ABSTRACT

Title: **EFFECT OF DIETARY SELENIUM STATUS ON T CELL IMMUNITY AND CANCER XENOGRAFT IN NUDE MICE**

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Selenium (Se) is known to regulate carcinogenesis and immunity at nutritional and supranutritional levels. Because the immune system provides one of the main body defenses against cancer, we asked whether T cell immunity can modulate selenium chemoprevention. Homozygous NU/J nude mice were fed selenium-deficient, torula yeast basal diet alone or supplemented with 0.15 or 1 mg Se/kg for 8 months in Experiment 1 and for 11 weeks in Experiment 2. Mice were inoculated with PC-3 prostate cancer cells followed by a 7-week tumor development. Peripheral T cells were analyzed at baseline, day 9, 19, 34 and 47 by flow cytometry. Tumor development in adult nude mice (Experiment 1) was suppressed whereas in young nude mice (Experiment 2) was promoted by feeding a high selenium diet. Dietary selenium deficiency does not affect tumor weight. After xenograft, dietary selenium status does not affect levels of CD4 and CD8 T cells in adult nude mice in Experiment 1, while high selenium resulted in significant decrease in CD4 T cells in young nude mice in Experiment 2. Taken together, there is an opposing role of excessive selenium on tumor xenograft development in adult and young nude mice carrying differential T cell profiles.

EFFECT OF DIETARY SELENIUM STATUS ON T CELL IMMUNITY AND CANCER XENOGRAFT IN NUDE MICE

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CHAPTER I: INTRODUCTION

Selenium

Essential trace element

Selenium as a chemical element was first discovered in 1818 by the Swedish chemist Jöns Jacob Berzelius (Weeks 1932). The first evidence of the importance of selenium in normal metabolic functions came in 1957 when selenium, even in very small amounts, was found to be the element that prevented necrotic liver degeneration in vitamin E-deficient rats fed torula yeast as protein source (Schwarz and Foltz 1957). The most effective compound of the element was found to be in the form of inorganic salt, sodium selenite. From these results, Schwarz and Foltz suggested that selenium is an essential trace element that may play a role in the oxidation reduction reactions. In fact, in 1973 glutathione peroxidase was the first selenium-containing enzyme to show antioxidant activity (Rotruck, Pope et al. 1973). By then, selenium has been recognized as an essential micronutrient of fundamental importance to human health (Driscoll and Copeland 2003). It is essential because its deficiency results in suboptimal biological function that is preventable and reversible if the element is added back to the diet. It is a micronutrient because only very small amounts are found in the body and the tissues and less than 100 mg per day is needed to sustain proper body and metabolic function (Gropper 2009). And it is important to human health because selenium functions in selenoenzymes that have antioxidant properties.

Besides its role involving antioxidant defense systems, it has also been shown to play important role in immune function, thyroid hormone metabolism, anti-carcinogenesis, anti-inflammatory and anti-viral activity (Brown 2001).

Chemical form, bioavailability and intake

Selenium is available from food in organic form primarily as selenocysteine (SeC) and selenomethionine (SeM) or inorganic form as selenite (H_2SeO_3), hydrogen selenide (H_2Se) or selenate (H_2SeO_4) (Gropper 2009). In the United States, the recommended dietary allowance (RDA) for selenium is 55 μg/day for men and women and the tolerable upper intake limit (UL) has been set at 400 μg/day. The daily intake and recommended values vary with geographical location of selenium-containing food consumption as selenium content in the food is dependent on the amount of selenium in the soil (Levander 1987). Data from selenium intakes for most regions in the United States show sufficient amount of selenium, however, some countries, particularly in Europe and some parts of China and New Zeeland, have selenium intakes bellow the recommended values. As a result, selenium deficiency or suboptimal intake can occur among the populations. On the other hand, very high selenium intakes as directly related to the amount of selenium in the plants which absorb it from the soil, have been observed in countries such as Venezuela, parts of China, Northern US and Canada (Rayman 2004).

Too much selenium consumption can be toxic and leads to selenosis (Rayman 2008). Forty μg of selenium per day is considered the minimal requirement to prevent adverse effects on human health as this is the amount to allow for full expression of most selenoproteins except selenoprotein P (Xia, Hill et al. 2005), whereas 200-400 μg is considered a supranutritional dose, a higher than nutritional dose but bellow toxic, which is above 800 μg per day. A study of selenium-deplete subjects showed that 66 μg selenium / day was not sufficient to reach optimal levels of selenoprotein P (Xia, Hill et al. 2005). On the other hand, selenium-replete subjects did not show increase in plasma selenoprotein P concentrations after supplementation with 200, 400, or 600 μg selenium /

day because the protein was already fully expressed (Burk, Norsworthy et al. 2006). So the required amount of selenium necessary to optimize selenoprotein P in selenium – deficient subjects (plasma selenium <110 ng/ml) was addressed in a study by Hurt et al. The results showed that 50 µg selenium /day was sufficient to increase selenoprotein P concentrations to a steady state after 10 weeks of supplementation. Additional selenium (100 or 200 µg/day) did not further increase plasma selenoprotein P concentrations (Hurst, Armah et al. 2010).

Saturation of selenoproteins occurs at the nutritional level as was shown in a study carried out in a selenium-deficient part of China, where Keshan disease was endemic. 120 subjects with an average intake of 10 µg selenium per day were given a supplement of either SeM or selenite. Maximum GPx activity was achieved by administering just 37 µg/day SeM or 66 µg/day selenite. Therefore, SeM has been shown to be twice as effective in maximizing GPx activity as selenite (Xia, Hill et al. 2005).

Daily selenium requirement for optimal health is dependent on many factors such as the chemical species ingested, selenium status of the individual (deplete vs. replete), mechanism of action (antioxidant, anti-carcinogenic, antiviral), health condition (immune, cognitive, reproductive, viral, malignant), and even genomic differences between individuals or populations (Rayman 2008). The bioavailability of selenium is dependent on the form of selenium species ingested. Brazil nuts are the richest natural source of selenium ranging from 10 to 42 µg per nut with the most prevalent form being SeM (Thomson, Chisholm et al. 2008). In dietary supplements, selenium is usually found as SeM, selenite or selenate.

The effect of supplementation on selenoprotein activity is dependent on the selenium status of the individual. In a study by Thomas et al, the effect of selenium-rich food and selenium supplement at the nutritional dose on selenium status was investigated in a randomized placebo-controlled trial of selenium-deplete adult males and females. Selenium status was determined by plasma selenium concentration and GPx activity at baseline and every 4 weeks for 12 weeks (Thomson, Chisholm et al. 2008).

Another randomized placebo-controlled intervention of selenium-replete adult males and females assessed the effect of three different forms of high doses of selenium supplements on selenium status. Three plasma selenium biomarkers and urinary selenium excretion were measured at baseline and every 4 weeks for 16 weeks. The studies showed that selenium-depleted individuals show increase in selenoprotein activity whereas selenium-repleted individuals do not (Burk, Norsworthy et al. 2006; Thomson, Chisholm et al. 2008). The latter study concluded that SeM is the most bioavailable form of selenium species in food and should be the chemical form in supplements to increase absorption and retention of selenium in the body (Burk, Norsworthy et al. 2006).

Though bioavailability of SeM seems to be higher than that of other selenium compounds, the metabolism is quite different depending on the chemical species, for instance, and as a result can have very different effects on the body as was shown in a study by Whanger et al (Whanger, Xia et al. 1993) conducted on selenium-depleted Chinese men supplemented with SeM or selenate. They showed that more selenium was associated with the GPx in plasma and red blood cells and extracted from the nails and hair in the group supplemented with selenate than SeM. SeM, however, was associated

with higher deposition in albumin protein. So it is important to include different selenium species when conducting studies as each can have varying effects.

The different chemical forms of selenium that have been used in previous clinical, mouse, and cell studies include selenium-enriched yeast which contains a mix of organic forms of selenium, predominantly SeM, as well as elemental selenium (Duffield-Lillico, Reid et al. 2002), L-selenomethionine (Lippman, Goodman et al. 2005; Li, Lee et al. 2008), seleno-L-methionine (Fischer, Mihelc et al. 2007) sodium selenate, Se methylselenocysteine, sodium selenite, and methyl seleninic acid (Li, Lee et al. 2008) among others.

Selenium metabolism

Selenium enters the food chain via plants that absorb it in the inorganic form from the soil (Papp, Lu et al. 2007) . Human enteric absorption of selenium differs for different chemical compounds but in general, it occurs in the lower intestine where they are metabolized to more reduced states. For example, selenate is reduced to selenite (Combs and Combs 1984). SeM has been found to be absorbed and stored the most efficiently (75%) whereas SeC and selenite as low as 45%. SeM actively competes for transport with methionine in cecum and colon as primary sites of absorption using transmucosal uptake via concentration gradient whereas SeC does not (Combs and Combs 1984).

It is interetesting to note that in both the rat and human balance studies the excretion is closely related to the dietary intake of selenium but below a certain threshold, the body seems to conserve selenium by limiting urinary excretion when selenium supply is sparse (Levander 1986).

The uptake of selenium can be increased by presence of vitamin A, E and C when dietary intake is higher than amounts normally required for these vitamins (Combs and Combs 1984). Transportation of selenium occurs primarily through association with plasma proteins. Since the organic forms of selenium are reduced to selenite by post absorptive catabolism, the metabolism of selenium is closely linked to selenite. Selenite is reduced to a readily transportable H_2Se through a series of reactions involving thiols and NADPH molecules and catalyzed by glutathione reductase. Hydrogen selenide can further be methylated for secretion, oxidized to elemental selenium, or used as the precursor for selenoprotein synthesis.

Since H_2Se is the most toxic intermediary metabolite, the methylation process serves as detoxification mechanism. Dimethyl selenide is volatile and excreted in the lungs under high levels of selenium intake, whereas trimethyl selenonium cation is highly soluble and excreted in the urine to maintain selenium homeostasis (Combs and Combs 1984). SeM is metabolized in the same pathway as methionine contributing to the methionine pool, and is interchangeable in non-specific protein synthesis (Sunde, Gutzke et al. 1981; Burk, Hill et al. 2001). When SeM is consumed in the form of food protein or supplements, it can be directly metabolized into SeC (Zeng 2009). Selenocysteine, when consumed from the diet or as a product of SeM metabolism, is cleaved into H_2Se. In turn, H_2Se can enter another metabolic pathway that results in selenoprotein synthesis (Finley 2006). Figure 1 bellow summarizes the metabolic pathways involved in selenium metabolism.

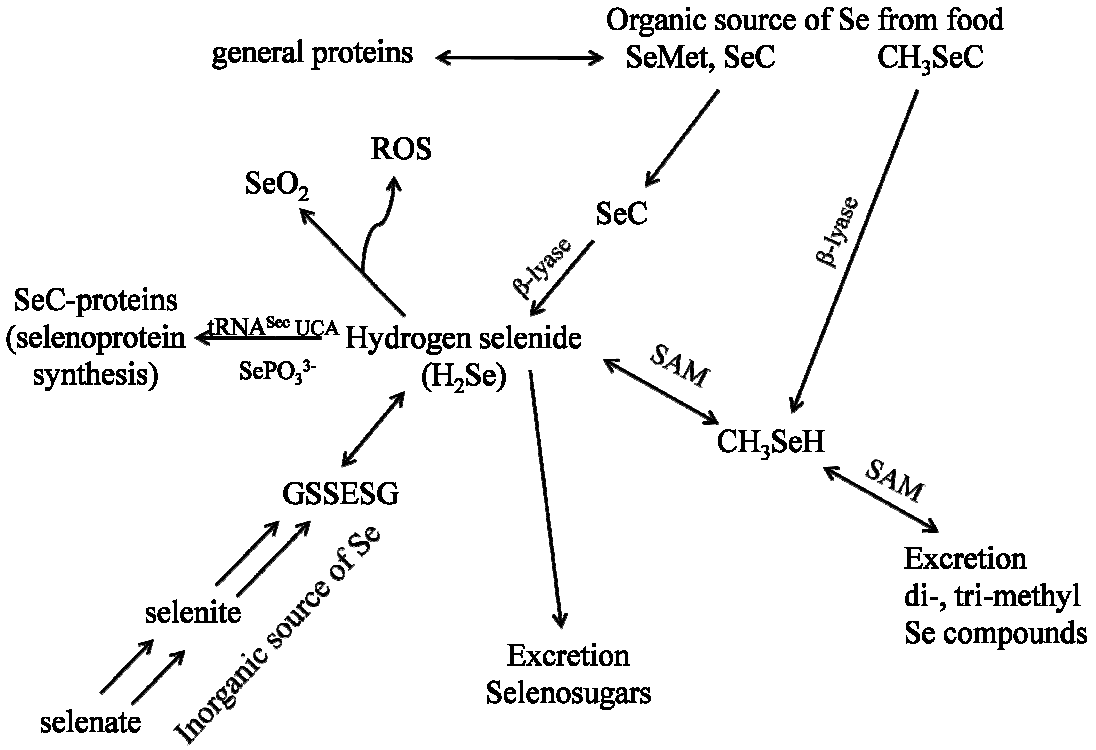


Figure 1. Biochemical pathways of selenium metabolism. Modified from (Zeng 2009).

Selenoproteins

Selenium is found in the body mainly bound to protein. Selenium is incorporated into proteins as selenocysteine (SeC) or selenomethionine (Papp, Lu et al. 2007). Selenoproteins are a special class of proteins containing specifically SeC (Mostert 2000). (Burk 1991). Selenocysteine is found in the catalytic site of the selenoenzymes (Papp, Lu et al. 2007). Selenocysteine has long been accepted by the research community as the 21st genetically encoded amino acid (Papp, Lu et al. 2007) The biosynthesis of selenoproteins requires a unique Sec-charged tRNA containing UCA anticodon. UGA is normally a stop codon but in selenoprotein synthesis, UGA codes for SeC (Burk 1991; Driscoll and Copeland 2003). The fact that UGA is not read as the stop codon on the selenoprotein mRNA is due to a special SECIS element, which forms a stem-loop structure located in

the 3'untraslated region (3'UTR) of the mRNA. Interestingly, SeC is synthesized on its tRNA. SeC does not occur as free amino acid. Rather, it is inserted co-translationally into polypeptides. SeC specific $tRNA^{Sec}$ is first charged with serine by seryl tRNA synthetase that converts the seryl residue to selenocysteine using monoselenophosphate as the selenium donor. Monoselenophosphate is synthesized from selenide (Driscoll and Copeland 2003; Allmang and Krol 2006). The physiological functions of selenium are exerted mainly through selenoproteins.

As identified by genome-wide search of SECIS, humans contain 25 selenoproteins (Kryukov, Castellano et al. 2003), Glutathione peroxidases-1 (GPx-1) was the first identified (Rotruck, Pope et al. 1973) and the most studied (Brown 2001) selenoprotein. The human selenoproteome consists of 17 selenoprotein families including glutathione peroxidases, thioredoxin reductases (TRs), iodothyronine deiodinases, and selenophosphate synthetases (Papp, Lu et al. 2007). GPx enzymes are involved in cellular antioxidant activity in the reduction of hydrogen peroxides and lipid peroxides, highly toxic molecules to the cell but by-products of normal metabolism. GPx is often used as a marker of selenium status, however, selenoprotein P is considered to be more representative as it contains almost 60% of the plasma selenium (Gropper 2009). In fact, together, the GPx3 and selenoprotein P contain over 90% of the total selenium in the plasma (Brown 2001).Therefore, they more or less represent overall selenium status in the body. Studies have also shown that the level of GPx decreases faster than that of selenoprotein P and other selenoproteins exhibiting hierarchy of synthesis (Driscoll and Copeland 2003). Saturation of selenoproteins occurs at below the supranutritional levels,

suggesting that anticarcinogenic properties come mainly from selenium metabolites rather than from selenoproteins (Combs and Gray 1998).

Selenium and human health

Though selenium deficiency is rare, it can occur in regions with selenium-poor soil (Combs and Gray 1998). Diseases associated with selenium deficiency are marked by increased oxidative stress, and changes in redox signaling (Papp, Lu et al. 2007). Perhaps the best known selenium deficiency-induced disease is Keshan disease, a cardiomyopathy, once endemic in northern-east part of China (Rayman 2008). Keshan disease can be fatal resulting in heart failure and pulmonary edema. The symptoms can be reversed most of the time and the disease cured by selenium supplementation. The time from selenium deficiency and start of supplementation is critical as the recovery depends on the extent of myocardial necrosis. Supplement of 150 µg/day is recommended for patients with heart failure associated with low plasma selenium levels (Saliba, El Fakih et al.).

Kashin-Beck disease is another selenium deficiency-related disorder. The underlying factor for the joint and cartilage osteoarthropathy seems to be increased oxidative stress due to combined selenium and iodine deficiency that can lead to poor immune response and viral susceptibility (Rayman 2008).

As is the case with any other chemicals, too much selenium can have adverse health effects as well. Selenosis, which is chronic selenium toxicity, occurs cumulatively and happens if too much selenium is consumed either in the diet, such as in seleniferous areas, or as supplement. Toxicity of selenium is directly related to the solubility of the compound ingested. The more soluble, the more toxic the compound is. Symptoms of

selenium poisoning include, hair loss, garlic breath (from the excretion of the volatile metabolites), brittle nails, diarrhea, vomiting, skin lesions, restlessness, tachycardia, fatigue, convulsions and death as observed in humans and animal studies (Yang and Zhou 1994; Rayman 2008). The amount of intake associated with selenium toxicity has been reported to be above 900 µg/day.

Though not as obvious and manifested with severe symptoms, selenium inadequacy which is expected to be quite prevalent in the United States due to mal- or poor nutrition can have serious deleterious effects on the human health. Suboptimal selenium intake can contribute to viral infections, male infertility, depressed immunity, adverse mood states, and higher risk of cancer incidence (Brown 2001; Rayman 2008).

Cancer prevention

Epidemiological studies

Numerous studies have contributed to the evidence that selenium has anti-carcinogenic properties. The cancer preventive properties of selenium were first proposed by Shamberger and Frost 40 years ago based on geographical data in a number of studies that indicated an inverse relationship between the incidence of mortality from cancer and the nutritional status of selenium in the population, as the amount of selenium in diet is closely related to the selenium content in the soil in that particular region or country (Shamberg.Rj and Frost 1969). The epidemiological study by Clark found negative correlation between cancer rates and the amount of Se in the forage crop and concluded that counties with high selenium had lower rates of cancer, especially that of colon, lung, esophagus and bladder (Clark 1985). Other studies support these results. Based on a prospective study that assessed the effect of selenium status analyzed from toenail

clippings on prostate cancer incidence, the results showed that higher levels of selenium intake were associated with lower risk of advanced prostate cancer (Yoshizawa, Willett et al. 1998). Recently, Vogt et al looked at serum selenium concentrations and the incidence of prostate cancer among American black and white men. The results again showed an inverse relationship between selenium status and cancer incidence (Vogt, Ziegler et al. 2003).

Clinical studies

Clinical trials have also shown correlation between selenium supplements and reduced risk of certain types of cancers, in particular prostate cancer (Duffield-Lillico, Reid et al. 2002). The results of a 13 year long multicenter, double-blind, randomized, placebo-controlled cancer prevention trial (NPC) in which 1312 men (75%) and women (25%) were supplemented with a daily dose of 200 μg of selenium in the form of brewer's enriched yeast, which contain 80% selenium as selenomethionine and the other 20% are other selenium compounds, have the strongest evidence in support of the hypothesis that supplemental selenium in excess of the nutritional amount, may reduce the incidence of and mortality from carcinomas. The study was initially designed to assess the effect of long-term selenium supplementation on recurrent non-melanoma skin cancer. There were no significant effects of the treatment on the skin cancer, however, secondary results showed that selenium treatment was associated with decrease in total cancer incidence (RR=63) and carcinomas of the prostate (RR=51), colon (RR =46) but no effect on colon (RR=70). It should also be noted that not all the subjects benefited from the selenium treatment, in fact, the largest protective effect of selenium was most noticeable in men who started the trial with lowest prostate specific antigen (PSA, < 4

ng/ml) and lowest tertile of plasma selenium status and these resulted in a 65% decrease of prostate cancer incidence suggesting an early stage protection (Duffield-Lillico, Reid et al. 2002).

Another even larger randomized, placebo-controlled double-blind prostate cancer prevention clinical trial (SELECT) involved participation of 35,535 men. This trial was a selenium (200 μg/day from *L*-selenomethionine) and/or vitamin E (400 IU/day of *all-rac*-α-tocopheryl acetate) or placebo supplementation for a minimum of 7 years, maximum of 12 years (2011-2014) (Lippman, Goodman et al. 2005). Preliminary data showed that after a follow up of about 5.5 years the results indicated non-significant effect of selenium in form of *L*-selenomethionine or Vitamin E or the combination of both supplements on prostate cancer prevention as compared to placebo (Lippman, Klein et al. 2009). This seemingly contradictory result of SELECT to NPC underlines the importance of the selection of selenium compound and the selenium status of the individual. It has been noted from the results of NPC that individuals with below optimal levels of selenium benefited the most from selenium supplementation. Men in SELECT had mostly selenium levels that were not low or suboptimal and as such it is possible that additional selenium was not beneficial in preventing prostate cancer.

