NOTE TO USERS

THE EFFECTS OF *ECHINACEA PURPUREA* ON IMMUNE AND HEMOPOIETIC CELL POPULATIONS IN A PLASMACYTOMA MOUSE MODEL

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To François, with love

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Preface

This thesis is prepared in accordance with the guidelines dictated by the Faculty of Graduate Studies and Research at McGill University. All the experimental work in this thesis was done by the candidate Mélanie Brousseau. Dr. Sandra Miller assisted in editorial help and analysis of data. Danielle Delorme and Annie Nguyen assisted in feeding procedures.

Abstract

Pristane, a pure alkane, is known to induce plasma cell dyscrasias in BALB/c mice. The present study aimed to investigate the effect of long-term dietary administration of *Echinacea purpurea* on immune and hemopoietic cell populations of the spleen and bone marrow, of normal and plasmacytoma-bearing mice. The results revealed quantitative cell lineage-specific changes in both the spleen and bone marrow of pristane-exposed animals. Moreover, the consumption of *Echinacea* resulted in cancellation of the pristane-mediated negative effects on NK cells and nucleated erythroid cells. *Echinacea* administration over the long-term resulted in a prolongation of the onset of old age mediated deaths by 120 days, and indeed resulted in a 30% increase in survival at age of 400 days when 55% of normal mice, consuming an untreated diet, were dead. In contrast, however, *Echinacea* did not improve the lifespan of plasmacytoma-bearing animals.

Résumé

L'alcane pristane est une substance responsable du développement de tumeurs des cellules produisant les anticorps chez les souris BALB/c. Ce projet de recherche consistait à étudier les effets à long terme de l'échinacée (*Echinacea purpurea*) sur les cellules immunitaires et hématopoïétiques de la rate et de la möelle osseuse d'animaux normaux ou affectés par un cancer de type plasmacytoma. Les résultats démontrent des changements spécifiques à certaines lignées de cellules au niveau de la rate et de la möelle des animaux traités avec l'alcane pristane. De plus, la consommation d'échinacée par les animaux souffrant d'un cancer cause une normalisation des effets négatifs du pristane sur les cellules NK et les cellules rouges immatures (avec un noyau). L'échinacée retarde aussi l'apparition des décès reliés à l'âge avancé des souris normales et entraîne une augmentation de 30% de la survie à la fin du projet et ce, par rapport aux animaux normaux, ne consommant pas d'échinacée, qui meurent dans une proportion globale de 55%. Finalement, l'échinacée ne joue aucun rôle dans l'amélioration de l'espérance de vie des souris cancéreuses.

Introduction : rationale, hypothesis, and objectives

The research project consisted of analyzing the role of *Echinacea purpurea* on the hemopoietic and immune cells of mice bearing plasmacytoma tumors. The disease was induced by pristane, a pure alkane previously shown to cause cancer in animals (Potter and Boyce, 1962; Anderson and Potter, 1969). It appears that pristane-induced plasmacytoma is an excellent mouse model for plasma cell tumors, human multiple myeloma, and inflammatory arthritis (Hopkins *et al.*, 1985; Gado *et al.,* 2001). Results of the project may potentially be extrapolated to these diseases. The functions of *Echinacea* in plasmacytoma-bearing mice were assessed using techniques currently in use in our laboratory (Sun *et al.*, 1999).

The hypothesis was that *Echinacea purpurea* would boost the non-specific resistance (NK-cell-mediated) in plasmacytoma-bearing animals, as it does in other disease conditions (Currier and Miller, 2001; 2002; Hayashi *et al.,* 2001), such that NK cells would recognize and kill plasmacytoma cells and this, increase the longevity of such *Echinacea*-consuming plasmacytoma-bearing mice vs control diet, plasmacytoma-bearing animals (Kurosawa *et al.*, 1993; 1995; Kawamura *et al.*, 1999).

The objectives of the project were to :

1. Develop a mouse model for plasma cell tumorigenesis with pristane.

   A modified protocol was created in order to increase the rate of development of plasmacytoma, in BALB/c mice, following pristane injections. This new protocol was based on previously used procedures (Potter and Wax, 1983).

