the General Linear Models method of SAS® (SAS Institute) and treatment group means compared by the method of least squares means applied at a significance level of p<0.05.

Results

The abbreviated results of *in vitro* exposure to selenium are presented in Table I. The lowest concentration of each form of selenium that inhibited greater than 50% of each response was reported as an inhibition threshold. These results reflect the relative toxicities of the three forms of selenium. Selenocystine and selenite were considerably more toxic than selenomethionine to lymphocyte function in *in vitro* assays.

Table I. Selenium inhibition thresholds (greater than 50% inhibition).

Selenium form	T-cell	B-cell*	Cytokine production
Selenomethionine	3 ppm	3 ppm	none
Selenocystine	0.25 ppm	0.125 ppm	0.5 ppm
Sodium selenite	0.125 ppm	0.25 ppm	0.25 ppm

* Dose responses in the elispot assay appeared to be biphasic. This is believed to be an artifact of the assay, which cannot distinguish between spots formed by lytic release of immunoglobulin and those formed because of immunoglobulin secretion. All forms inhibited this function at 3 ppm, with selenocystine and selenite again showing inhibition at about 0.25 ppm.

Immunotoxicities in mice

Water consumption by mice in the selenocystine and selenite groups was significantly reduced. This is perhaps reflected in the differences between treatment groups in weight gained over the seven week period. Weight gain for controls was 21% over initial weight, selenocystine 15%, selenomethionine 9%, and selenite 8%. Because of low animal numbers, these differences in gains are not significant, but may represent a trend.

Liver selenium accumulation (9ppm) in the selenomethionine group was considerably higher than controls and the other treatment groups ($\leq$ 2.1 ppm) (Fig. 1). T-cell blastogenesis assays did not reveal significant differences between groups, although a trend in decline of proliferative ability was observed. Selenite inhibited proliferative ability the most, while selenomethionine inhibited T-cell proliferation the least (data not presented).
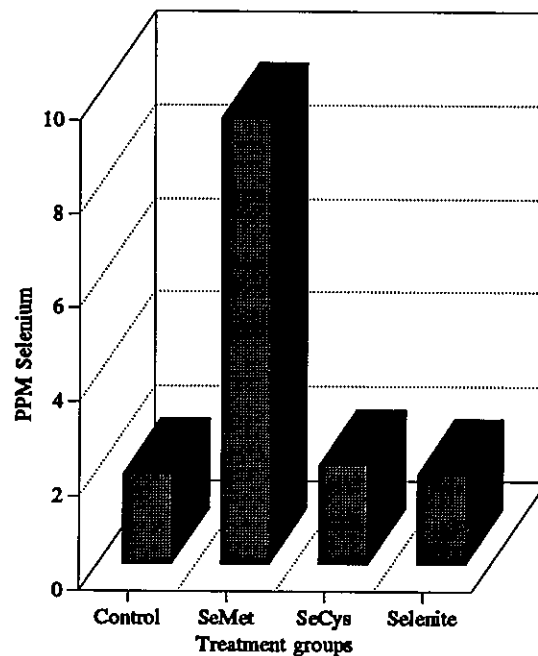

Figure 1. Mouse liver selenium concentrations.

There were no discernable differences between groups in total immunoglobulin at the end of the trial. Primary antibody responses to ova antigen were significantly lower in the treatment groups, which indicates an impairment of *in vivo* immune function (Fig. 2). Again, sodium selenite inhibited the primary response
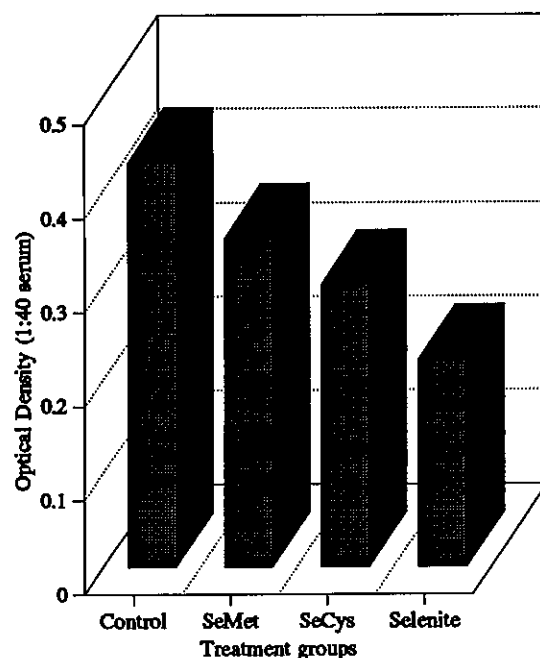
Figure 2. Mean mouse primary antibody responses to OVA antigen 33 days post injection.