In vivo studies

Since human clinical studies require much longer time, and for ethical reasons are not always possible to implement, numerous animal studies have shown selenium efficacy against carcinogenesis in many tumor models. Li et al in 2008 tested four selenium compounds, specifically methyl seleninic acid (MSeA), MSeC, SeMet and sodium selenite (Na_2SeO_3) in xenograft of two human prostate cancer cell lines (DU145

and PC-3) using athymic nude mice as the animal model. The results showed oral dose-dependent response with MSeA exhibiting the highest inhibitory efficacy against human prostate cancer. The doses of selenium were 1 mg/kg body weight (b.w.) and 3 mg/kg b.w. and only sodium selenite showed evidence of genotoxicity at the 3 mg/kg dose. The efficacy of the compounds was lower against the PC-3 xenograft compared to the DU145 xenograft. PC-3 cells are a later stage of prostate cancer cell line and are more aggressive and metastatic compared with the DU145 cell line. PC-3 cells are also p53-deficient and androgen-independent whereas DU145 cells are p53-proficient and androgen-dependent accounting for alternate signaling pathways. These factors possibly play role in the tumorigenesis and angiogenesis during cancer development and account for the different responses to selenium treatment in these two cancer cell lines.

Erdman et al explored the effect of selenium, vitamin E and lycopene on growth of prostate tumors in male rats. The experimental diets were fed 4-6 weeks prior to tumor injections and contained either selenium as methylselenocysteine (1 mg/kg diet), lycopene (250 mg/kg diet), vitamin E as γ-tocopharol (200 mg/kg diet) or combinations of these. Only MSeC significantly reduced the tumor area, tumor weight and tumor weight/body weight ratio. However, the mechanism of action remains unknown (Lindshield, Ford et al. 2010).

Bioavailability of selenium compounds may not play as important role in cancer prevention as it does in enhancement of antioxidant activity of selenoproteins. Finley and Davis conducted an experiment testing the anti-carcinogenic efficacy of high selenium-broccoli in rats with chemically induced colon cancer. The rats were depleted for six weeks and then repleted for 60 days and samples analyzed at intervals. The results

showed that Se-methyl selenocysteine (SeMSeC), the chemical form found in the high selenium-broccoli, has low bioavailability (no significant accumulation in tissues nor enhanced GPx activity) compared to SeM, selenite and selenate but had the highest reduction in cancer incidence. The bioavailability of high selenium-broccoli was determined by analyzing the repletion of selenium in tissues and GPx acitivity (Finley and Davis 2001).

Mechanisms of anticarcinogenic activity

In vitro models have been used extensively to elucidate possible mechanisms by which selenium exerts its anticarcinogenic effects. Hypotheses on pathways of action are still emerging and more research is needed in this area. It seems that anticarcinogenic activity can come from both the antioxidant role of selenoenzymes as well as prooxidant role of selenium metabolites of low molecular weight (Combs and Gray 1998; Drake 2006) .

In order to better understand these ideas, it is helpful to be familiar with the process of carcinogenesis and DNA damage. Carcinogenesis can be broadly classified in three-stages: initiation, promotion and progression. Initiation is the first stage of carcinogenesis and seems to occur quite rapidly. It is also an irreversible step. It is the initial insult by a carcinogen that if not eliminated by detoxification can lead to formation of reactive molecules or DNA adducts creating a damaged or pre-initiated cell. Unless the damage is repaired by the cell's DNA damage repair machinery, the cell will divide and become an "initiated cell". The second stage is the promotion, which can take several years but is reversible and depends on the status of immune response, altered gene expression and enhanced cell division, which will affect the speed and degree of

accumulation of benign tumor cells also called pre-neoplastic cells. The third and last stage of carcinogenesis is progression, which can take up to ten years or longer but eventually leads to neoplastic cells, invasive growth and metastasis, which is often incurable. Progression is affected by additional mutations and expression of oncogenes or silencing of tumor supperessor genes (Surh 2003).

Selenium plays a role in the maintenance of genome stability but the pathways of mechanism are still to be elucidated. Previous studies have shown that selenium, at the supranutritional levels, can induce oxidative stress *in vitro.* Oxidative stress in turn activates DNA damage response (DDR) and the cascading signaling pathways that lead to cell cycle arrest to allow time for repair and if irreparable lead to cellular senescence or apoptosis. Both cellular senescence and apoptosis are considered early barriers of tumorigenesis (Bartkova, Rezaei et al. 2006). Therefore it is logical to hypothesize that selenoproteins such as GPx and/or TRs with antioxidant and redox regulation activity could protect against oxidative damage caused by reactive oxygen species (ROS), such as singlet oxygen, hydrogen peroxide, and hydroxyl radical, and prevent the initiation stage of carcinogenesis. In this case, selenium deficiency could increase cancer risk due to ROS accumulation.

Since selenium doses above those sufficient for maximum selenoprotein activity have resulted in anticarcinogenic effect, selenium metabolites, other than selenoproteins, may also be responsible. Studies have shown that both organic and inorganic forms of selenium can have antitumorigenic activity (Combs and Gray 1998). The inorganic selenite is known to induce apoptosis in various cell models. Other studies pinpointed to methyl selenol acting as the main anticarcinogenic metabolite through activation of

caspase proteinases, a key family of proteins in the apoptotic pathway (Ganther 1999). The cancer protective property of MSeC was mentioned earlier (Finley and Davis 2001). So it is likely that different selenium metabolites can act through different mechanisms during different stages of carcinogenesis.

An emerging of selenium is radioprotection of normal tissue. In a randomized trial, women with post-operative endometrial cancer undergoing radiotherapy (RT) were given high dose of sodium selenite (500 or 300 µg/day). Not only did selenium supplementation prevent selenium deficiency, common side effect of RT, but it also reduced incidence of RT-induced diarrhea and improved overall 5-year survival rate (Micke, Schomburg et al. 2009; Muecke, Buentzel et al. 2009).

Selenium and the immune system

Selenium has been shown to play a functional role in the immune system. Early studies with mice demonstrated enhanced primary immune response with above nutritionally adequate dose of selenium (0.1 µg/g) (Levander 1986). On the other hand, supplementation of selenium-deficient Finnish men had no effect on the leukocyte migration inhibitory factor production by lymphocytes, even though plasma selenium concentrations increased more than 2-fold from 74 to 169 ng/ml (Levander 1986). More recently, immunologic responses attributed to selenium comes mainly from selenoproteins with antioxidant activity but the mechanistic pathways are not fully understood (Arthur, McKenzie et al. 2003). Selenium functions in the immune system as peroxide-reducing agents, enzymes in the hydroperoxide metabolism, and in the modulation of the respiratory burst among others (Spallholz, Boylan et al. 1990). Four selenoproteins (GPx1, GPx4, TR2, 15 kDa selenoprotein) have been isolated from T cells

with functions in the cell yet to be identified. Patients infected with HIV have been shown to have low levels of selenium (Gladyshev, Stadtman et al. 1999), suggesting a role of selenium in viral infection. Experimental evidence suggests that selenium deficiency leads to less responsive immune system, selenium adequate diet boosts immune response, and selenium toxicity causes immunosuppression (Spallholz, Boylan et al. 1990). As mentioned above, selenium deficiency weakens the immune system and makes it more susceptible to viral infections such as the case with Keshan disease.

Selenium affects immunity through antioxidant and redox mechanism

The mechanisms by which selenium affects the immune system are not clear (Shrimali, Irons et al. 2008). However, several studies point to a mechanism involving antioxidant activity of selenium in T cells. Recently, one such study proposed a mechanism of action that involves selenoproteins and their antioxidant activity in ROS modulation and T cells of the immune system. This model has been proposed based on the results from a study where mice were generated lacking selenoprotein expression in T cells. Activation and proliferation of T cells by T cell receptor signaling was greatly reduced by the absence of selenoprotein expression. It was also discovered that the mature T cells were functionally defective due to selenoprotein deficiency. ROS production was increased in the T cells isolated from the mice lacking selenoprotein expression and this was reversed by the addition of an antioxidant, *N*-acetyl cysteine (NAC), greatly supporting the antioxidant effect of these selenoproteins (Shrimali, Irons et al. 2008). Similar study was carried out by the same group on macrophages devoid of selenoproteins (Carlson, Yoo et al. 2009), which showed that selenoproteins were necessary for the antioxidant effect.

A very recent study by Hoffmann et al showed that dietary selenium ranging from 0.08 to 1.0 mg/kg effects CD4+ T cell activation and differentiation through modulation of free thiols in a response induced by peptide/adjuvant using mouse model. High selenium diet (addition of 1.0 mg/kg sodium selenite to casein-based diet) increased proliferation and differentiation of CD4+ T cells while low selenium diet (0.08 mg/kg) reduced the activity of these T helper cells. There was also a shift toward the Th1 effector cell phenotype in the high selenium diet which can explain its role in an antiviral and antitumor robust immune response. They reported increased T cell receptor signaling in response to high dietary selenium that involves free thiol concentrations in CD4+ T cells. In conclusion, this study showed the importance of understanding the effect of selenium supplementation on Th1 immunity in association with declining immunity during aging and immune suppression during cancer. (Hoffmann, Hashimoto et al.).

Because there is a very close association between cancer and the immune system, the status of immune system plays an important role in combating cancer. In a study by Roy and Kiremidjian-Schumacher, 33 patients with head and neck squamous cell carcinomas were given 200 µg/day of sodium selenite or placebo starting on the day of surgery and radiotherapy for 8 weeks. The lymphocyte response to antigens was significantly increased in subjects with selenium supplementation. The enhancement of lymphocyte response has been linked to increased production of α, β and γ subunits of interleukin 2 (Kiremidjian-Schumacher and Roy 2001).

Adaptive immune system

B cells and T cells are lymphocytes involved in adaptive immunity. B cells play important roles in the humoral immunity. T cells play major roles in cell-mediated immunity. Even though, both B and T cells are produced in the bone marrow by the hematopoietic stem cells. The main difference between B cells and T cells is that B cells differentiate and mature in the bone marrow, whereas T cells travel to and mature in the thymus. There are more than 350 subsets of leukocytes, each of which is assigned a cluster differentiation (CD) number indicating a cell phenotype-determining antigen (Chaplin 2010).

All mature leukocytes express specific cell surface antigens. Lymphocyte subsets (B cells, T cells, Natural Killer (NK) cells and NK-T cells) can be further discriminated by surface phenotype. T cells express T cell receptors (TCR). The majority of T cells expresses αβ TCR; those further differentiate into subsets, such as CD8+ T cells, are primarily involved in killing microbe-infected cells and tumor cells through their cytotoxic activity. In contrast, CD4+ T cells mainly mediate humoral and immune responses. CD8+ T cells also contain regulatory cells (suppressor cells) that can down-regulate immune response (Chaplin 2010). Figure 2 depicts T cell differentiation and maturation designating the functionally distinct subpopulations and subsets.

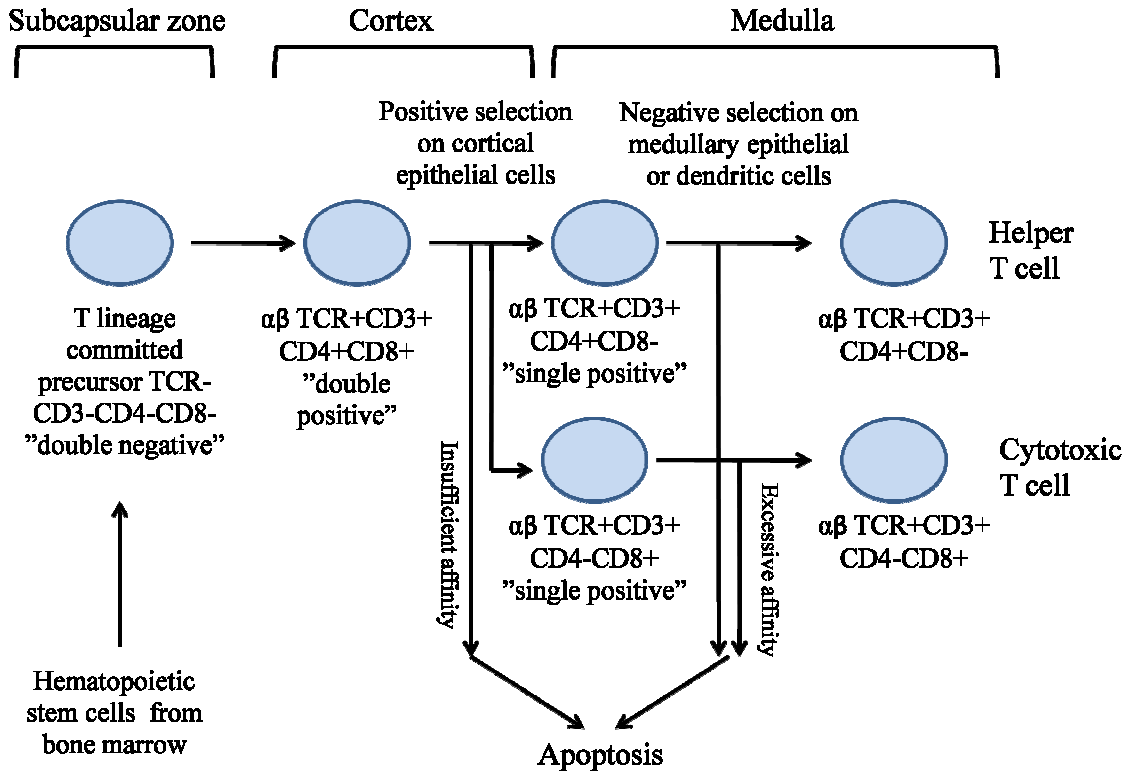


Figure 2. Maturation and differentiation of T cells in the thymus. Modified from (Chaplin 2010).

Some circulatory CD4+ T cells, known as Treg cells, play important roles in suppressing the immune response. These Treg cells are further divided into two groups: natural Treg cells (regulatory function developed in thymus) that express CD4, CD25 and sometimes Foxp3 antigens, and induced Treg cells that are thought to develop regulatory functions in the periphery from naïve CD4+ cells. These cells sometimes express Foxp3 antigen. They are marked by excretion of interleukin (IL)-10. Other clusters of differentiation relevant to this study are CD69 and CD44. CD69 expression is associated with activation and proliferation of T-lymphocytes. CD44 expression is an indicative marker of effector-memory T cells (Chaplin 2010).

Animal model

Nude mice have been used extensively in xenograft tumor models and are the animal model used in the present study (Watrach, Milner et al. 1984; Lin, Boylan et al. 1991; Li, Lee et al. 2008; Yang, Huang et al. 2009; Bai, Mao et al. 2010). The nude mice are immunosuppressed by virtue of lacking functional T lymphocytes and as such are well suited for tumor xenograft studies as they do not readily reject human cancer cells. The initial studies with nude mice were conducted using skin grafts from different animals and have been shown to be maintained indefinitely for the life of the animal (Manning, Reed et al. 1973; Reed and Manning 1973). The purpose of the present study is to determine the effect of dietary selenium on tumor growth and extrathymic T cell maturation in adult nude mice inoculated with human prostate cancer (PC-3) cells. The effect of aging on extrathymic T cell maturation in nude mice has been observed previously (Kennedy, Pierce et al. 1992). Immunodeficient nude mice start to develop T cells after about 8 weeks of age and the maturation increases linearly thereafter. This might interfere with the xenograft in older nude mice.

CHAPTER II: MATERIALS AND METHODS

Animals and Diet

Forty seven-week-old and nine three-week-old NU/J homozygous male nude mice were obtained from the Jackson Laboratory (Bar Harbor, Maine) for Experiment 1 and Experiment 2, respectively. The animals were randomly chosen into three groups (see Table 1 for experimental design) and fed the AIN-93G Torula yeast purified rodent diet purchased from Dyets Inc (Bethlehem, Pennsylvania). Se-deficient (Se-) diet (see APPENDIX B for detailed composition) was the basal Torula yeast diet and contained less than 0.03 ppm selenium by analysis as certified by the company (APPENDIX E). Se-adequate (Se+) diet was supplemented with 0.15 mg Se/kg (see APPENDIX C for composition) and Se-supranutritional (Se++) diet supplemented with 1 mg Se/kg as sodium selenate (see APPENDIX D for composition). The diet was stored at 4˚C. Mice were kept under pathogen-free conditions in a 12-hour dark/light cycle with controlled temperature of 24˚-25˚C and humidity between 40-65%. Animals were housed three to five mice per cage in disposable individually ventilated cages. The bedding was irradiated and mice had *ad libitum* access to food and sterilized water. The selenium-modified diet was given throughout the whole course of the experiment, i.e. before and after the xenograft administration. The body weight was measured once a week.

Table 1. Experimental design.

Group	sodium selenate (ppm)	Number of animals (n)		Length of time on diet (weeks)	
		Experiment 1	Experiment 2	Experiment 1	Experiment 2
Se-	0.0	5	3	28-32	11
Se+	0.15	5	3		
Se++	1.0	5	3		

Cell line

All cell culture supplies were purchased from Mediatech, Inc unless otherwise specified. The PC-3 human prostate cancer cell line was obtained from American Type Culture Collection (Manassas, VA). This PC-3 cell line was originated from a 62-year-old male taken from a bone metastasis with a stage IV adenocarcinoma. Cells were grown and maintained in an incubator at 37°C, 5% CO_2 in RPMI1640 medium containing 10% fetal bovine serum (FBS) (Aldrich-Sigma), 1% penicillin-streptomycin antibiotics and 1:5000 plasmocin (Invivogen) to prevent mycoplasma. Cells were subcultured in 1:4 ratio every 3-4 days when reaching 80% confluent.

Blood collection

Peripheral T cells were analyzed from blood collected from the tail of the animals. Animals were warmed up for five minutes under a heat lamp and placed in a restraining tube. The site of withdrawal was disinfected with alcohol before making a small cut with

a scalpel about 1/3 distance down from the top of the tail. Five drops (approximately 100 µl in total) of blood were collected into blood collection tubes containing heparin to prevent coagulation. T cell analysis of xenografted animals was performed at baseline (day before xenograft), day 9, 19, 34 and 47 for experiment 1. T cell analysis for experiment 2 was done only at termination, day 46.

Human tumor xenograft

To prepare cells for inoculation, cells were harvested from passage 23 to 26 cells. After trypsinization, cells were counted by the use of disposable hemocytometer (IN CYTO, C-chip, SKC). Each large flask (175 cm^2) grown to 100% confluency yielded an average of $1x10^7$ cells. After counting, cells were centrifuged at 500 x g for 5 min. Cells were resuspended with 10 mL SFM and centrifuged again. After removing the supernatant, cells were resuspended and 0.5 ml of cells in media were added to cold 0.5 ml Matrigel™ Basement Membrane Matrix (BD Biosciences, Cat.#356234) on ice, mixed with prechilled pipette tips and 100 µl aliquoted into eppendorf tubes on ice. Right before inoculation the mixture was drawn into syringes and injected subcutaneously on each side of shoulder blade (left or right dorsal thoracic region). Each injection contained 2×10^6 cells for Experiment 1 and 8 $x10^5$ cells for Experiment 2. The size of the tumor was measured every other day using a plastic ruler. Length (L), width (W) and height (H) were measured for 6-7 weeks and the volume estimated using the following formula: W x L x H x 0.5236 (mm^3) (Lee, Chun et al. 2006). At the termination of the experiments mice were euthanized using CO_2, tumors and organs harvested and washed in PBS, and terminal bleeding was performed by cardiac puncture with a 21gauge 1½ needle and collected into a 3 mL syringe. Blood was then transferred into an eppendorf tube with

heparin and kept on ice. Tumors and spleen were blotted dry and weighed. Small sections of heart, liver, kidney, tumor tissue, and one whole lung were fixed in 15 mL of freshly made 4% paraformaldehyde for 24 hours for histology processing. The remaining tissues and organs were flash frozen in liquid nitrogen and stored in -80˚C until further analyses.

Analytical methods

Whole blood was centrifuged at 12000 g for 10 minutes at 4˚C (Schweizer, Michaelis et al. 2004) and plasma collected by aspiration and stored at -80˚C until analysis. Plasma selenium content were analyzed by a hydride-generation atomic absorption spectrometry method (Davis, Zeng et al. 2002).

Peripheral T cell phenotyping

Circulating peripheral T cells from blood collected from the tail were stained with antibodies and analyzed by flow cytometry. The following antibodies conjugated to specific fluorophores were used: anti-CD4 (Percp), anti-CD8 (PE), anti-CD44 (APC), anti-CD25 (FITC), and anti-CD62L (pacific blue) (from Biolegend, San Diago, CA).The whole blood was incubated with antibodies for 30 minutes at 4˚C, then fixed with 4% paraformaldehyde for 15 minutes at 4˚C, and washed with staining buffer (2% fetal calf serum in phosphate-buffered saline). Lysis buffer was used to lyse the red blood cells (NH_4Cl, $KHCO_3$, 0.25 M EDTA) at room temperature for 10 minutes. The cells were then centrifuged at 12000 g for 12 minutes and supernatant aspirated. The pellet was resuspended and washed with staining buffer and centrifuged at 12 000 g for 10 minutes. Cells were resuspended in 100 µl staining buffer. The samples were then analyzed by FACSCalibur flow cytometer (BD Bioscience) and FLOWJO software (BD Biosciences) was used to analyze the data.