2. Compare the hemopoietic and immune (lymphoid) cell populations of pristane-injected mice and saline-injected control animals.

   The effect of pristane on spleen and bone marrow cellular populations was measured by obtaining the absolute numbers of lymphocytes, NK cells, nucleated erythroid cells, granulocytes, granulocyte precursors, and monocytes in both organs. To investigate if pristane had a specific lymphoid cell target, immunocytochemistry was used to quantify the absolute numbers of NK cells in the bone marrow (site of origin of NK cells) and in the spleen (site of most NK cell cytolytic functions).

3. Quantify the effects of *Echinacea*, a previously demonstrated NK cell enhancer and a powerful agent for leukemia treatment, on hemopoietic and lymphoid cells as well as on the lifespan of plasmacytoma-bearing mice.

Review of the literature

Introduction

In the past years, it has been demonstrated that the administration of *Echinacea purpurea* daily had significant effects on the non-specific resistance in mice. Our laboratory had shown that *Echinacea* feeding resulted in a considerable increase in the NK cell population, in normal mice (Sun *et al.*, 1999). A similar conclusion was obtained in mice affected by a virus-derived leukemia (Currier and Miller, 2001). In this model, the innate immune system was significantly boosted by *Echinacea*, resulting in a major increase in splenic NK cell numbers as well as significant lifespan prolongation. The leukemic condition was therefore greatly ameliorated by administration of this herb. The fact that *Echinacea* appeared to have potential applications for the treatment of some tumors gave rise to the present research project.

Plasmacytoma in mice has been achieved using pristane, a well-known ascitogenic agent (Anderson and Potter, 1969). Potter had reported, via an elaborate strategy, that pristane was indeed a chemical useful for developing plasma cell tumors in mice (Potter, 1986; Felix *et al.,* 1997). This fact was followed by the elaboration of a procedure to use pristane as the priming agent in the *in vivo* production of monoclonal antibodies (Silverman, 1987; Jackson *et al.*, 1999; Peterson, 2000). BALB/c mice are susceptible to pristane injections and they react by forming plasmacytomas. Not all BALB/c substrains react the

same way. It was shown that BALB/cAnPt mice are highly susceptible to oil, whereas BALB/cJ are more resistant to plasmacytoma induction (Potter and Wax, 1981; 1983; Potter, 1984; Potter *et al.*, 1985b).

Analysis of pristane-induced tumors in animals had shown that there was a depression in the immune system functions. Pristane was responsible for the decrease in lymphocyte (both T and B) responses as well as in the NK cell immunosurveillance (Freund and Blair, 1982; Wilson and Munson, 1996). It was also demonstrated that priming agents such as pristane and mineral oil have a negative effect on antibody production (B cells) but have a positive effect on graft rejection (T cells) (Kripke and Weiss, 1970). The decrease in immunity caused by pristane administration might contribute greatly to the proliferation of cancer cells (Potter, 1986).

Pristane

Pristane (2, 6, 10, 14-tetramethylpentadecane, norphytane) is an isoprenoid pure alkane frequently used in the induction of plasma cell dyscrasias. It can be obtained from phytane, which is metabolically transformed to pristane by marine copepods, and animal livers concentrate the final product (Potter, 1986). This chemical is able, when injected intraperitoneally, to form an extensively reactive tissue that becomes progressively vascular and invaded by lymphocytes, macrophages and neutrophils (Cancro and Potter, 1976; Anderson