the most, while selenomethionine inhibited the response the least. Although the selenomethionine group had considerable liver assimilation of selenium, while the other treatment groups exhibited liver selenium concentrations less than the control group, the OVA specific antibody production was least inhibited by selenomethionine treatment.

In concurrence with the *in vitro* immunotoxicity studies, results support the conclusion that sodium selenite is the most toxic to the parameters examined, followed by selenocystine and then selenomethionine. These mouse studies demonstrate that simply measuring the total amount of selenium in the tissues of an animal will not serve to predict the degree of immunotoxicity. The form of selenium in the animal, presumably partially determined by the form to which the animal is exposed, and reflecting its relative bioavailability to ongoing synthetic processes, needs to be determined before an assessment of potential immune impairment can be made.

Steers

Blastogenesis responses to T-cell mitogens are presented for days 0, 42, and 120 of the study. Because selenium acquired by cells in the animal can be diluted away from the cytoplasmic milieu when cells are placed in culture, blastogenesis assays were done on day 120 both in the absence of additions of selenium and with additions of selenium at 1/10th the mean blood concentrations determined for the previous bleeding (Fig. 3). Selenium additions, based on blood selenium concentrations for the previous bleeding, were calculated to be 0.17, 0.34 and 0.65 ppm for the three dose levels of selenomethionine. Each of these concentrations is below the inhibition threshold of selenomethionine determined from the *in vitro* toxicity studies. Sodium selenite additions were calculated to be 0.08, 0.08 and 0.27 ppm, respectively, for the steers fed selenite. The highest concentration (0.27 ppm) is above the threshold level of sodium selenite determined from *in vitro* studies. The other two concentrations are below the threshold level.

T-cell proliferation responses did not differ between treatment groups and controls for the days where
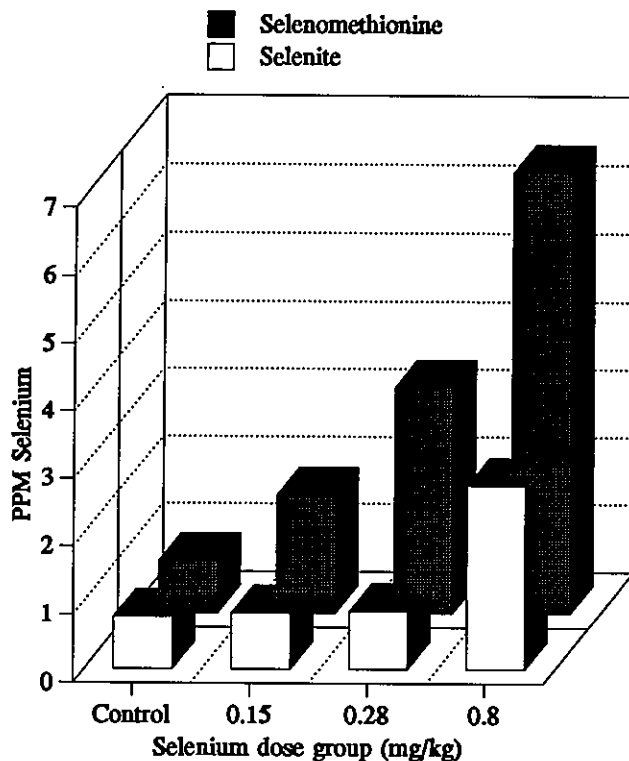
Figure 3. Steer blood total selenium concentrations.