Histopathology

Liver, kidney, heart, and lung, as well as tumor tissues fixed in 4% paraformaldehyde were submitted to histology laboratory (Histoserv, Germantown, MD) for tissue processing. Processed tissues were embedded in paraffin and sectioned on a microtome 5 µm thick. Sections were put on uncoated slides, stained with H&E stain (hematoxylin and eosin stain) and coverslipped. Slides with tumor tissue sections were examined and histopathologically evaluated by a Board-certified Veterinary Pathologist, Jerrold M. Ward, DVM, PhD, in the Histoserv, Inc. The evaluation was based on the presence or absence of inflammation, necrosis, and hyalinization. Grading was done using the following scale: +, minimal; ++, mild; +++, moderate; ++++ severe.

Statistical analysis

Statistical analysis of the data obtained from body weight, tumor volume estimation, tumor weight, and plasma selenium concentration were performed by Student *t* test analysis and Analysis of Variance (ANOVA) using GraphPad Prism 5.03 software. Tukey's post-test was used to compare between groups. T cell profile data were graphed and analyzed using a two-tailed Student's *t* test (GraphPad Prism 3.0 software). The significance level was set at $p < 0.05$.

RESULTS

CHAPTER III: ADULT NUDE MICE XENOGRAFT STUDY

Effect of long-term dietary selenium-deficiency on body weight and survival in male nude mice

Forty eight-week-old male nude mice were randomly selected into two dietary groups (selenium-adequate and selenium-deficient). Four weeks later, six or seven mice were randomly taken out of the two groups and assigned to a third dietary group (selenium-supranutritional). One mouse from the selenium-adequate group died within the first four weeks on the diet of unknown causes, most likely unrelated to the diet, and was removed from all data and analyses. All mice ate the same selenium-modified diets. Selenium–adequate diet (Se+) was supplemented with 0.15 ppm sodium selenate, which is at the nutritional range, and served as the control group. Selenium-deficient diet (Se-) is the basal Torula yeast diet without additional supplementation with selenium. The third group is the selenium-supranutritional diet (Se++) which was supplemented with 1 ppm. sodium selenate. The average amount of feed consumed per day per animal was observed to be approximately 4 g. Based on this information, the selenium-deficient group consumed about 0.12 µg, selenium-adequate group 0.6 µg, and selenium-supranutritional group 4 µg of selenium per day. Assuming that the average body weight of nude mice is 30 g, the equivalent amounts correspond to an average of 4 µg Se/kg body weight, 20 µg Se/kg body weight, and 130 µg Se/kg body weight in Se-, Se+, and Se++ dietary group, respectively. These calculations are summarized in Table 2.

Body weight was measured every week. The initial body weights at the age of nine weeks, ranged from 22 g to 32 g and the final weights at the age of ten months were in the range of 29.7 g to 34.7 g. Five fittest nude mice from each group were removed for

xenograft experiment after 5-6 months on the experimental diet and the body weights were recorded and analyzed separately. The mean body weights for nude mice in the control (selenium-adequate) group (32.97 ± 0.45g) compared to the selenium-deficient (30.90 ± 0.37g) and selenium-supplemented (31.68 ± 1.07g) groups were not significantly different ($p = 0.10$) among the three dietary groups after 7 to 8 months on the selenium-modified diet. The changes in body weights over time are shown in Figure 3.

The body weights of nude mice fed the selenium-deficient diet were marginally lower than the selenium-adequate or selenium-supranutritional groups. Based on these results, the selenium given at the three doses (0, 0.15, 1.0 mg Se/kg diet) did not have negative effect on the body weight for the length of time used in this study.

Survival rate seemed to be effected by the selenium modified diet and is depicted in Figure 4. Selenium deficiency seems to have negative effect on survival of nude mice. Selenium deficiency can be induced after about eight weeks. The survival data were recorded for twelve months and all surviving animals were sacrificed at that point. The survival was lower in selenium-deficient mice compared to the other groups especially during the first nine months. Only 3/8 mice in the selenium-deficient group survived beyond twelve month compared to 6/8 on the selenium-adequate diet and 4/8 on the selenium-supranutritional diet.

Table 2. Amounts of selenium in each dietary group based on an average of 4 g of food consumed by experimental animal per day with the average body weight of 30 g. The basal selenium-deficient, Torula yeast basal diet contains 0.03 ppm selenium by analysis.

Dietary group	ppm (mg Se/kg diet, by analysis)	µg Se/4g diet	µg Se/kg body weight
Se-	0.03	0.12	4
Se+	0.18	0.6	20
Se++	1.03	4	130

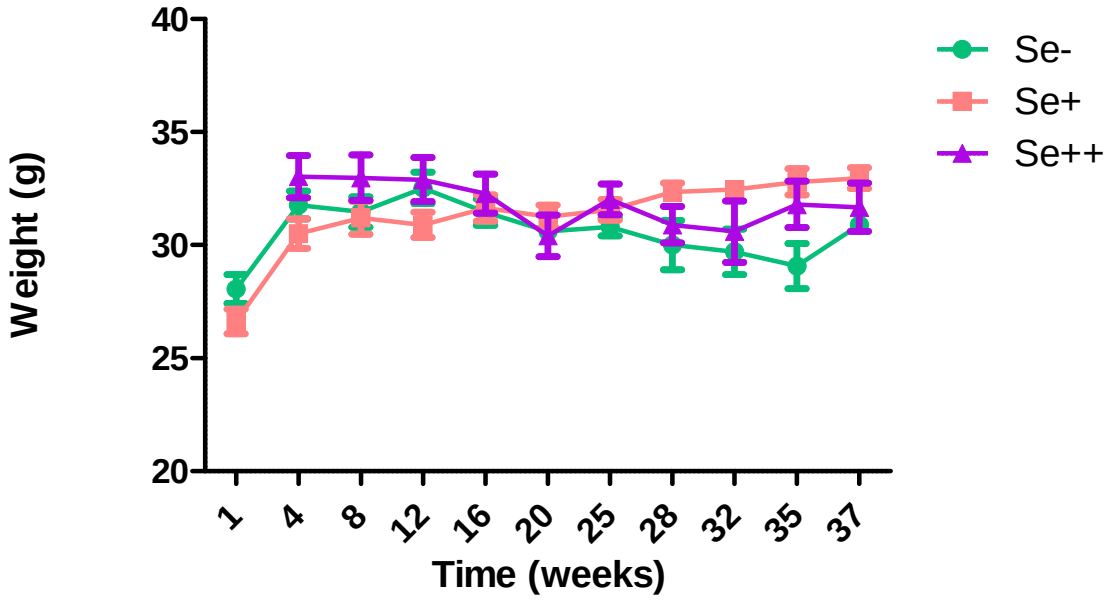


Figure 3. Effect of long-term selenium supplementation on changes of the mean body weights of the male nude mice fed *ad libitum* selenium-modified AIN-93G purified rodent diet for 37 weeks. The five animals from each group chosen for Experiment 1 (xenograft) were not included after 25 weeks on this graph. Those animals are presented separately in the Experiment 1 section. Se-, selenium deficient; Se+, selenium adequate; Se++, selenium supranutritional. Data are presented as mean ± SEM. SEM, standard error of the mean.

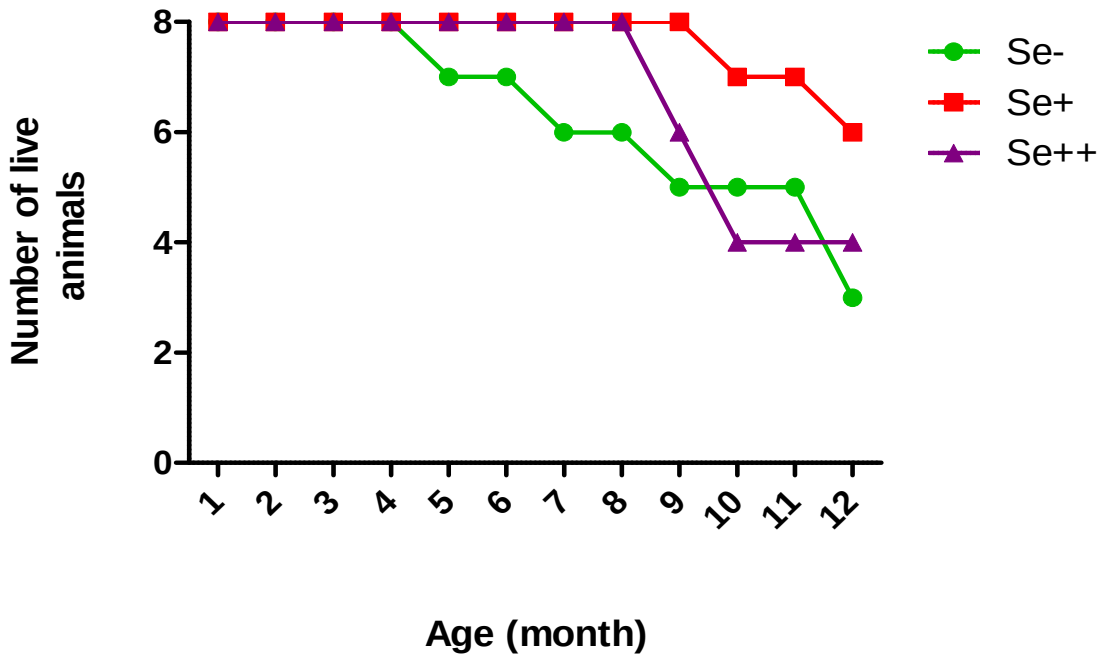


Figure 4. Effect of long-term supplementation of male nude mice with selenium on survival. Animals were fed *ad libitum* selenium-modified AIN-93G purified diet for 10 months. Se-, selenium deficient; Se+, selenium adequate; and Se++, selenium supranutritional.

Plasma selenium analysis of nude mice in xenograft Experiment 1

Five mice from each group were chosen based on their weight and health status in order to increase chance of survival throughout the xenograft study. The average lifespan of nude mice is much lower than the wild type mice due to their suppressed immunity. Animals with good health and of steady weight were chosen to minimize losing a subject during the experiment due to poor health. In order to induce severe deficiency, we allowed for 6 months on selenium-deficient diet. At the same time, this period was necessary for extrathymic T cell maturation. It has been observed that extrathymic T cell maturation increases with age in nude mice, especially a dramatic increase after 32 weeks of age (Kennedy, Pierce et al. 1992). At the time of administration of the xenograft, the

animals were 33 weeks of age. This age was chosen in order for the animals to satisfy two criteria: long-term selenium deficiency and increased extrathymic T cell maturation. The effect of selenium supplementation on selenium status was analyzed by measuring the plasma selenium concentrations for nude mice at 32 weeks on selenium-deficient and selenium-adequate diet at 28 weeks on selenium-supranutritional diet. The data for plasma selenium concentrations are in Table 3. The results of the mean plasma concentrations for each dietary group are shown in Figure 5. The mean plasma selenium concentration was 2.5- and 2.8-fold lower in the Se- group (143.4 ± 13.0 ng/ml) compared to Se+ (353.2 ± 52.7 ng/ml, $p = 0.0048$) and Se++ (400.6 ± 41.0 ng/ml, $p = 0.0003$) group, respectively. The difference between Se+ and Se++ group was not statistically significant ($p=0.498$). Plasma selenium concentrations were observed to increase with higher selenium content in the diet.

Table 3. Plasma selenium concentrations (ng/ml, mean ± SEM, n = 5) of the 3 dietary groups at termination (32 weeks on the diet).

Animal ID and group	Total selenium concentration in plasma (ng/ml)	Animal ID and group	Total selenium concentration in plasma (ng/ml)	Animal ID and group	Total selenium concentration in plasma (ng/ml)
Se-		Se+		Se++	
205	110	216	385	211	300
207	130	226	355	212	396
209	145	227	406	236	324
213	189	228	155	238	468
214	143	229	465	240	515
mean ± SEM	**143.4 ± 13.0**		**353.2 ± 52.7**		**400.6 ± 41.0**

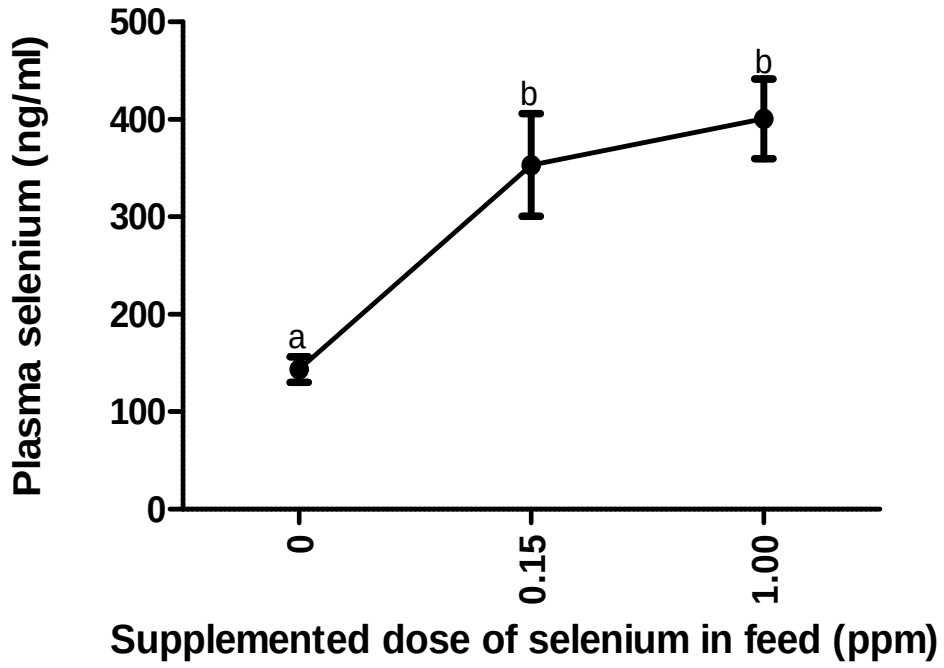


Figure 5. Mean plasma selenium concentrations in the post-mortem nude mice after termination of the xenograft experiment. The mice were fed on the Torula yeast basal diet (Se-) or the diet supplemented with 0.15 mg Se/kg feed (Se+) and 1 mg Se/kg feed (Se++) in the form of sodium selenate. The amount is expressed as mean ± SEM (n=5 mice).

Body weights of adult male nude mice during xenograft study

The body weights of study animals were measured every other day. The initial body weights at the beginning of the xenograft experiment ranged from 29.5 g to 35.4 g and the final weights after 7 weeks were in the range of 26.7 g and 32.0 g. The mean body weights for nude mice in the control (selenium-adequate) group (28.7 ± 0.9 g), the selenium-deficient (29.1 ± 0.9 g) and the selenium-supranutritional (30.2 ± 0.6 g) groups were not significantly different ($p = 0.41$) at the end of the study (40 weeks of age). The changes in the body weights over time are shown in Figure 6 and Figure 7. The mean body weights of study animals at baseline (32.0 ± 0.4 g, n = 15) compared to those at

termination (29.4 ± 0.5 g, n = 15) were significantly different (p = 0.0001). This result implies that the statistically significant decrease in body weights was due to the xenograft itself and not due to the selenium supplementation. To further investigate the effect of xenograft on the changes in body weights, age-matched nude mice fed the same diets for the same length of time were compared to the study mice and the data are shown in Figure 8. The mean body weights at 33 weeks (initial, 31.2 ± 0.5 g, n = 16) were not statistically different (p = 0.45) from the mean body weights at 40 weeks (final, 30.6 ± 0.7 g, n = 16) of the age-matched control nude mice.

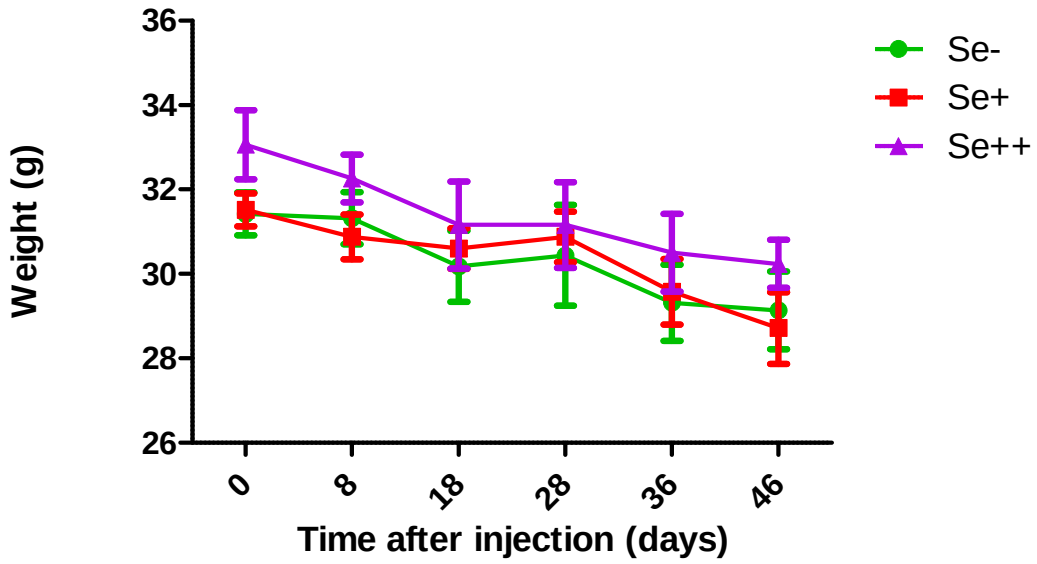


Figure 6. Changes in the mean body weights (g, mean ± S.E.M., n = 5) for adult male nude mice during the 7-week xenograft study. Se-, selenium-deficient; Se+, selenium-adequate; Se++, selenium-supranutritional.

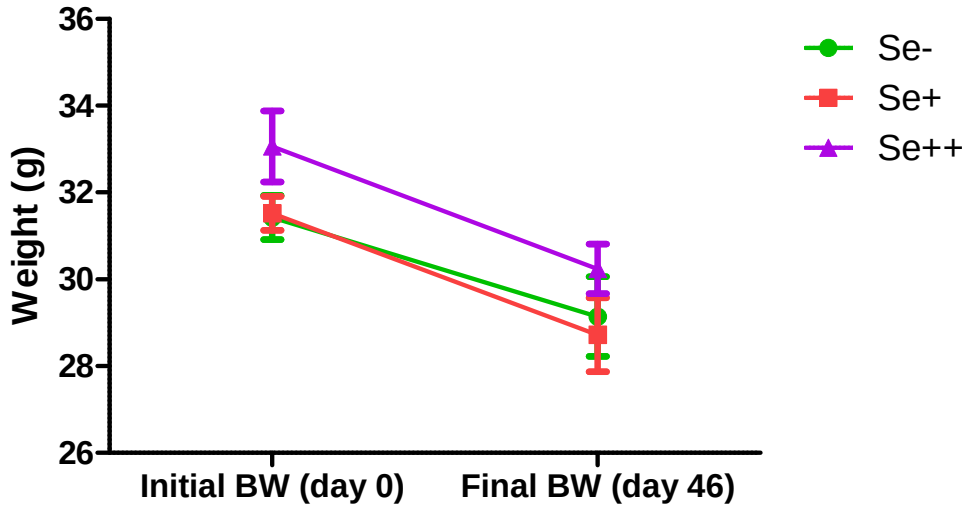


Figure 7. Changes in the initial and final mean body weights (g, mean ± S.E.M., n = 5) of adult male nude mice during 7-week xenograft study. Se-, selenium-deficient; Se+, selenium-adequate; Se++, selenium-supranutritional.

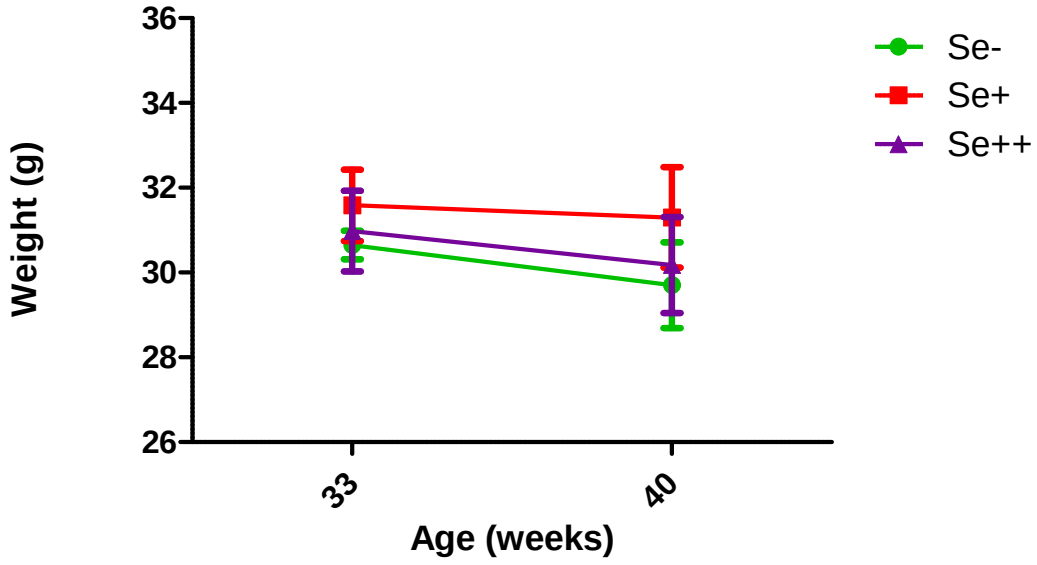


Figure 8. Changes in the initial (age 33 weeks) and final (age 40 weeks) mean body weights (g, mean ± SEM, n = 5) of control age-matched adult male nude mice. Se-, selenium-deficient; Se+, selenium-adequate; Se++, selenium-supranutritional.

Effect of selenium on tumor volume and tumor weight in adult male nude mice in xenograft Experiment 1

Estimated tumor volume was plotted as a function of time (Figure 9). The experiment was terminated when the average estimated tumor volume reached the greatest difference between groups (46 days after inoculation). At that point, the weight of the dissected tumors was measured and the mean as well as total weight for each dietary group were calculated and plotted as shown in Table 4 and depicted in Figure 10. Animals in selenium-deficient and selenium-adequate group were on the experimental diet for 25 weeks and in selenium-supranutritional group for 21 weeks prior to cancer cell inoculation. The experimental diet continued to be given during the 7 weeks of xenograft study. 2×10^6 cells of human prostate cancer of the cell line PC-3 were injected subcutaneously on either side of the shoulder blade of each experimental animal (2 injections/mouse). The success of the xenograft was recorded as the incidence of tumor growth at site of injection and is reported in Figure 12. The xenograft had nearly 100% success rate (30/31). Selenium deficiency enhances the initial tumor growth in the first 4 weeks, but did not impact on tumor growth thereafter. Supranutritional level of dietary selenium greatly suppressed the tumor growth at all stages of the xenograft study (Figure 9). At day 45 of xenograft (the last day to measure volume before sacrificing experimental animals) the mean tumor volume was significantly lower in selenium-supranutritional group compared to selenium-deficient group ($p < 0.05$) but not selenium-adequate group due to large within-group variance ($p = 0.09$) (Figure 10).

The control/selenium-adequate group had the largest tumor growth but also the largest within-group variability. The tumor volume estimation is a great tool in monitoring the tumor growth progression and is only as accurate as the observer's measurement abilities. Tumor weight measured in post-mortem samples is therefore more accurate and is useful to verify the tumor volume data.

The mean tumor weights differed between groups: selenium-deficient (0.29 ± 0.10 g), control/selenium-adequate (0.64 ± 0.38 g), and selenium-supranutritional (0.21 ± 0.06 g). However, due to the great within-group variability in the control group, the differences were not statistically significant ($p = 0.37$). The difference in tumor weight was evidenced from the total tumor weight depicted in Figure 11B. Table 4 gives a summary of the combined tumor weights for each animal as well as the mean ± S.E.M. and the total tumor weight figures. The total tumor weight in the control group (2.56 g) was 3- and nearly 2-fold greater than that in the selenium-supranutritional (0.83 g) or selenium-deficient (1.46 g) groups, respectively. In order to account for differences in body weight in mice bearing tumors, we also explored the effect of the tumor weight to body weight ratio and found that the difference between groups was not statistically significant ($p = 0.39$) (Figure 13).

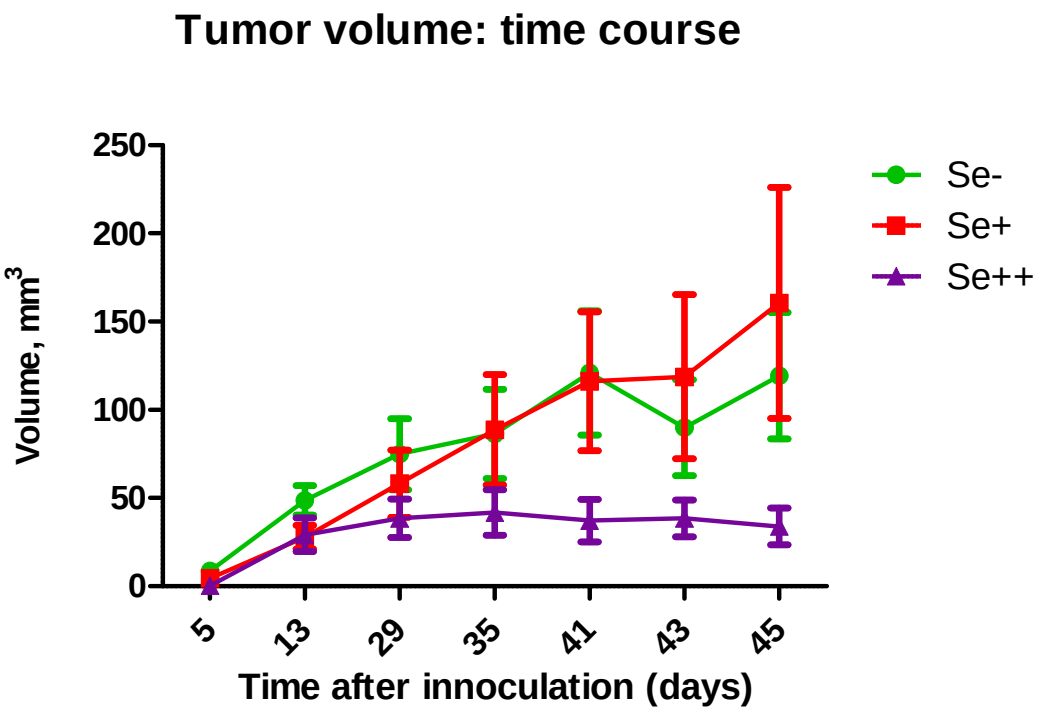


Figure 9. Effect of selenium-modified diet on changes in the mean tumor volume. 2 x 10^6 cells of the PC-3 cell line of human prostate cancer were subcutaneously injected in adult male nude mice. Se-, selenium deficient; Se+, selenium adequate; Se++, selenium supranutritional. Data are presented as mean ± SEM. Volume was measured in mm^3, n = 5.

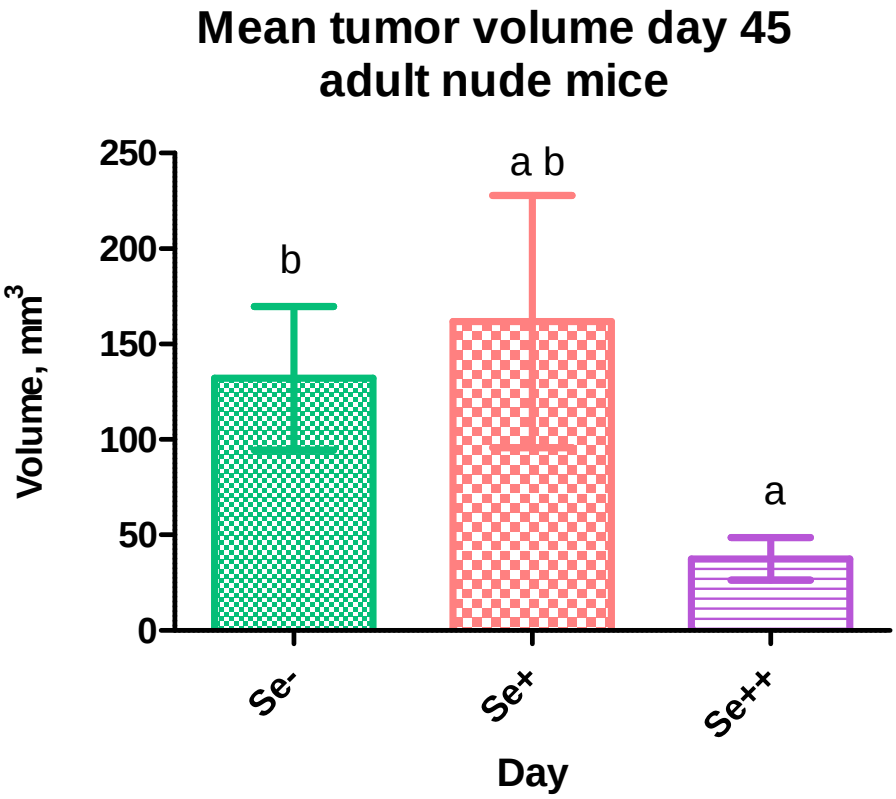
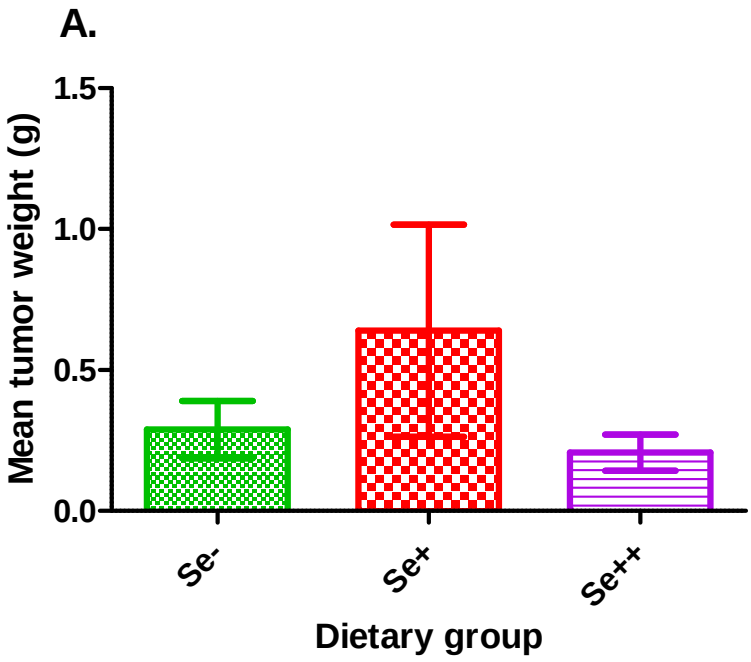


Figure 10. At day 45 of xenograft (the last day to measure volume before sacrificing experimental animals) the mean tumor volume was significantly lower in selenium-supranutritional (Se++) compared to selenium-deficient (Se-) ($p < 0.05$) but not selenium-adequate (Se+) due to large within-group variance.

Table 4. Tumor weights data (g, mean ± SEM, n= 5) of the 3 dietary groups at termination (day 47 after inoculation) Tumor weight was calculated as total weight of multiple tumors per mouse, if more than one found. Se-, selenium deficient (0.03 ppm); Se+, selenium adequate (0.15 ppm); Se++, selenium supranutritional (1 ppm). ND = none detected

Animal ID and group	Total tumor weight (g)	Animal ID and group	Total tumor weight (g)	Animal ID and group	Total tumor weight (g)
Se-		control/Se+		Se++	
205	0.15	216	1.70	211	0.02
207	0.47	226	0.20	212	0.29
209	0.59	227	ND	236	0.29
213	0.22	228	0.63	238	0.23
214	0.03	229	0.03	240	ND
Total	**1.46**		**2.56**		**0.83**
mean ± SEM	**0.29 ± 0.10**		**0.64 ± 0.38**		**0.21 ± 0.06**



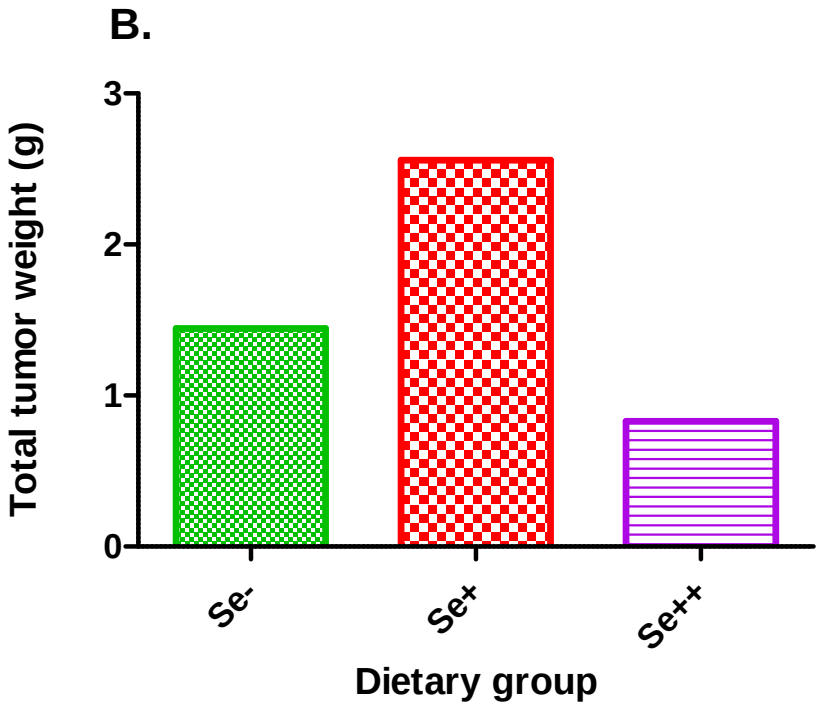


Figure 11. Final mean tumor weight (A) and total tumor weight (B) at termination (day 47 after inoculation) compared between dietary groups. The values are mean ± SEM, n=5. Tumor weight was calculated as total weight of multiple tumors per mouse.

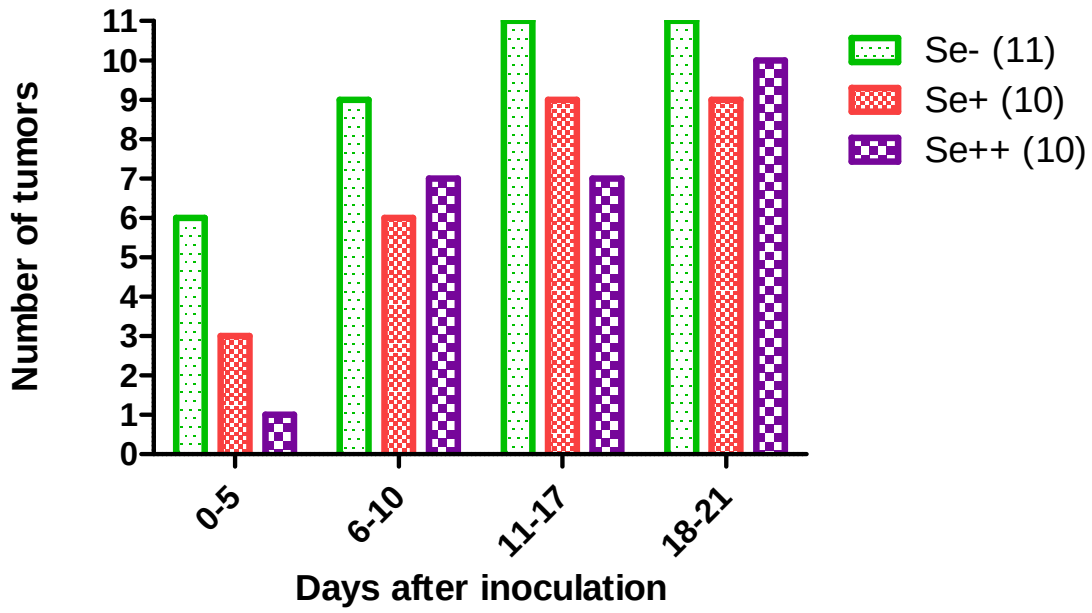


Figure 12. Incidence of tumor growth at site of cancer cell inoculation for the first 3 weeks of xenograft study in adult male nude mice. Se-, selenium deficient; Se+, selenium adequate; Se++, selenium supranutritional. Numbers in parentheses represent the number of tumor inoculations per group.

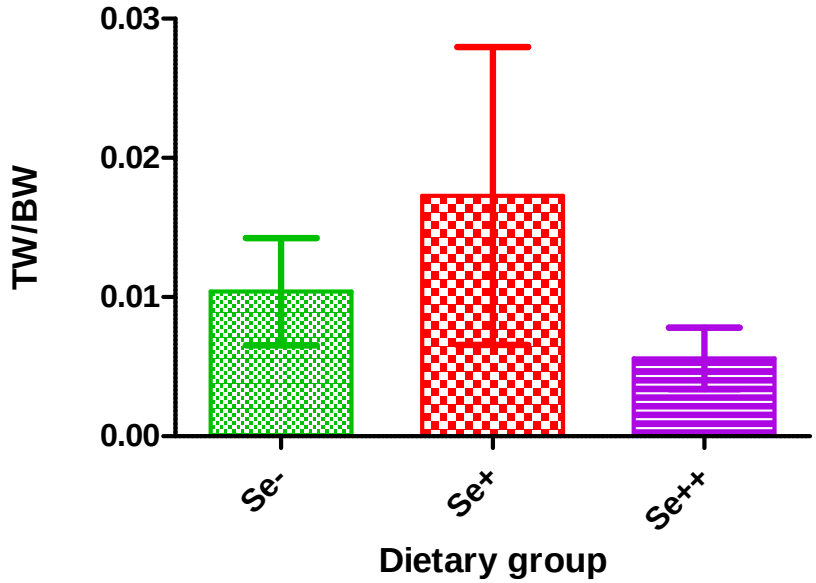


Figure 13. Tumor to body weight ratio compared between dietary groups. The values are mean ± SEM, n=5. Tumor weight (TW) was calculated as total weight of multiple tumors per mouse and the body weight (BW) was the last recorded BW before sacrifice.

It is interesting to note that one animal (ID #240) from the selenium-supranutritional group grew 2 tumors of maximum estimated volume 70 and 40 mm^3 but the tumors regressed and completely disappeared by week 5 and 6. Another animal (ID #227) from Se-adequate group grew 2 tumors of maximum estimated volume 20 and 45 mm^3 but the tumors regressed and were undetectable by week 3.

Circulating peripheral lymphocyte analysis for adult male nude mice in control animals

Preliminary B and T cell profiles were performed for 30-week-old nude mice that did not receive xenograft. Flow cytometric analysis was employed to separate and quantify T cell subsets. Wild type B6 mice served as the control since these mice have normal functional thymus. The results show that the T cell numbers are about ten times

lower in nude mice than in the control B6 mice, confirming that immune deficiency in nude mice is closely linked to T cells but not B cells. However, no significant differences were observed between the dietary groups (Figure 14). Surprisingly, B cells were found to be about 10% higher ($P < 0.05$), in nude mice than in the control B6 mice. B cells were also significantly lower in selenium-deficient ($p < 0.05$) and selenium-supranutritional (p close to 0.05) groups compared to selenium-adequate group (Figure 15). To further analyze the T cell phenotypes represented by CD3-expressing cells, CD4 and CD8 T cell subsets were measured and the results are depicted in Figure 16 and Figure 17. CD8 T cells were found in higher numbers than CD4 T cells. CD8 T cells were approximately 10 times lower in nude mice than in the control B6 mice and did not differ significantly between dietary groups. CD4 T cells were observed to be 10-20% lower in nude mice than the control B6 mice. The CD4 T cell numbers were significantly lower in the selenium-adequate group compared to selenium-deficient group ($p<0.05$) but not selenium-supranutritional group. Both selenium-deficient and selenium-supranutritional diets seemed to increase the CD4 T cell numbers. CD8 T cells did not seem to be affected by selenium supplementation or deficiency.

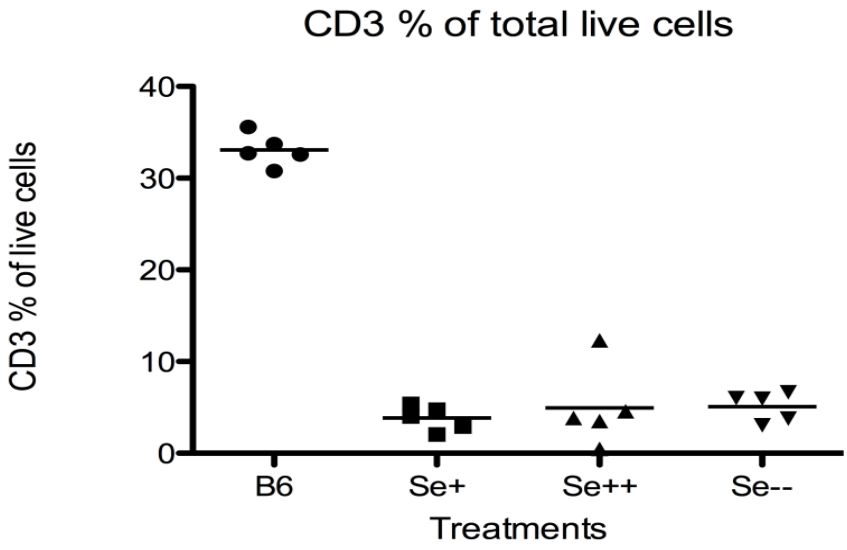


Figure 14. Percentage of total live T cells (CD3 positive) in 30-week-old nude mice with B6 wild type mice serving as control. Se-, selenium deficient; Se+, selenium adequate; Se++, selenium supranutritional. N=5 for all groups, except Se++ n=4 due to one sample lacking sufficient events.