no selenium additions were made. Selenomethionine additions resulted in no significant differences from those responses where no additions were made. Conversely, sodium selenite additions resulted in responses which were significantly smaller than those responses where no additions were made (Fig. 4). In the high selenite dose group, where the selenite addition was 0.27 ppm, a toxicity comparable to that found in the *in vitro* studies was observed. However, even for the other selenite dose groups which received selenium additions at concentrations which were less than the threshold of *in vitro* toxicity, T-cell proliferation was substantially inhibited compared to the same dose groups where selenium additions were not made.

These results justify the effort to more closely represent an *in vivo* selenium environment in cell culture studies. Addition of noninhibiting selenite concentrations to the cultures may have prevented equilibrium loss of selenium from the cells, resulting in demonstrable T-cell proliferation inhibition in cells from sodium selenite-dosed animals.

No significant differences between treatment groups and controls were found with the elispot assay (data not presented). This was true both with and without selenium additions; all additions being below the *in vitro* inhibition level for this assay.

Total immunoglobulin levels did not vary within treatment groups across the treatment period. However, very importantly, primary antibody responses to ovalbumin antigen did differ significantly between treatment groups. As indicated in Fig. 5, the inhibition of antibody response was dose related, with increasing dose levels causing increased inhibition. Consistent with the mouse studies, sodium selenite exposure resulted in greater inhibition of antibody formation than did selenomethionine.
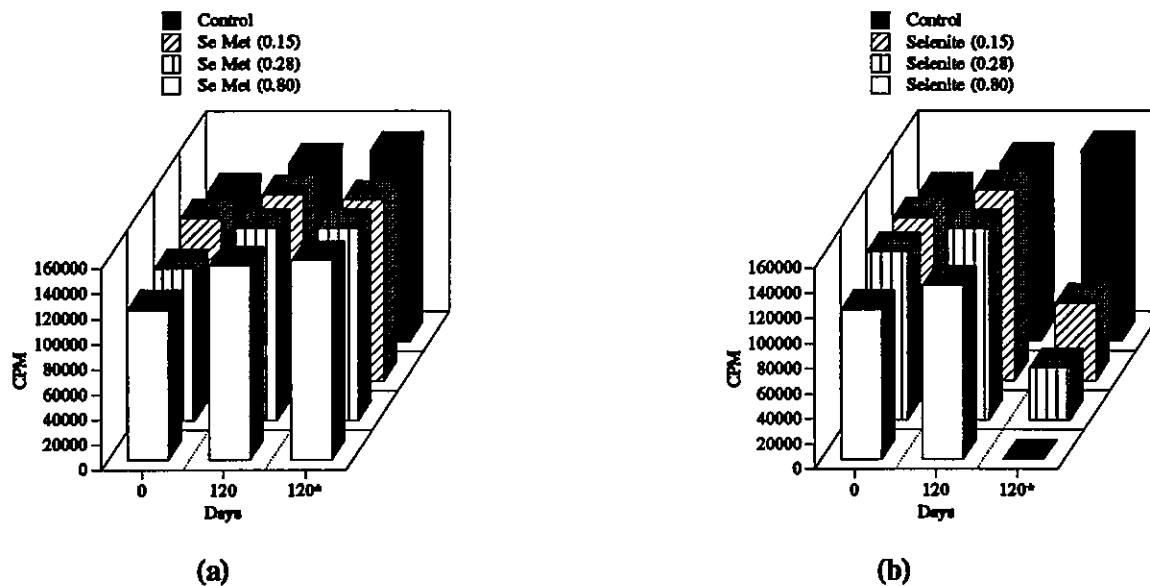
Figure 4. Steer lymphocyte proliferation in response to Concanavalin A (2.5 ug/ml). 4a) Mean proliferation (CPM) responses at day 0, 120, 120* (*selenium additions to cell culture) for animals exposed to selenomethionine; 4b) Animals exposed to sodium selenite.
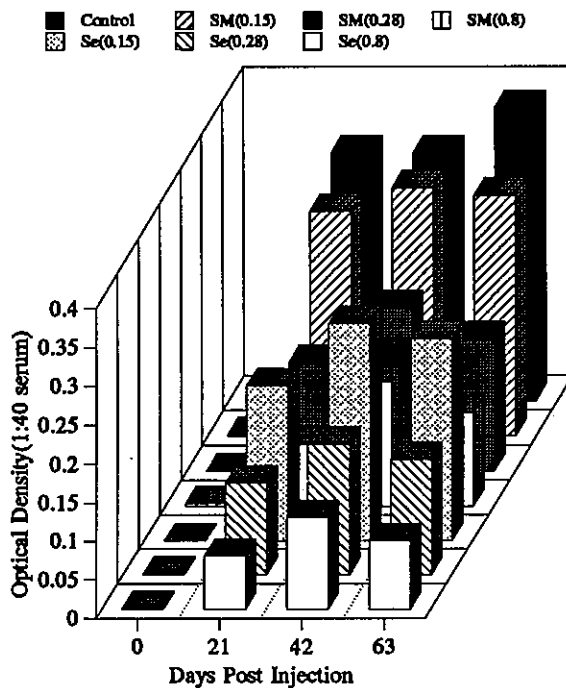