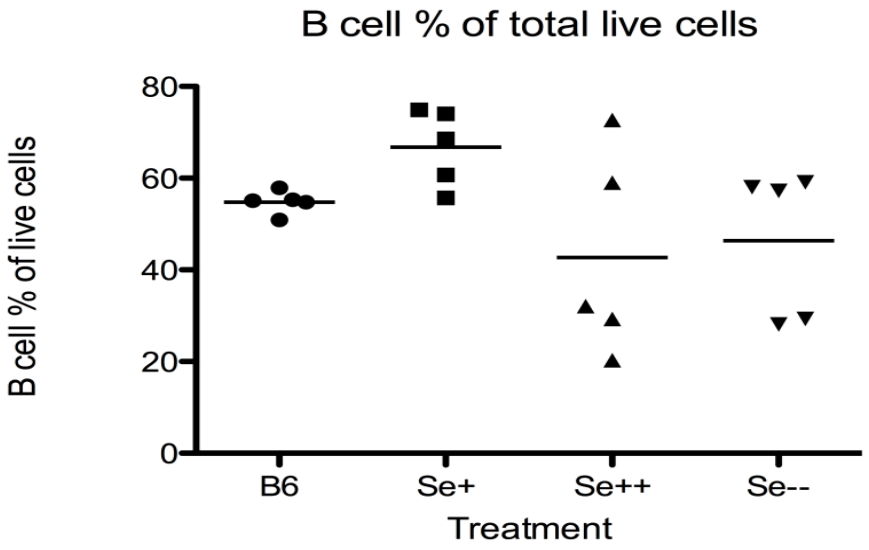


Figure 15. Percentage of total live B cells (CD220) in 30-week-old athymic nude mice with B6 wild type mice serving as control. Se-, selenium deficient; Se+, selenium adequate; Se++, selenium supranutritional. N=5.

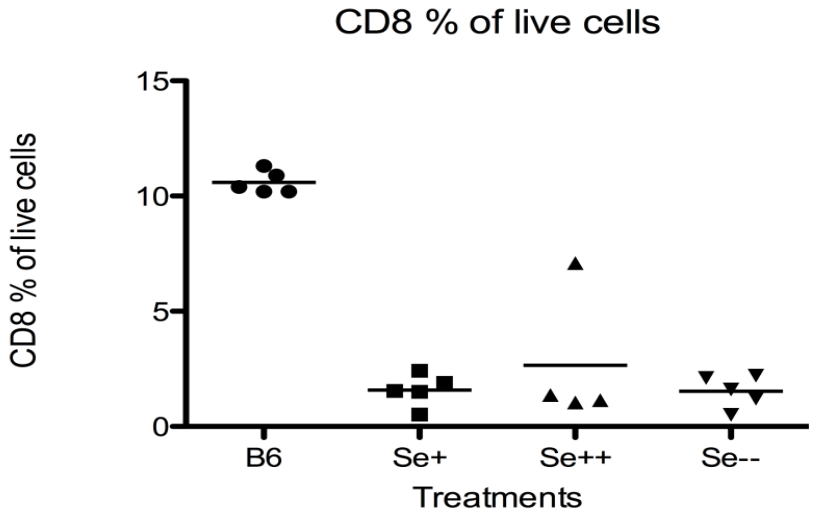
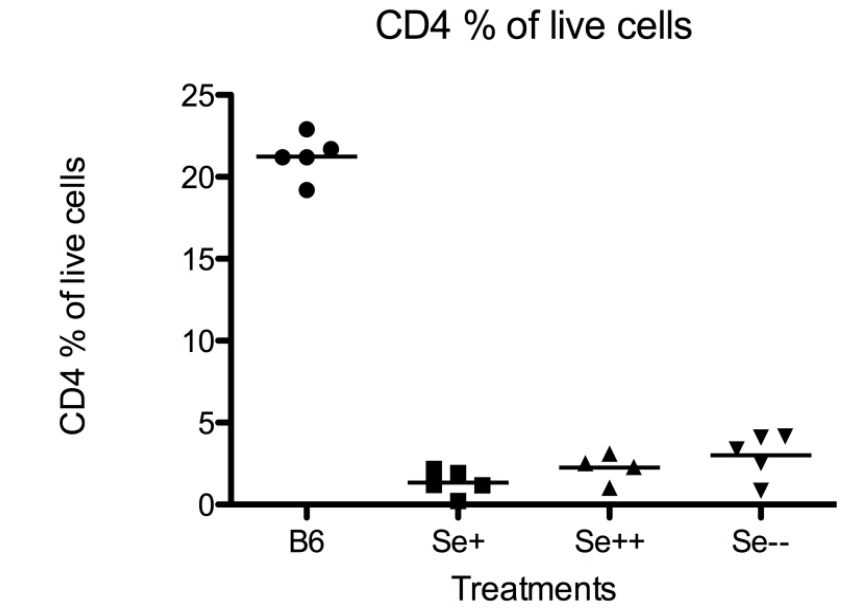


Figure 16. Percentage of live CD8+ cells (T cell subset) in 30-week-old nude mice with B6 wild type mice serving as control. Se-, selenium deficient; Se+, selenium adequate; Se++, selenium supranutritional. N=5 for all groups, except Se++ n=4 due to one sample lacking sufficient events.



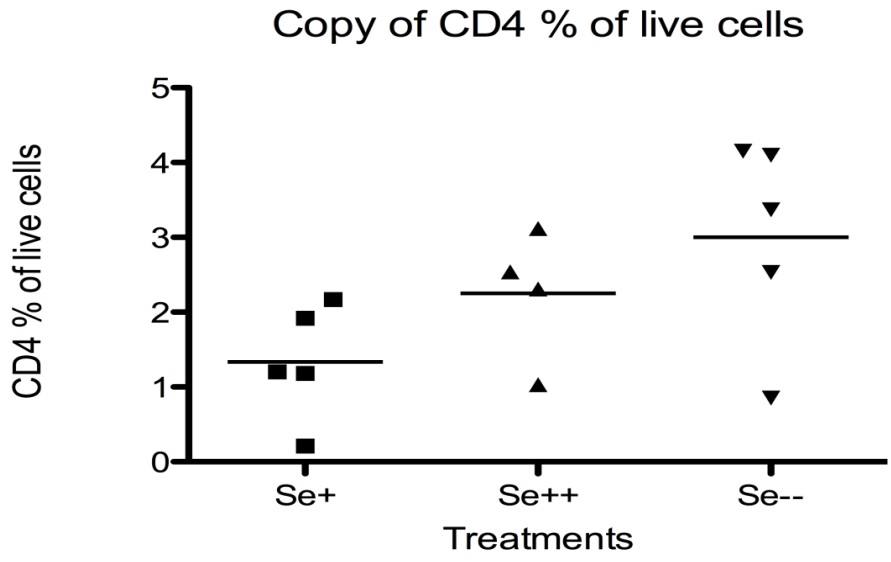


Figure 17. A. Percentage of live CD4+ cells (T cell subset) in 30-week-old nude mice with B6 wild type mice serving as control. Se-, selenium deficient; Se+, selenium adequate; Se++, selenium supranutritional. N=5 for all groups, except Se++ n=4 due to one sample lacking sufficient events. B. Close up of A without control B6.

In addition, the expression of CD62L cells, a homing to peripheral lymph nodes T cell receptor, was significantly lower in nude mice compared to B6 mice in both CD8 and CD4 T cells but was not significantly different between selenium dietary groups. CD4 T cells, in fact, expressed extremely low levels of CD62L (bellow 3%). Results are shown in Figure 18 and Figure 19. This could indicate a potentially low activation and ability to perform function, especially for CD4 T cells. On the contrary, CD44 expression was generally high in the CD4 and CD8 T cells. This result indicates memory-like phenotype and could be involved in tumor immunity.

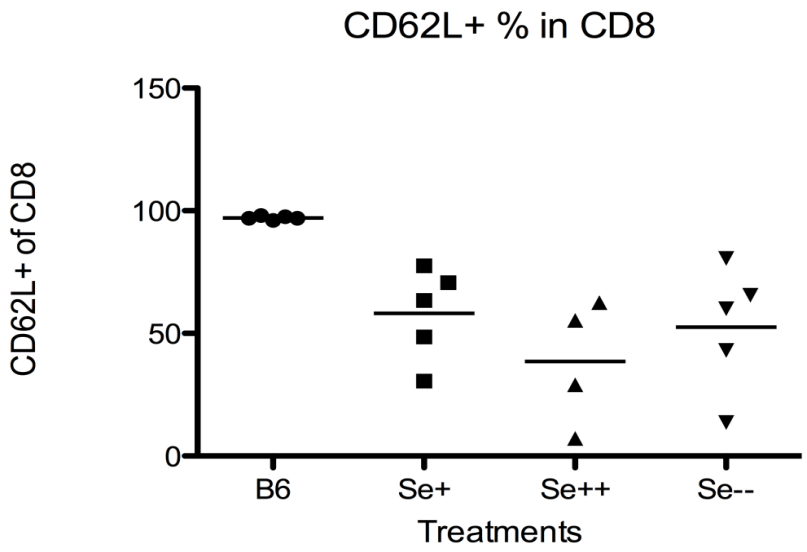


Figure 18. Percentage of CD62L expression in CD8 T cells in 30-week-old nude mice with B6 wild type mice serving as control. Se-, selenium deficient; Se+, selenium adequate; Se++, selenium supranutritional. N=5 for all groups, except Se++ n=4 due to one sample lacking sufficient events.

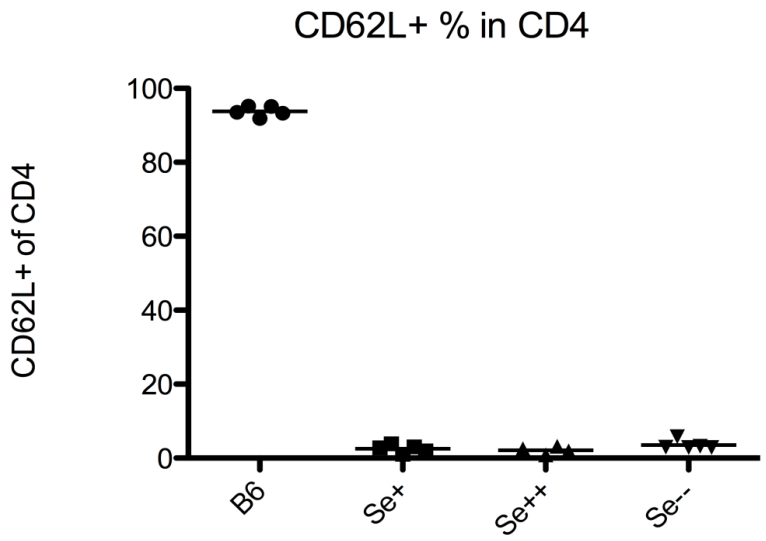


Figure 19. Percentage of CD62L expression in CD4 T cells in 30-week-old nude mice with B6 wild type mice serving as control. Se-, selenium deficient; Se+, selenium adequate; Se++, selenium supranutritional. N=5 for all groups, except Se++ n=4 due to one sample lacking sufficient events.

Circulating peripheral lymphocyte analysis for adult male nude mice in xenograft Experiment 1

T cells were measured at baseline, day 9, 19, 34, and at termination of the xenograft experiment 1. The major phenotypes were CD4+ and CD8+ T cells. Expression of CD25 and CD44 was also measured and analyzed. Figure 20 summarizes the results for percent of CD4 positive live cells at the specified time points. Selenium status did not have significant effect on CD4 T cell maturation and the xenograft seemed to suppress these cells. However, as expected, the CD4 T cell numbers increased significantly with age of the animals as compared at baseline and at termination for all three dietary groups.

Similarly, percent of circulating live CD8 positive T cells were analyzed at the specified time points during the xenograft and the results show a trend of decrease after exposure to tumor cells (Figure 21). CD8 T cells were slightly enhanced by high

selenium diet before the xenograft. Selenium-deficient diet also seemed to increase the CD8 T cells after the xenograft (day 47). Selenium-adequate diet resulted in lower CD8 T cells compared to selenium-deficient (p = 0.8) and selenium-supranutritional diet (p = 1.2); however, the difference was only marginally significant. Figure 22 depicts both CD4 and CD8 T cells combined. The trend follows the previous result with no significant differences between dietary groups, but with significant increase in the CD4 and CD8 T cells when comparing baseline numbers to those at termination.

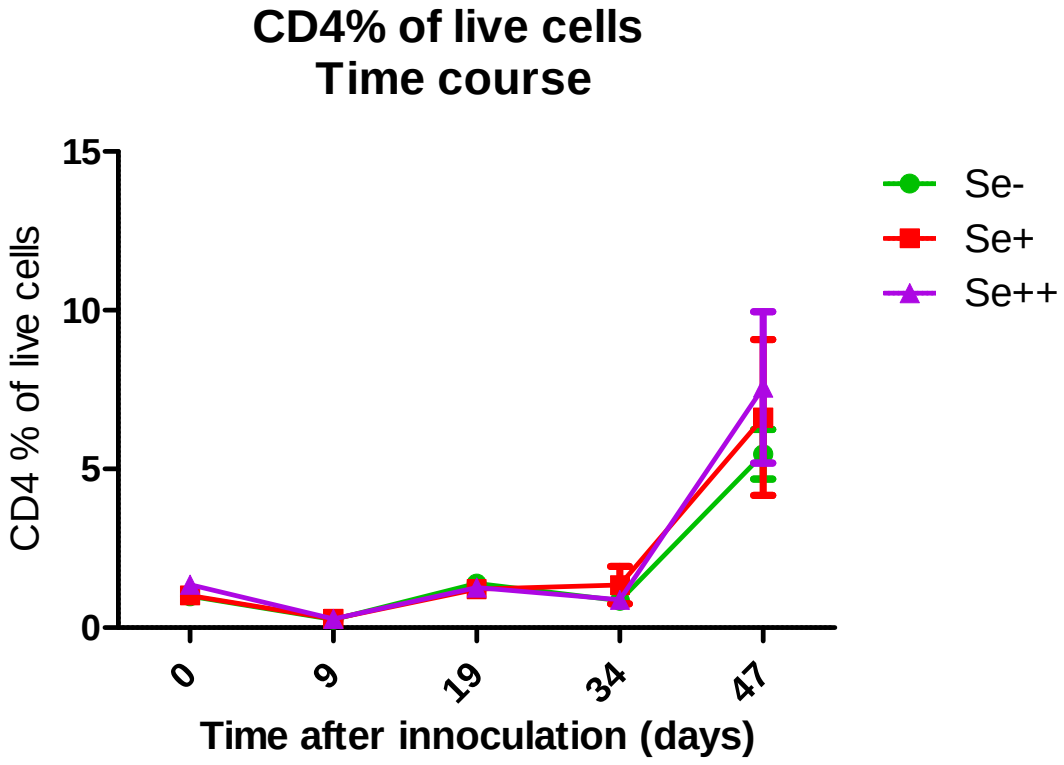


Figure 20. Mean percent peripheral live CD4 positive T cells measured at baseline, day 9, 19, 34 and termination of xenograft experiment. N=5, mean ± SEM for each dietary group, where Se-, selenium deficient; Se+, selenium adequate; Se++, selenium supranutritional.

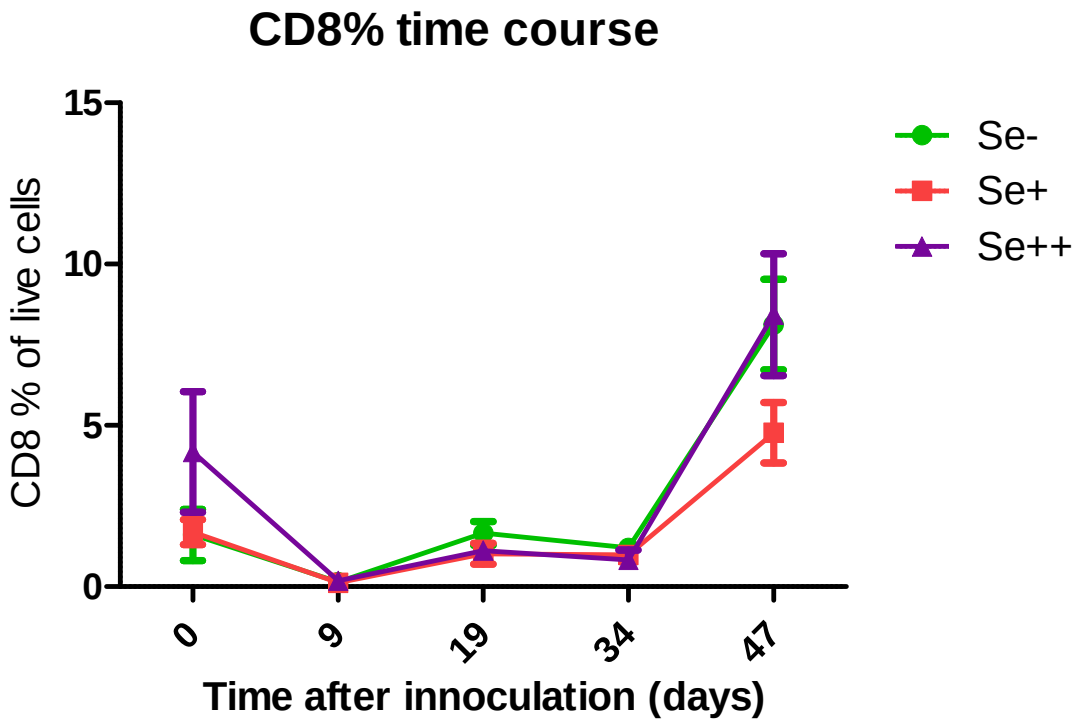


Figure 21. Mean percent peripheral live CD8 positive T cells measured at baseline, day 9, 19, 34 and termination of xenograft experiment. N=5, mean ± SEM for each dietary group, where Se-, selenium deficient; Se+, selenium adequate; Se++, selenium supranutritional.

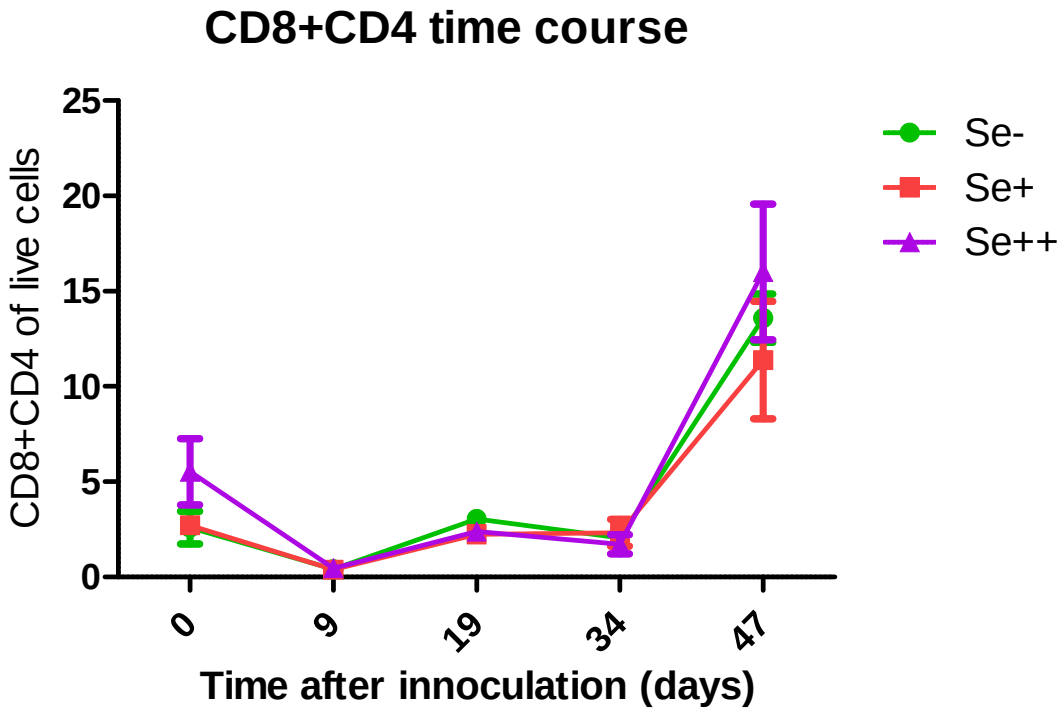


Figure 22. Mean percent peripheral live CD4 and CD8 positive T cells together measured at baseline, day 9, 19, 34 and termination of xenograft experiment. N=5, mean ± SEM for each dietary group. Se-, selenium deficient; Se+, selenium adequate; Se++, selenium supranutritional.

Expression of CD25 is a measure of activity and ability to perform functions of T cells. A marginally significant difference was observed on day 9 of the xenograft in CD25+CD4+ cells ($p = 0.1$) between selenium-adequate and selenium-supranutritional groups. Selenium-adequate group had lower CD25 expression than selenium-deficient and selenium-supranutritional groups. The expression of CD25 in either CD4 or CD8 T cells at any other time points was not significantly different between dietary groups.

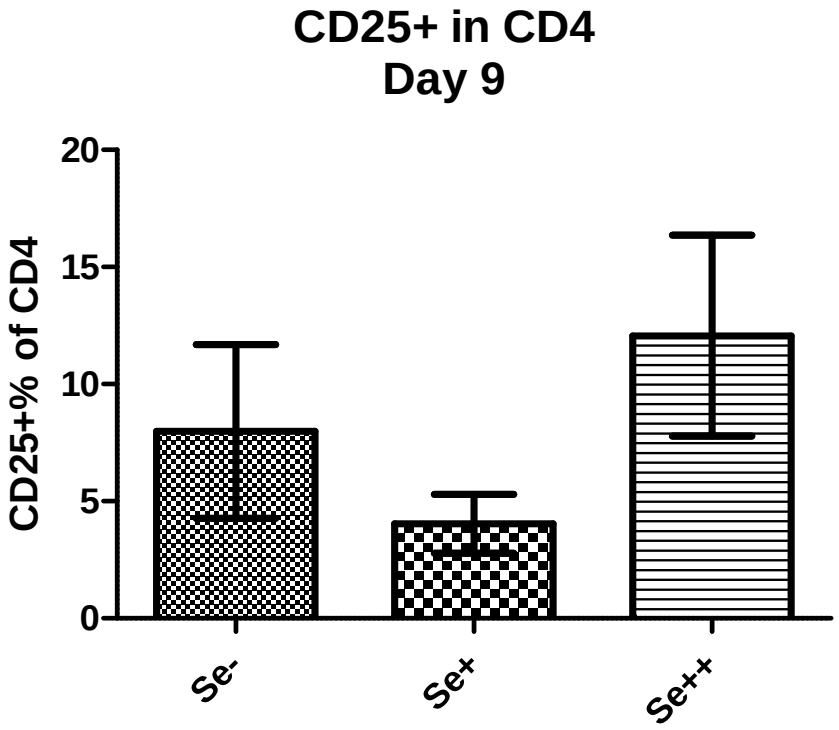


Figure 23. Mean percent CD25+CD4+ cells measured at day 9 of xenograft experiment. N=5, mean ± SEM for each dietary group. Se-, selenium deficient; Se+, selenium adequate; Se++, selenium supranutritional.

Since spleen is the main site for T cell accumulation, the weight of the organ could be a relative indicator of the amount of T cells in the body. The mean spleen weight for each dietary group was calculated and is shown in Figure 24. The difference in spleen weight was not significant between the dietary groups. However, correlation between the weight of the spleen and CD4 positive T cells indicated strongly positive result (Pearson r = 0.83, p = 0.0001) (Figure 25). The absolute numbers of T cells isolated from the spleen and the T cell numbers measured in the periphery as measured in the blood are closely related. This has been observed in unpublished research from Dr. Xiao's laboratory.

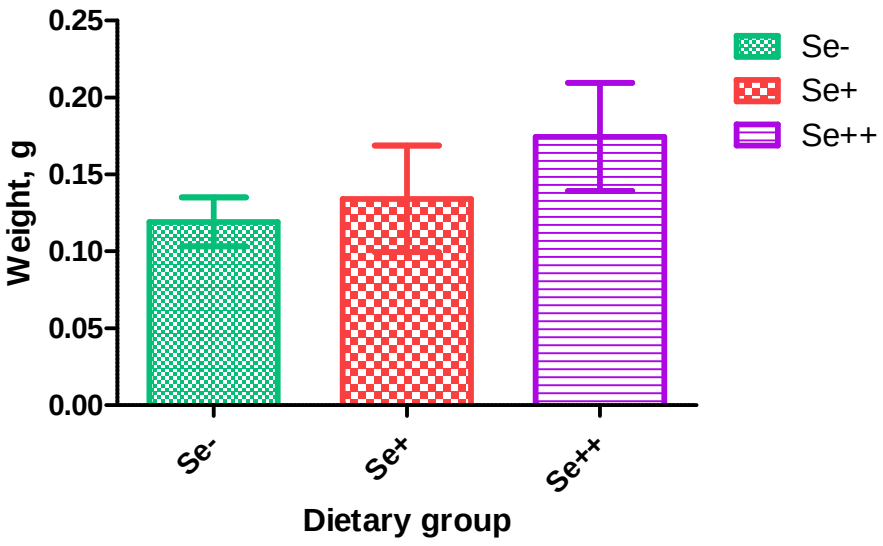


Figure 24. Mean weight of spleen for adult male nude mice comparing the three selenium dietary groups. N=5, mean ± SEM.

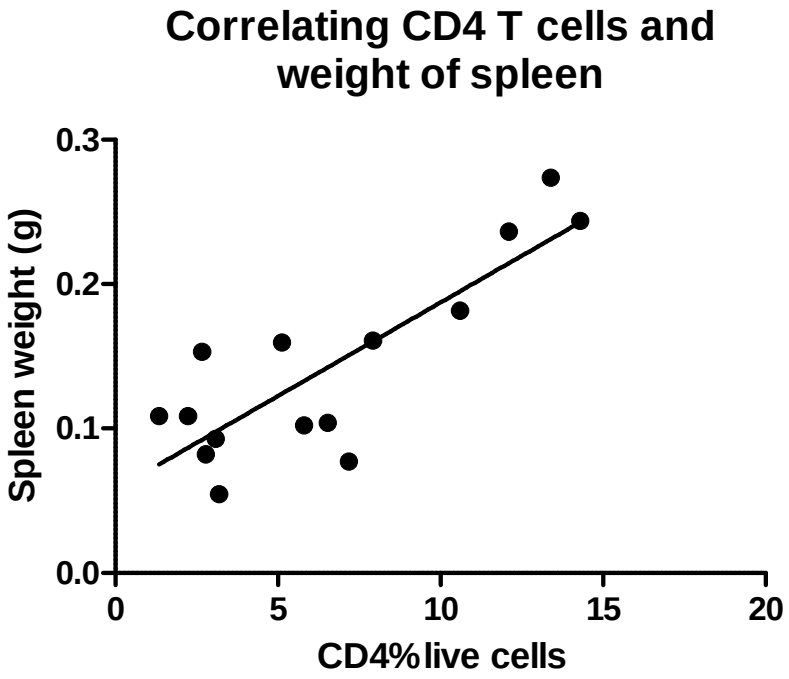


Figure 25. Correlation between CD4 T cell numbers and weight of spleen for adult male nude mice. N=15, Pearson r = 0.83, p = 0.0001.

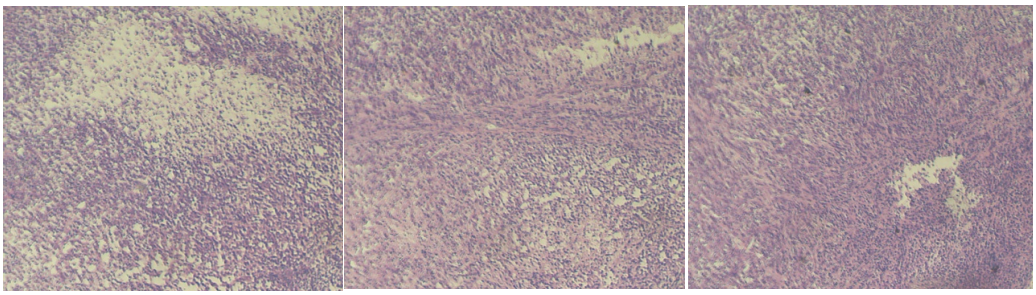
Histopathology of tumor tissues for adult nude mice

Histopathology was evaluated using H&E stained tumor and tissue sections fixed in paraformaldehyde and embedded in paraffin. The results are summarized in Table 5 and representative pictures are shown in Figure 26. The grading was done using the following scale: +, minimal; ++, mild; +++, moderate; ++++ severe by a board certified pathologist (Jerrold M. Ward, DVM, PhD). Of note, one tumor from the selenium-supranutritional group was hyalinized. The hyalinized tumor indicates that the tumor regressed and tumor cells may have undergone necrosis or apoptosis. The histopathological results indicate inflammation in necrotic lymphocytes and tumor cells. Tumors coming from the selenium-supranutritional group were found to have less

inflammation. Inflammation promotes tumor growth. This result is in accordance with the observation that the selenium-supranutritional group had the smallest tumors.

Table 5. Findings of histopathology for tumor sections from adult nude mice

Dietary group	Tag	Inflammation	Tumor Necrosis
Se-	205	++	+
	207	++	+
	209	+	+
	213	++	++
	214	++	+
Se+	216	-	-
	226	+	++
	228	+	++
	229	++	-
Se++	211	-	+ hyalinized
	212	-	-
	236	++	-
	238	++	+



(#207 (#216) (#212)

Figure 26. Representative hematoxylin and eosin (H&E) stained slides of tumor tissue from adult nude mice from the different dietary groups.

CHAPTER IV:

YOUNG NUDE MICE XENOGRAFT STUDY

After performing the first experiment with adult nude mice, we sought comparison of the data to young-aged subjects. Experiment 2 was therefore performed to mirror Experiment 1 with some minor changes that will be highlighted in this section of the thesis.

Body weight of young male nude mice on selenium-modified diet before and during xenograft study

Nine three-week-old male nude mice were randomly selected and were fed with a Se- basal diet, or the diet supplemented with 0.15 (Se+, adequate) or 1 (Se++, supranutritional) mg Se/kg as sodium selenate. Body weight was measured every week. The initial body weights at 3 weeks of age ranged from 9.2 g to 13.8 g and the final weights at the age of 14 weeks were in the range of 26.4 g to 32.5 g. The mean body weights for nude mice in the control (selenium-adequate) group (29.7 ± 1.4 g) compared to the selenium-deficient (29.6 ± 0.9 g) and selenium-supranutritional (27.2 ± 0.5 g) groups were not significantly different ($p = 0.21$) among the three dietary groups after 10 weeks on the selenium-modified diet. The changes in body weights over time are shown in Figure 27. The body weights of nude mice fed the selenium-supranutritional diet were marginally lower than the selenium-adequate or selenium-deficient groups. Based on these results, selenium given at the three doses (0, 0.15, 1 mg Se/kg feed) did not have negative effect on the body weight for the length of time used in this study. All nude mice survived the xenograft experiment until sacrificed at termination of the study.

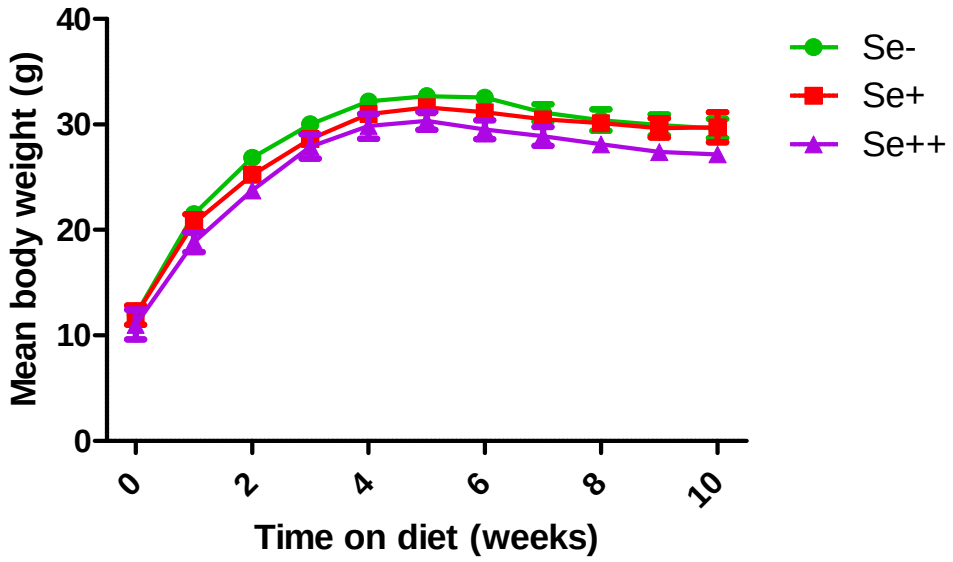


Figure 27. Effect of dietary selenium on changes of the mean body weights of young male nude mice fed *ad libitum* selenium-modified AIN-93G purified rodent diet for11 weeks. Se-, selenium deficient; Se+ ,selenium adequate; Se++, selenium supranutritional. Data are presented as mean ± SEM, n = 3. SEM = standard error of the mean.

The nude mice in this experiment were gaining weight until about 8 weeks of age, which is equivalent to 5 weeks on the experimental diet. After that point, the mice were assumed to have reached their maximum weight. In order to examine the effect of the xenograft itself on the body weight, the initial body weight was established as the maximum weight reached at 5 weeks on the diet (8 weeks of age) because the young mice were still gaining weight after xenograft. This corresponds to one week after xenograft administration. The xenograft study was carried out for 7 weeks to be consistent with experiment 1. Figure 28 and Figure 29 show the graphed results of changes in the mean body weights. The body weights of the animals were measured

every other day. The initial body weights at baseline of the xenograft experiment (week 7) ranged from 28.8 g to 32.9 g and the final weights at week 14 were in the range of 26.4 g and 32.5 g. The mean body weights for nude mice in the control (selenium-adequate) group (29.7 ± 1.4g) compared to the selenium-deficient (29.6 ± 0.9g) and selenium-supplemented (27.2 ± 0.5 g) groups were not significantly different (p = 0.21) among the three dietary groups after 10 weeks on the selenium-modified diet (14 weeks of age). However, the mean body weights of study animals at baseline (31.2 ± 0.5 g, n = 9) compared to at termination (28.8 ± 0.7 g, n = 9) were significantly different (p = 0.01). This result implies that the statistically significant decrease in body weights was due to the xenograft itself and not due to the selenium supplementation.

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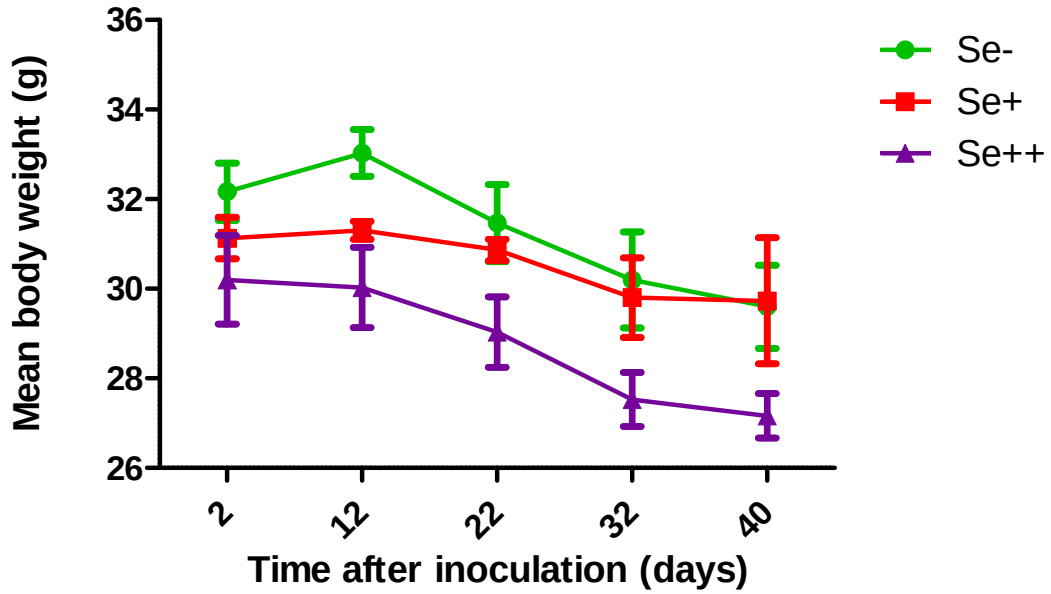


Figure 28. Changes in the mean body weights (g, mean ± SEM, n = 3) for young male nude mice during 7-week xenograft study. Se-, selenium deficient; Se+, selenium adequate; Se++, selenium supranutritional.

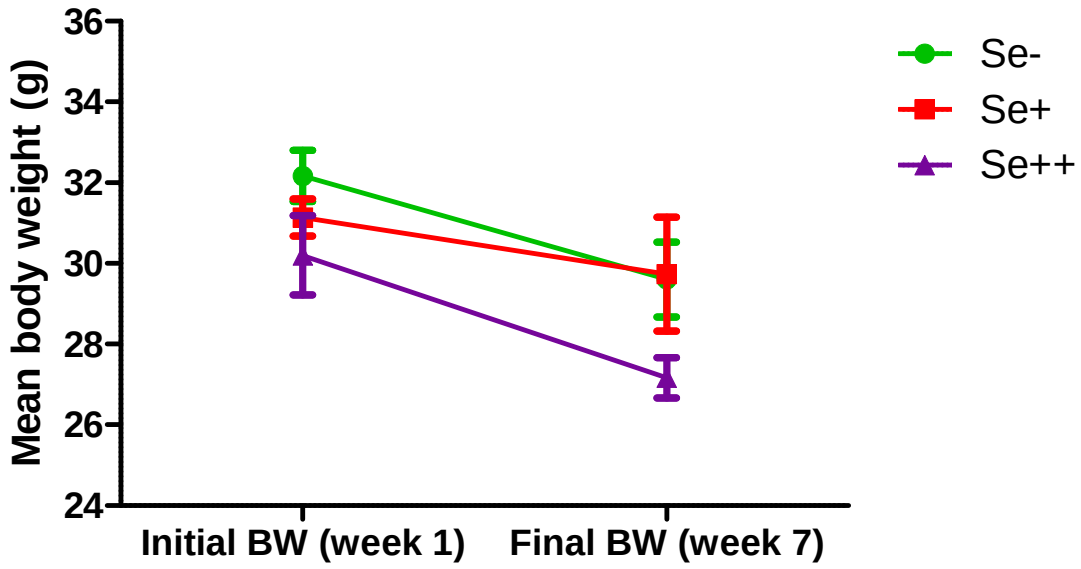


Figure 29. Changes in the initial and final mean body weights (g, mean ± SEM, n = 3) of young male nude mice during 7-week xenograft study. The initial weight was determined by the maximum body weight reached at week 1 after inoculation. Se-, selenium deficient; Se+, selenium adequate; and Se++, selenium supranutritional.

Plasma selenium analysis of young male nude mice in xenograft Experiment 2

The effect of selenium supplementation on body selenium status was analyzed by measuring the plasma selenium concentrations for nude mice at 11 weeks on the Se-modified diets. The data for plasma selenium concentrations are in Table 6. The results of the mean plasma concentrations for each dietary group are shown in Figure 30. The mean plasma selenium concentration was nearly 4-fold lower in the selenium-deficient group (90.0 ± 11.0 ng/ml) compared to selenium-adequate (348.7 ± 18.0 ng/ml, p = 0.0003) and

selenium-supranutritional (340.3 ± 12.5 ng/ml, p = 0.0001) group, respectively. The difference between selenium-adequate and selenium-supranutritional group was not statistically significant (p=0.723).

Table 6. Plasma selenium concentrations (ng/ml, mean ± SEM, n = 3) for young male nude mice in the 3 dietary groups at termination (10 weeks on the diet).

Animal ID and group	Total selenium concentration in plasma (ng/ml)	Animal ID and group	Total selenium concentration in plasma (ng/ml)	Animal ID and group	Total selenium concentration in plasma (ng/ml)
Se-		Control Se+		Se++	
260	79	265	313	263	365
262	112	266	362	268	331
270	79	267	371	269	325
mean ± SEM	**90.0±11.0**		**348.7±18.0**		**340.3±12.5**

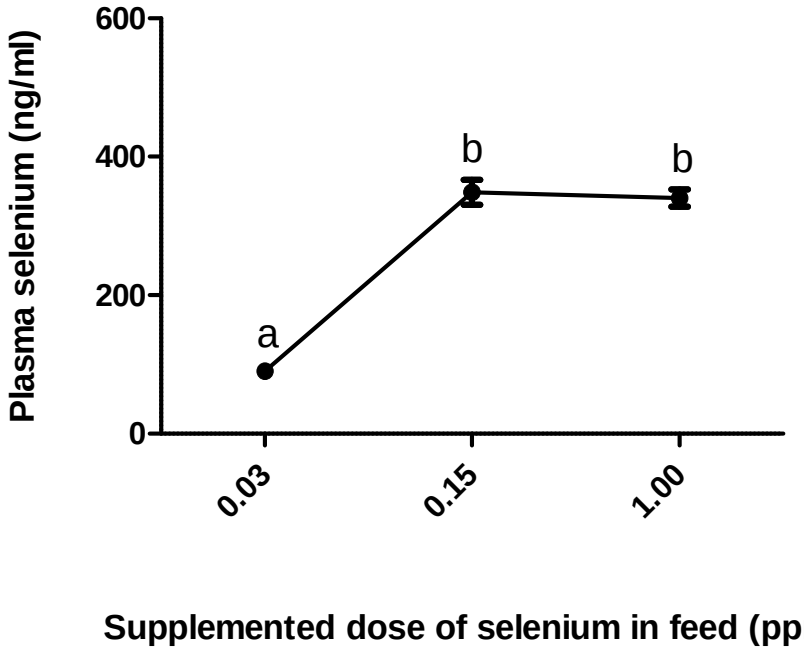


Figure 30. Mean plasma selenium concentrations for young male nude mice after 14 weeks on diet containing 0.03 ppm (Se-), 0.15 ppm (Se+), and 1 ppm (Se++) selenium in the form of sodium selenate. The amount is expressed as mean ± SEM (n=3 mice).

Effect of selenium on tumor volume and tumor weight in young male nude mice in xenograft Experiment 2

Estimated tumor volume was plotted as a function of time (Figure 31). Consistent with xenograft study 1 (46 days after inoculation), mice in Experiment 2 were terminated 7 weeks after the xenograft. At that point, the weight of the dissected tumors was measured and the mean as well as total weight for each dietary group were calculated and plotted as shown in Table 7 and depicted in Figure 32. All animals were on the experimental diet for the same amount time (4 weeks) prior to cancer cell inoculation. The experimental diet continued to be given during the 7 weeks of the xenograft study. 8 x 10^5 cells of human prostate cancer of the cell line PC-3 were injected subcutaneously on either side of the shoulder blade of each experimental nude mouse. We injected less

PC-3 cells in Experiment 2 (8×10^5 vs. 2×10^6) because young nude mice have stronger immune system as no extrathymic T cell maturation occurs yet.