Figure 5. Mean steer primary antibody responses to OVA antigen at day 0, 21, 42 and 63 days post-injection.

Antelope

Feeding high selenium diet (13-16 ppm) resulted in elevation of blood selenium levels (Fig. 6). By day 28, when antigen was administered, treatment group blood levels averaged 0.73 ppm compared to 0.28 ppm in control animals. Control animal concentrations remained between 0.22 ppm and 0.29 ppm throughout the study. Treatment group blood selenium concentrations increased from 0.24 ppm to a maximum of 1.28 ppm on day 142.
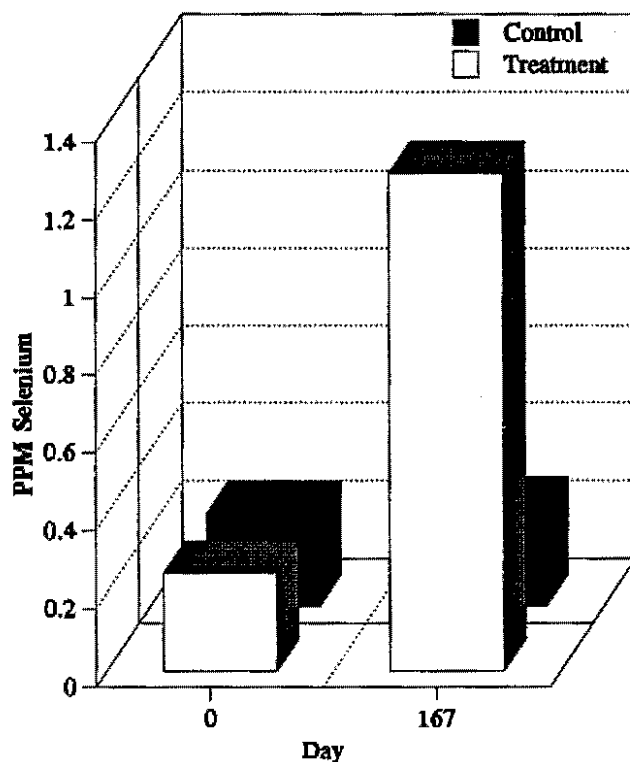

Figure 6. Antelope blood total selenium concentrations at days 0 and 167.

No consistent results were found with any of the *in vitro* assays. This partially reflects problems in obtaining, transporting, and then processing blood for lymphocyte harvest.

Peak antibody responses occurred by day 56 post-immunization. During this period of active immune response, blood selenium concentrations in the treatment group were about 3 times higher than the control group. Animals with elevated blood selenium had significantly inhibited primary *in vivo* antibody responses (Fig. 7).