The success of the xenograft was recorded as the incidence of tumor growth at site of injection and is reported in Figure 34. Selenium deficiency seemed to enhance the initial tumor growth, especially during the first few weeks. Selenium supplementation, on the other hand, seemed to delay tumor growth in the initial stages. The xenograft had a 100% success rate (20/20). Selenium deficiency seemed to enhance tumor growth in the first week, but did not enhance tumor growth, which was measured by estimating the volume, throughout the total time course of the xenograft experiment. Contrary to expectation and what was observed in experiment 1, supplementation with 1 mg Se/kg diet, did not suppress the tumor growth at all; rather, it enhanced tumor growth (Figure 31, Figure 32, Figure 33, and Table 7). Interestingly, the control/selenium-adequate group that had the largest tumor growth in the first experiment, had the smallest tumor volume and weight. The mean tumor weights did not differ significantly ($p = 0.47$) between groups: selenium-deficient (1.2 ± 0.1g), control/selenium-adequate (1.1 ± 0.6 g), and selenium-supranutritional (1.5 ± 0.1 g). The difference in tumor weight can be visually seen from the total tumor weight depicted in Figure 32B. Table 7 gives a summary of the combined tumor weights for each experimental rodent as well as the mean ± S.E.M. and the total tumor weight figures.

A nearly 2- and 1.6-fold decrease was observed in the total tumor weight between the control group (2.3 g) and the selenium-supranutritional (4.5 g) and selenium-deficient (3.7 g) groups, respectively. We also explored the effect of the tumor weight to body weight ratio and found that the difference between groups was not statistically

significant, however the difference between Se- and Se++ was marginally significant (p = 0.052) (Figure 33).

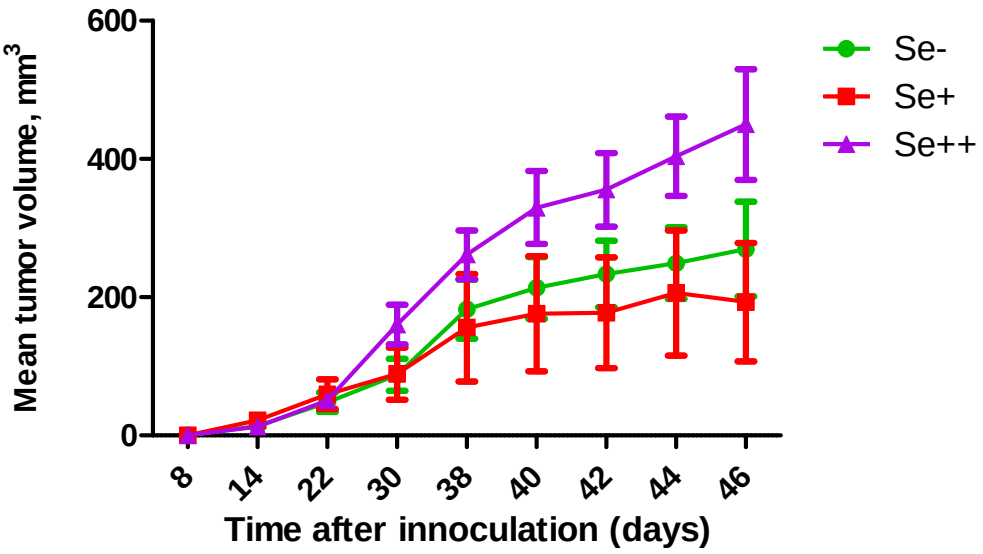
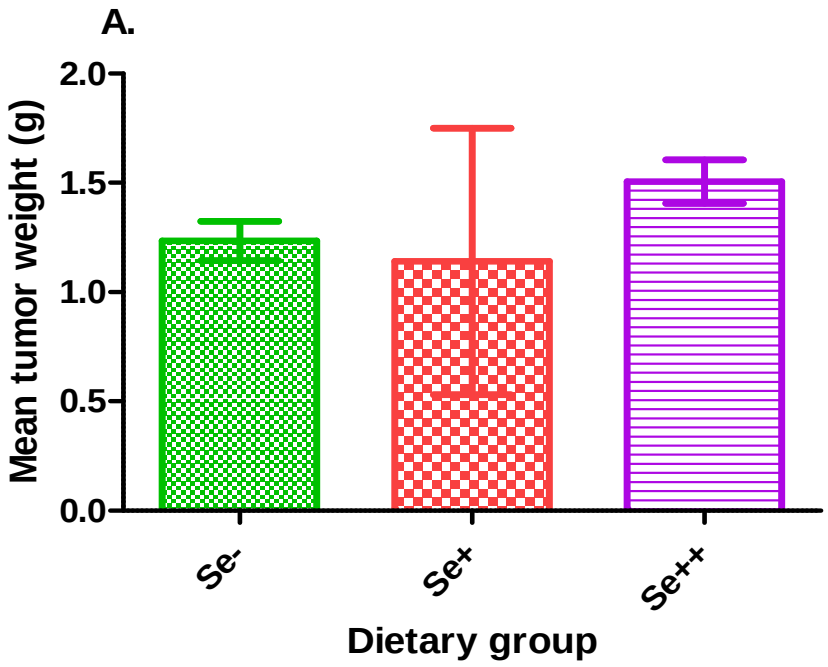


Figure 31. Effect of selenium-modified diet on changes in the mean tumor volume. $8x10^5$ cells of the PC-3 cell line of human prostate cancer were subcutaneously injected in young male nude mice. Se-, selenium deficient; Se+, selenium adequate; Se++, selenium supranutritional. Data are presented as mean ± SEM. Volume was measured in mm^3, n = 3.

Table 7. Tumor weights data (g, mean ± SEM, n= 3) for young nude mice in the 3 dietary groups at termination (day 46 after inoculation) Tumor weight was calculated as total weight of multiple tumors per mouse, if more than one found. Se-, selenium deficient (0.03 ppm); Se+, selenium adequate (0.15 ppm); Se++, selenium supranutritional (1 ppm) dietary group. ND = none detected

Animal ID and group	Total tumor weight (g)	Animal ID and group	Total tumor weight (g)	Animal ID and group	Total tumor weight (g)
Se-		control/Se+		Se++	
260	1.14	265	ND	263	1.71
262	1.15	266	1.75	268	1.40
270	1.42	267	0.53	269	1.41
Total	**3.70**		**2.28**		**4.52**
mean ± SEM	**1.24±0.09**		**1.14±0.61**		**1.51 ± 0.10**



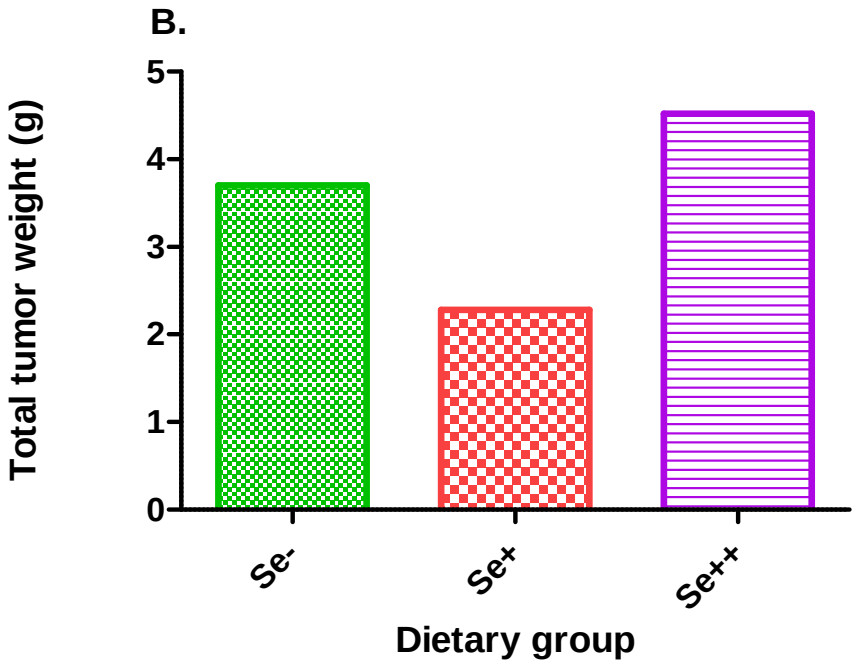


Figure 32. Final average tumor weight (A) and total tumor weight (B) at termination (day 46 after inoculation) for young male nude mice compared between dietary groups. A. The values are mean ± SEM, n = 3. Tumor weight was calculated as total weight of multiple tumors per mouse.

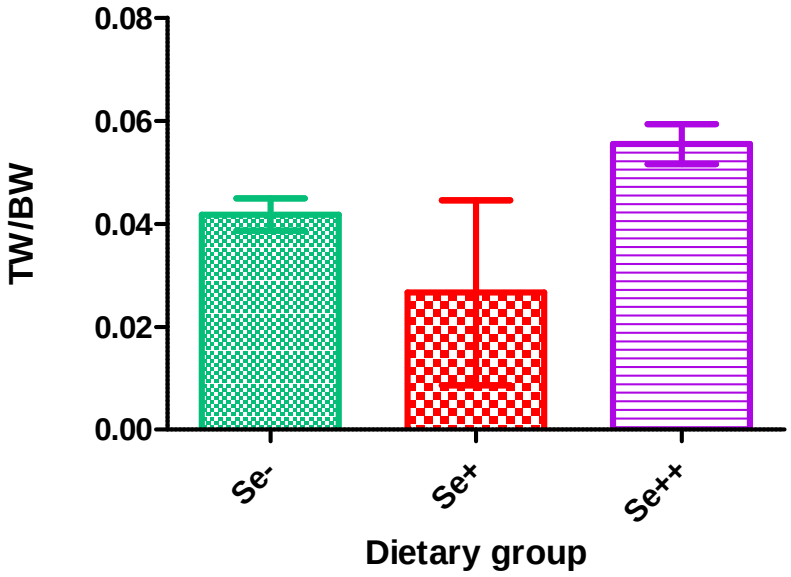


Figure 33. Tumor to body weight ratio compared between dietary groups. The values are mean ± SEM, n=3. Tumor weight was calculated as total weight of multiple tumors per mouse and the body weight was the last recorded BW before sacrifice.

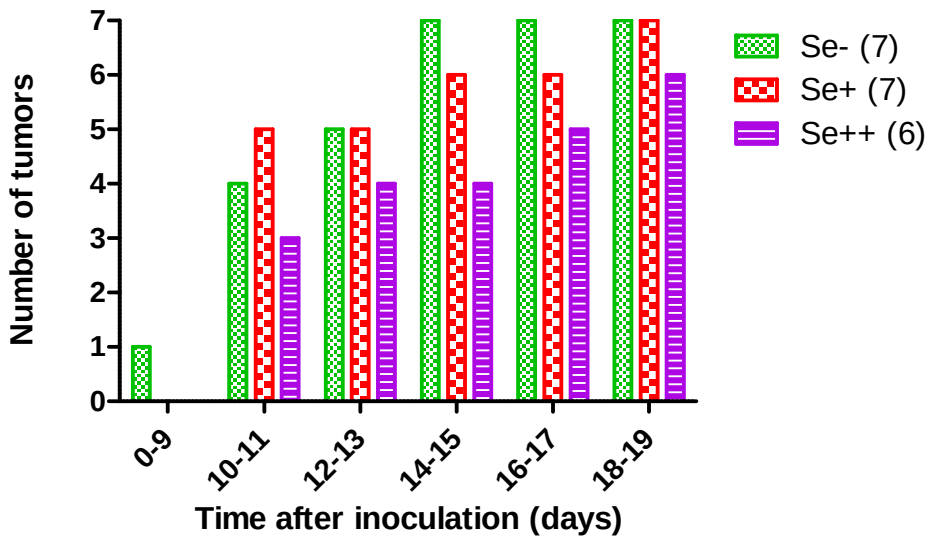


Figure 34. Incidence of tumor growth at site of cancer cell inoculation for the first 3 weeks of xenograft study in young male nude mice. Se-, selenium deficient; Se+, selenium adequate; Se++, selenium supranutritional. Numbers in parentheses represent the number of tumor inoculations per group.

It is interesting to note, that one animal in the control (Se+) group grew 3 tumors for 3-4 weeks and then all 3 tumors regressed, one by one, to undetectable size and were not even found during dissection. The estimated largest volumes for these tumors were 4, 40 and 70 mm^3.

Circulating peripheral lymphocyte analysis for young male nude mice in xenograft Experiment 2

T cells were measured only at termination as it was assumed that the numbers of circulating peripheral T cells were too low to be detectable in significant amounts for the young nude mice. The results for young nude mice were inconsistent with what was seen in adult nude mice, when analyzing the effect of dietary selenium dose on T cell

maturation. The percent of live CD8+ cells in young nude mice was highest for the selenium-supranutritional group but there was also very high within-group variability so difference was not significant (Figure 35). The mean percent of CD4 T cells in selenium-supranutritional group, on the other hand, was significantly lower than in selenium-adequate group ($p < 0.05$) and marginally lower than selenium-deficient group ($p = 0.9$). This result is shown in Figure 36. Figure 37 and Figure 38 depict the combined CD4+ and CD8+ T cells and the CD8/CD4 T cell ratio, respectively.

Figure 35. Percentage of live CD8+ T cells in 14-week-old athymic nude mice. Se-, selenium deficient; Se+, selenium adequate; Se++, selenium supranutritional. N=3 for all groups.

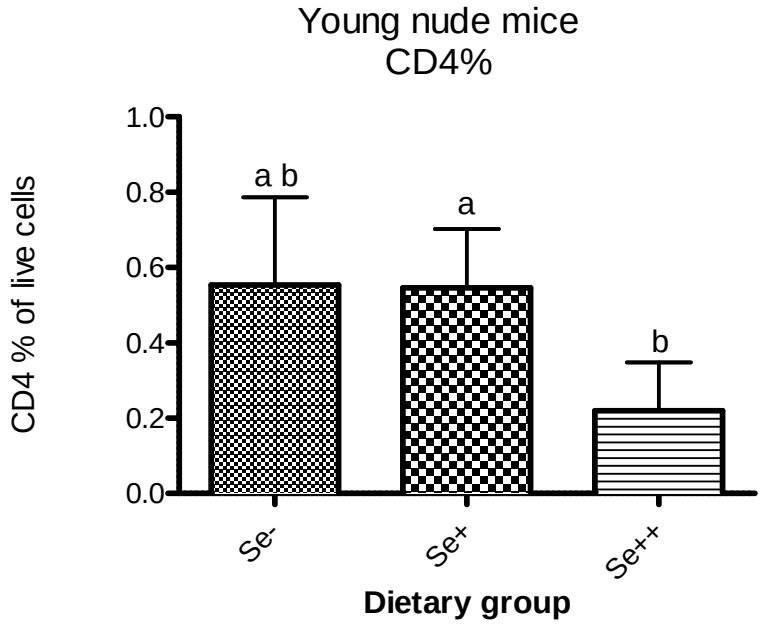


Figure 36. Percentage of live CD4+ cells in 14-week-old athymic nude mice. Se-, selenium deficient; Se+, selenium adequate; Se++, selenium supranutritional. N=3 for all groups.

Figure 37. Percentage of live CD8+ and CD4+ cells combined together in 14-week-old athymic nude mice. Se-, selenium deficient; Se+, selenium adequate; Se++, selenium supranutritional. N=3 for all groups.

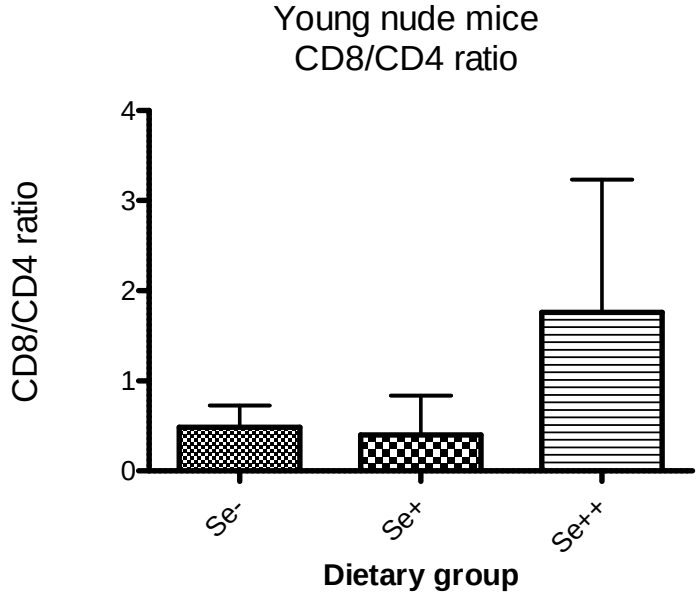


Figure 38. Ratio of CD8/CD4 T cells in 14-week-old athymic nude mice. Se-, selenium deficient; Se+, selenium adequate; Se++ selenium supranutritional. N=3 for all groups.

Since spleen is the main site for T cell accumulation, the weight of the organ could be a relative indicator of the amount of T cells in the body. The mean spleen weight for each dietary group was calculated and is shown in Figure 39. Correlation between the weight of the spleen and CD4 positive T cells indicate fairly positive result (Pearson r = 0.62, p = 0.07) (Figure 40).

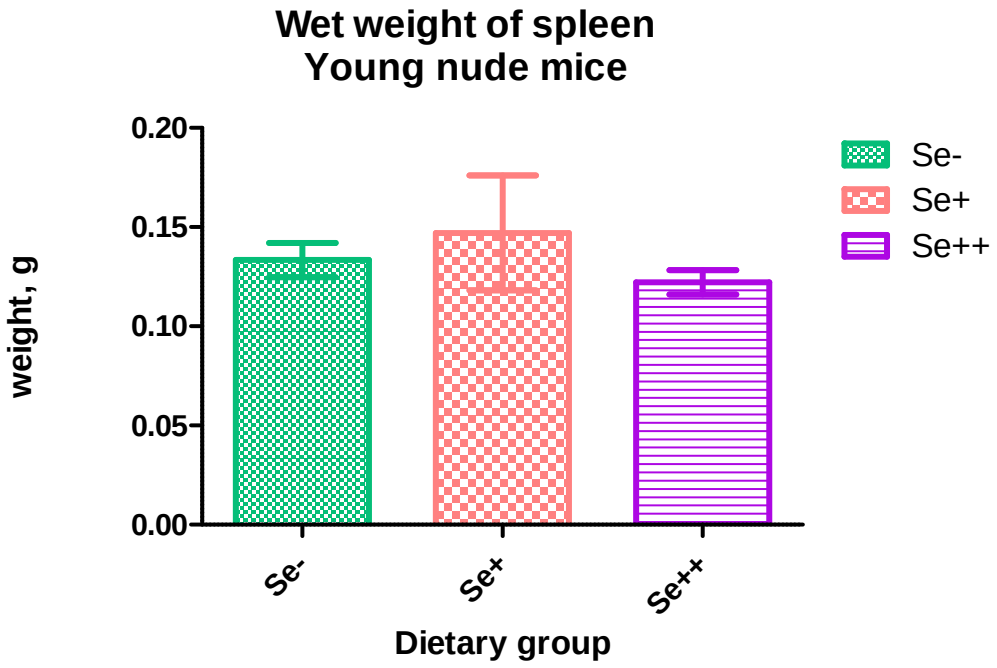


Figure 39. Mean weight of spleen for young male nude mice in comparing the three selenium dietary groups. N=3

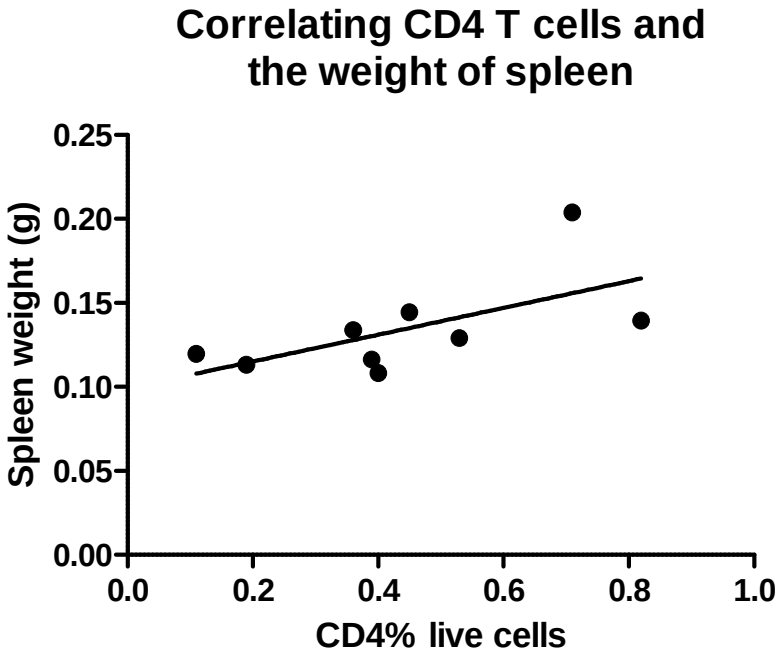


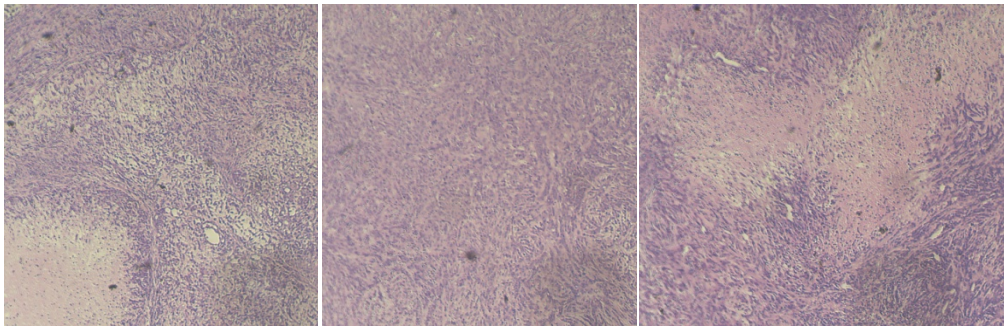
Figure 40. Correlation between CD4 T cell numbers and weight of spleen for young male nude mice. N=9, Pearson r = 0.62, p = 0.07.