Discussion and Conclusions

In all three species studied, increases in dietary selenium resulted in impairment of primary antibody responses *in vivo*. Inhibition of antibody responses in mice by sodium selenite is in agreement with reports of others (Spallholz et al, 1973; Shackelford and Martin, 1980). Dietary exposure of mice to equivalent concentrations of selenium as selenomethionine or selenocystine also inhibited primary antibody responses, but to a lesser degree. Even though selenite and selenocystine resulted in less liver accumulation of selenium,
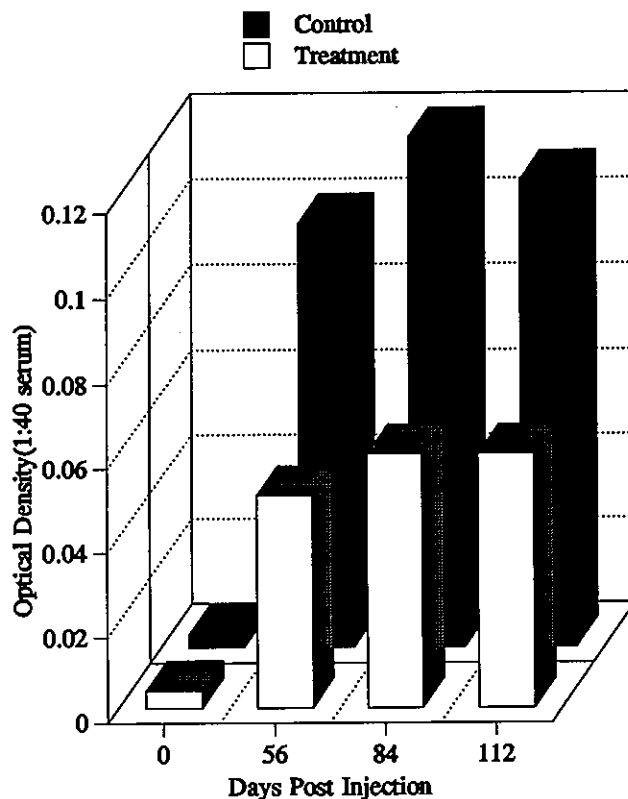
Figure 7. Mean antelope treatment group primary responses to OVA antigen at day 0 and days 56, 84, and 112 post-injection.

these forms caused greater inhibition. Although macrophage function in the role of antigen processing and presentation was not examined in this study, it is not unlikely that selenium toxicity at this level contributed to impairment of antibody responses. Alternatively, interference with cell interactions or inhibition of macromolecular cell signal exchange could cause this impairment.

In the steer studies, the most evident immune impairment was detected as *in vivo* reduction of antibody responses. As with mice, the greatest inhibition of antibody responses in steers occurred with animals fed sodium selenite. Selenomethionine exposure also caused significant inhibition. Inhibition was dose related with both forms and appeared to be related to blood selenium concentrations in these animals. Only in the steer study was an attempt made to more closely represent an *in vivo* environment in cell culture studies by the addition of the different selenium forms to the culture media. Selenite additions at concentrations below the toxic threshold impaired T-cell proliferative responses. These results support the conclusion that sodium selenite is more toxic than selenomethionine. The results also support the hypothesis that interference with cell and cell signal interactions is a cause of the observed *in vivo* impairment of antibody responses.

Feeding antelope high selenium content forage over a 167 day period resulted in the elevation of blood selenium. The forms of selenium in the forage were unknown. However, as with the mice and steer studies, dietary exposure to higher levels of selenium resulted in reduction of primary *in vivo* antibody responses.

392

The most conclusive results obtained from these studies were the consistently observed reductions in primary antibody responses. This decrease in immune competency could potentially cause an increase in susceptibility to infectious diseases, and validates concerns about exposure to chronic toxicity concentrations of selenium. Although these results confirm the immunotoxicity of selenium, studies of in vitro toxicity and examination of the functions of cells from chronically exposed animals failed to provide a more definitive understanding of the mechanisms of this immunotoxicity.

Acknowledgments

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