Histopathology of tumor tissues for young nude mice

Histopathological evaluation was done on H&E stained tumor tissue sections fixed in paraformaldehyde and embedded in paraffin. The results are summarized in Table 8. The grading was done using the following scale: +, minimal; ++, mild; +++, moderate; ++++ severe. A large number of the tumors had hyalinized stroma, an aberrant deposition of basement membrane material produced by tumor cells. The hyalinized tumor indicates that the tumor regressed and tumor cells may have undergone necrosis or apoptosis. Representative pictures from each group are shown in Figure 41.

Table 8. Findings of histopathological evaluation of tumor sections for young nude mice.

Dietary group	Tag	Inflammation	Necrosis	Hyalinized stroma
Se-	260	-	++	+++
	262	+	++	+
	270	-	+	-
Se+	266	-	+	+
	267	-	++	++
Se++	268	-	++	+
	269	-	++	-
	263	-	++	+



(#262) (#26 (#26

Figure 41. Representative pictures of hematoxylin and eosin (H&E) stained tumor tissue sections for young nude mice from the different dietary groups.

CHAPTER V: DISCUSSION

These experiments were undertaken in order to determine the effect of dietary supplementation with selenium on T cell immunity in response to human prostate cancer xenograft in male nude mice. Male nude mice animal model was applied in this study because they do not reject human xenografts (Manning, Reed et al. 1973) and because prostate cancer is a malignancy afflicting men only.

Prior to this work, no studies have looked at chemopreventive role of selenium in conjunction with T cell immunity in nude mice bearing xenografted tumors. To date, few studies have been conducted to explore the effect of various selenium compounds in nude mice bearing tumor xenograft coming from various human tissues (Lin, Boylan et al. 1991; Li, Lee et al. 2008; Yang, Huang et al. 2009). Here, we also aimed to explore the efficacy of selenium, at higher than nutritional dose, as a potential chemopreventive agent against prostate cancer. Conflicting results have been reported from two large intervention studies: the NPC trial that employed supplementation with 200 µg of selenium as enriched yeast and showed significant decrease in some cancers, especially prostate cancer (Duffield-Lillico, Reid et al. 2002); whereas the SELECT trial that used supplementation with 200 µg of selenium as selenomethionine failed to show decrease in prostate cancer incidence (Lippman, Klein et al. 2009).

In vivo studies of selenium as anticarcinogenic agent also show conflicting results. Study by Lin et al showed no effect of diet deficient in selenium or supplemented with 4 mg Se/kg (as sodium selenite) for 4 weeks on human mammary tumor xenograft in nude mice (Lin, Boylan et al. 1991). The tumor xenograft result for adult nude mice in this study is in accordance with other studies that used organic or inorganic forms of selenium

(Li, Lee et al. 2008) and we confirmed the anti-tumorigenic efficacy of sodium selenate at the supranutritional level but failed to show the same effect in young subjects. Similar to the result for young nude mice in this study, Finley and Davis showed an increase in chemically-induced aberrant crypt and aberrant crypt foci in rats depleted and then repleted with sodium selenate at 0.1 ppm and 1 ppm even more, compared to no addition (Finley and Davis 2001).

To our knowledge, no data is available concerning the effect of long-term dietary supplementation with sodium selenate on human prostate cancer xenograft in nude mice. The long-term supplementation was chosen to allow for observation of possible adverse effects of supranutritional dose supplementation as well as deficiency. It also allowed for extrathymic T cell maturation, which usually peaks at around 32 weeks of age in nude mice, so that the effect of dietary selenium dose on the maturation and activity could be examined (Kennedy, Pierce et al. 1992).

Supplementation with high selenium has opposing effect on prostate tumor xenograft in adult versus young nude mice

The most interesting result was observed from high supplementation of selenium on tumor growth in adult versus young nude mice. Why did selenium supplementation at the supranutritional level suppress tumor formation in adult nude mice but promoted tumor growth in young nude mice? In order to attempt to answer this question, it is important to look at the differences in plasma selenium levels and T cell phenotypes. In adult nude mice, the tumor volume/weight overall was 2-3-fold lower than in the young nude mice (Figure 10, Figure 11, Figure 31, Figure 32). Although, adult mice were on selenium-modified diet 6 months longer than the young mice, plasma selenium concentrations were similar and the differences between dietary groups were comparable

(Figure 5 and Figure 30). So the difference in tumor size in the young versus adult animals could be more likely attributed to the effect of T cell immunity. The T cell (CD4+CD8) maturation increased with the age of the animals as seen in the graph bellow (Figure 42), from bellow 0.5% at 14 weeks to 3-5% at 33 weeks to 10-15% of CD4+CD8 percent live cells at 40 weeks of age. When selenium-supranutritional dietary groups are evaluated closely, there are differences between the numbers of CD4+ T cells of adult and young nude mice. In fact, the CD4 + T cells in young nude mice are significantly lower compared to the other selenium dietary groups (Figure 36) and the tumor volume/weight are significantly higher. From this observation, it seems that high dietary selenium causes a decrease in CD4 T cells which may be associated with tumor formation in young nude mice. Conversely, why does high selenium in older nude mice not suppress CD4 T cells? Is it possible that other functional T cells are stimulated in the older mice that are lacking in the young ones? The pathway or mechanism to explain this observation is beyond the scope of this study and could be the groundwork for future research. Selenium has been shown to affect cell-mediated immunity. Specifically, combined selenium -vitamin E deficient diet depressed T-lymphocyte-mediated cytotoxicity in rats after 7 weeks on diet (Meeker, Eskew et al. 1985). Thus we could speculate, that the toxic effect of selenium could be compromised in young nude mice whose T-lymphocytes are mostly immature and non-functional. This phenomenon, in turn, could explain why the older nude mice, with increased T cell maturation, exhibit the tumor suppressive effect of selenium given at supranutritional level.

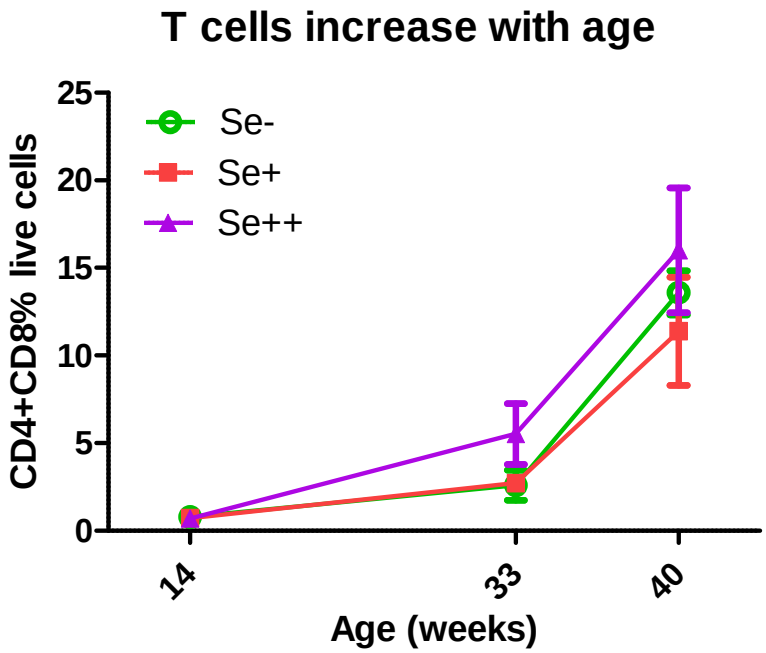


Figure 42. T cell maturation increases with age

Plasma selenium concentrations are not related to tumor growth in adult and young nude mice supplemented with 0, 0.15 and 1 mg Se/kg diet

Although, dietary supplementation with sodium selenate was 5- and 35-fold higher in selenium-adequate and selenium-supranutritional diet, respectively, compared to selenium-deficient diet, plasma selenium levels of adult mice from selenium-deficient group were 40% of those of selenium-adequate and 35% of those mice in selenium-supranutritional group (Figure 5). In young mice from selenium-deficient group, plasma levels were 25% of those mice from both of the supplemented groups (Figure 30). Thus, it seems that plasma selenium levels are related to age and there seems to be a cut off value at which plasma selenium does not increase with increasing dietary intake. Similar to our study, plasma selenium values of 0.9, 5.1, and 4.5 µmol/L were observed by Cheng et al, where mice were fed selenium deficient, adequate, excessive diet containing < 0.02,

0.5, 3.0 mg Se/kg feed, respectively, as sodium selenite for 6 weeks. The plasma selenium concentrations in our study are very similar even with lower adequate (0.15 ppm) and supranutritional (1.0 ppm) doses. Specifically, in adult nude mice plasma selenium values were 1.8 µmol/L (143 ng/ml), 4.5 µmol/L (353 ng/ml) and 5.0 µmol/L (400 ng/ml) and in young mice 1.4 µmol/L (90 ng/ml), 4.4 µmol/L (348 ng/ml) and 4.3 µmol/L (340ng/ml) (Cheng 1998). The explanation could be that selenium excretion also increases with increased dietary intake but is reduced under selenium deficient conditions to preserve body's reserves (Levander 1986). Therefore, plasma selenium concentration values in mice on selenium-adequate diet are very similar to values of mice on selenium-supranutritional diet in both studies. This observation leads to another question. Why does such small difference in plasma selenium (adequate vs. supranutritional) give such drastic result in tumor growth suppression in adult nude mice? First, Finley and his group showed that high-selenium broccoli was the most efficacious against colon aberrant crypts in spite of having the lowest bioavailability (measured as GPx activity) when compared to other selenium compounds (Finley and Davis 2001). Next, selenium can act through selenoproteins, which have antioxidant role, and through intermediary metabolites at higher doses, which are toxic and capable of inducing DNA damage leading to cell death or senescence (Combs and Gray 1998; Drake 2006; Wu, Kang et al. 2010). It appears that in our study selenium-deficient and –adequate levels did not have effect on tumorigenesis but the higher selenium-supranutritional level was sufficiently high to have a significant effect on tumor growth suppression. From our knowledge of selenium metabolism, our results support that selenium metabolites play pivotal role in cancer prevention.

Selenium-deficient status does not have effect on tumor growth

It was observed, that selenium-deficient diet greatly reduces plasma selenium concentrations. This result has been reported in other studies as well (Burk, Norsworthy et al. 2006; Wang, Wu et al. 2009). However, selenium deficiency did not significantly enhance tumor growth in either adult or young animals. This result has been observed elsewhere in a similar model using dietary supplementation with selenite for 2 months and human mammary tumor xenograft in nude mice for 4 weeks and measuring GPx activity instead of plasma selenium for indication of selenium status (Lin, Boylan et al. 1991). Although selenium-deficient diet did not enhance final tumor volume and tumor weight, it did promote the initial tumor growth and incidence (Figure 12 and Figure 34) in both age groups. Selenoprotein activity is affected by selenium-deficient status (Combs and Gray 1998; Driscoll and Copeland 2003). Therefore, it is possible that the antioxidant activity of selenoproteins plays role during the initial stage of tumorigenesis as seen in our results of tumor growth and incidence.

Limitations and Strengths of the study

This study could have been improved by measuring T cell profiles at baseline in addition to at termination for young nude mice xenograft experiment in order to compare the effect of short-term selenium supplementation on T cell maturation and activation in younger subjects. Measuring the selenium content in the tumor tissue in addition to in the plasma only, would also provide valuable information regarding the distribution of selenium during tumorigenesis. However, the tumor tissues collected were not sufficiently large to perform this analysis on all samples.

On the other hand, this study has some noteworthy strengths. Nude mice have been shown to be good models for cancer studies. Mice also develop cancer like humans do. The use of older mice allowed for a good mimic of practical situation: the incidence of cancer increases with age. The supranutritional dose (1 ppm) allowed us to study the long-term effect of supplementation with higher selenium without the toxic effects that could be associated with higher doses. And at the same time, this period was necessary for T cell maturation to occur as well.

SUMMARY AND IMPLICATIONS

Selenium given as sodium selenate at the three doses (0, 0.15, 1 mg selenium/kg diet) does not have negative effect on the body weight for the length of time used in this study. However, long-term selenium deficiency seems to have negative effect on survival of nude mice. Plasma selenium increases with higher dietary intake but does not differ significantly between adequate and supranutritional level. Tumor xenograft significantly decreased body weight of mice regardless selenium supplementation.

Dietary supplementation with sodium selenate for 8 months at the supranutritional level suppresses tumor formation in adult nude mice but promotes tumor growth in young nude mice. Tumor size was greatly affected by the age of the animals and could be likely attributed to the effect of T cell immunity. The T cell (CD4+CD8) maturation increased with the age of the nude mice. After xenograft, dietary selenium status does not affect levels of CD4 and CD8 T cells in adult nude mice in Experiment 1, while high selenium resulted in significant decrease in CD4 T cells in young nude mice in Experiment 2. Taken together, there is an opposing role of excessive selenium on tumor xenograft development in adult and young nude mice carrying differential T cell profiles. These results indicate that the anticarcinogenic activity of selenium could be correlated with the age of the subject. It seems that with age, selenium plays more significant role in tumor growth suppression, whereas in the young mice, selenium seems to promote tumorigenesis. The importance of age considerations and the appropriate dose as well as chemical from of selenium are critical when selenium is used in cancer prevention.

APPENDICES

APPENDIX A. Approval from Institutional Animal Care and Use Committee

UNIVERSITY OF MARYLAND
GRADUATE STUDIES AND RESEARCH
Institutional Animal Care & Use Committee

Jim Dietz
IACUC Chair
jmdietz@umd.edu
Phone: (301)405-6949

February 18, 2009

Dr. Wen-Hsing Cheng
Department: Nutrition & Food Sciences
University of Maryland
whcheng@umd.edu
Phone: (301)405-2940

Dr. Cheng,

This letter is to inform you that on **February 18, 2009**, the members of the Institutional Animal Care & Use Committee (IACUC) reviewed and approved the annual review for the protocol :

Selenium, DNA Repair & Aging

R-07-87

Please note that an approved protocol is valid for three (3) years unless there is a change in the protocol. Thus, this protocol is valid until **February 5, 2011**. Federal laws indicate that protocols must be reviewed yearly. All work extending beyond the approval date of the protocol must be submitted to the IACUC as a new protocol.

Sincerely,

Jim Dietz
Professor, Biology
Chair, IACUC

CC: Doug Powell, Amanda Underwood

APPENDIX B. Composition of modified AIN-93G Torula yeast diet for selenium studies in rodents to provide selenium deficient diet. Dyet # 116053

Ingredients	kcal./gm	grams/kg	kcal./kg
Torula Yeast	3.59	300	1077
Sucrose	4	100	400
Corsntarch	3.6	318.861	1147.8996
Dyetrose	3.8	107	406.6
L-Cystine	4	3.52	14
Cellulose, microcrystalline	0	50	0
Soybean Oil	9	70	630
t-Butylhydroquinone	0	0.014	0
Mineral Mix#216269, Rx	0.34	35	11.9
Vitamin Mix #310025	3.87	10	38.7
L-Methionine	4	2.91	11.64
L-Tryptophan	4	0.16	0.64
Choline Bitartrate	0	2.5	0
		1000	**3738.5996**

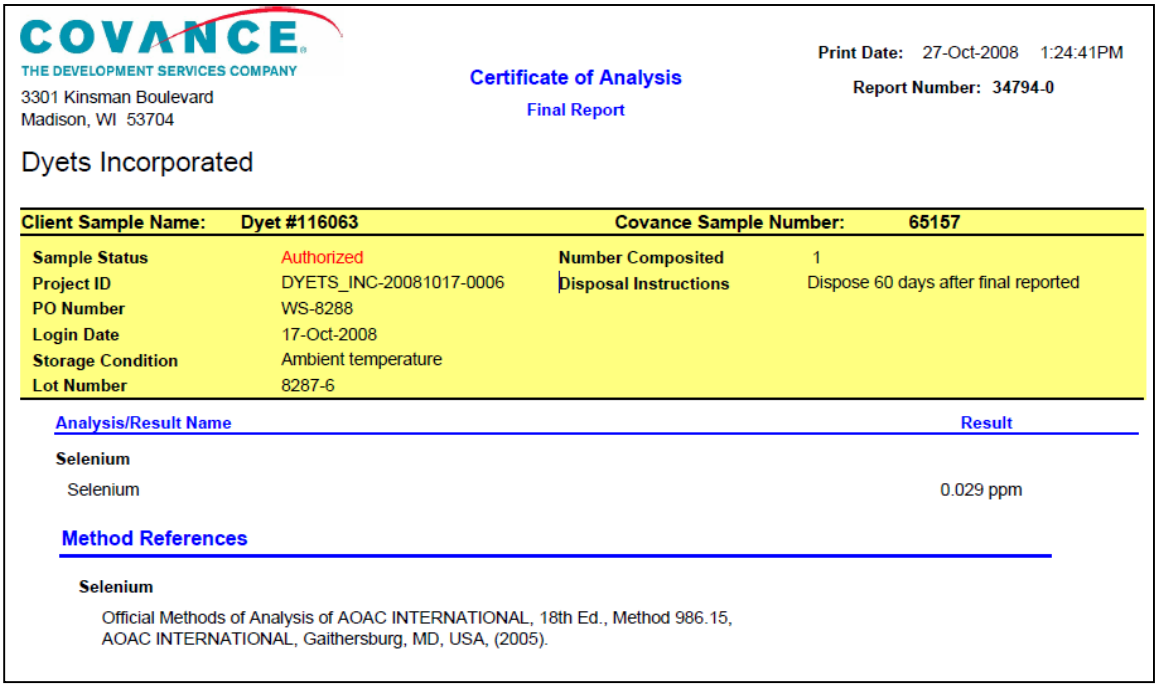
APPENDIX C. Composition of modified AIN-93G Torula yeast diet for selenium studies in rodents to provide 0.15 ppm selenium as sodium selenate. Dyet # 116053

Ingredients	kcal./gm	grams/kg	kcal./kg
Torula Yeast	3.59	300	1077
Sucrose	4	100	400
Corsntarch	3.6	318.861	1147.8996
Dyetrose	3.8	107	406.6
L-Cystine	4	3.52	14
Cellulose, microcrystalline	0	50	0
Soybean Oil	9	70	630
t-Butylhydroquinone	0	0.014	0
Mineral Mix#216269, Rx	0.34	35	11.9
Vitamin Mix #310025	3.87	10	38.7
L-Methionine	4	2.91	11.64
L-Tryptophan	4	0.16	0.64
Sodium Selenate Premix (10 mg/g)	4	0.035	0
Choline Bitartrate	0	2.5	0
		1000	**3738.5996**

APPENDIX D. Composition of modified AIN-93G Torula yeast diet for selenium studies in rodents to provide 1 ppm selenium as sodium selenate. Dyet # 116054

Ingredients	kcal./gm	grams/kg	kcal./kg
Torula Yeast	3.59	300	1077
Sucrose	4	100	400
Corsntarch	3.6	318.861	1147.8996
Dyetrose	3.8	107	406.6
L-Cystine	4	3.52	14
Cellulose, microcrystalline	0	50	0
Soybean Oil	9	70	630
t-Butylhydroquinone	0	0.014	0
Mineral Mix#216269, Rx	0.34	35	11.9
Vitamin Mix #310025	3.87	10	38.7
L-Methionine	4	2.91	11.64
L-Tryptophan	4	0.16	0.64
Sodium Selenate Premix (10 mg/g)	4	0.25	0
Choline Bitartrate	0	2.5	0
		1000	**3738.5996**

APPENDIX E. Certification for selenium analysis in torula yeast basal rodent diet.



COVANCE
THE DEVELOPMENT SERVICES COMPANY
3301 Kinsman Boulevard
Madison, WI 53704

Certificate of Analysis
Final Report

Print Date: 27-Oct-2008 1:24:41PM
Report Number: 34794-0

Dyets Incorporated

Client Sample Name:	Dyet #116063	Covance Sample Number:	65157
Sample Status	Authorized	Number Composited	1
Project ID	DYETS_INC-20081017-0006	Disposal Instructions	Dispose 60 days after final reported
PO Number	WS-8288		
Login Date	17-Oct-2008		
Storage Condition	Ambient temperature		
Lot Number	8287-6		

Analysis/Result Name	Result
Selenium	
Selenium	0.029 ppm

Method References

Selenium

Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Method 986.15, AOAC INTERNATIONAL, Gaithersburg, MD, USA, (2005).

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