et al., 1985; Potter and Wax, 1986). This tissue is known as oil granuloma. For this tissue to become cancerous, a latent period of 120 days is required (Potter, 1984; 1986). Little is known about the factors affecting susceptibility to pristane carcinogenesis. It was suggested that susceptibility was genetically defined. Pristane was proven to be mutagenic to B cells and fibroblasts *in vitro* and the mutations were probably caused by defective DNA repair mechanisms (Beecham, 1991; Felix *et al.*, 1997; 1999; Silva *et al.*, 1997). Pristane is most likely inert and it does not attack DNA directly. Also, plasmacytomas are related to the presence of non-random chromosomal translocations on the chromosome 15 (*myc* locus) as well as on the immunoglobulin gene chromosomes 6 (kappa light chains) and 12 (heavy chains) (Ohno *et al.*, 1979; 1984; 1999; Potter *et al.*, 1987; Weissinger *et al.*, 1990; Kovalchuk *et al.*, 2000). Once the *myc* gene is deregulated by over-promotion or decreased repression (common effect is the constitutive expression of the *myc* gene), loss of mitotic control and inhibition of the maturation-elimination process occur and lead to cancer promotion (Potter, 1984). Yet unknown secondary genetic lesions seem to be required to complete plasmacytomagenesis (Sugiyama *et al.*, 1995; Mai and Wiener, 2002). Since most strains of mice are totally resistant to pristane, it is assumed that some sensitive genes are segregating in the BALB/c strain (and its substrains), which make those animals more prone to plasma cell cancer (Mock *et al.*, 1993; 1997; Zhang *et al.*, 1998). A possible role for retroviruses had been postulated but significant evidence is still lacking (Armstrong *et al.*, 1978; Hayward *et al.*, 1981; Payne *et al.*, 1982; Potter *et al.*, 1984; Kumar *et al.*, 1999). Some substrains are

partially resistant to oils, they develop arthritic joints but no evidence of plasmacytoma. The joints are highly invaded by inflammatory cells, and these joints may be inflammed for short to long periods. Pristane-induced arthritis is a disease characterized by its high degree of variability (Potter and Wax, 1981; Hopkins *et al.*, 1985).

Plasmacytoma and associated diseases

Plasmacytomas are malignancies affecting B-lymphocytes as well as Ig-producing plasma cells. They are usually the result of an inductive process such as the injection of pristane in the abdominal cavity. As indicated previously, pristane has the ability to create a reactive tissue characterized by acute and chronic inflammation (Leak *et al.*, 1985). The oil granuloma is composed of inflammatory cells responding to the signals for the clearance of emulsified oil droplets. The immune and other cells, i.e., neutrophils, monocytes-macrophages, lymphocytes, and plasma cells, accumulate in this zone of high inflammation. The phagocytic cells produce oxygen radicals that are likely to damage the surrounding cells. It is presumed that anions, radicals, and peroxides contribute to the sensitivity of plasma cells to genomic translocations (Potter, 1986). This fact is supported by the inhibition of cancer incidence when an anti-inflammatory drug such as indomethacin is given to animals (Potter *et al.*, 1985a; 1997; McDonald and Degrassi, 1993; Potter, 1999; Potter and Kutkat, 1999). As more and more alkane is added and processed, the granuloma expands and angiogenesis occurs. The neovascularization brings additional

reactive immune and related cells, thus increasing the chronic irritation process (Anderson *et al.,* 1985). Inside the granuloma, plasma cells start proliferating to form aggregates of medium (less than 25 cells) or large (more than 50 cells) size (Kovalchuk *et al.*, 2001). Most of these cells are abnormal and have some metastatic characteristics. As more and more foci are developed, some of them fuse to form plasma cell tumors that begin to release free plasma cells into the peritoneal cavity (Hentschel and Kolsch, 1983). Those cells will most likely remain in the abdomen since they exhibit little ability for further invasion. Tumors found in the diseased animals are readily transplantable into similar, pre-pristane primed mice (Ruiz-Bravo *et al.*, 1995). To be diagnosed as having a plasmacytoma cancer, animals must be submitted to paracentesis, and a smear of the intraperitoneal fluid must be analyzed. A smear of 1000 nucleated cells with 10 or more plasma cells that usually appear as normal to slightly abnormal cells is considered as a positive diagnosis (Potter, 1986). Apart from indomethacin and similar anti-inflammatory drugs, no other means of treatment have been investigated.

Plasmacytomagenesis is seen as a good murine model for the human condition called multiple myeloma (Gado *et al.*, 2001). The latter disease is characterized by the proliferation of plasma cells in the bone marrow resulting in multiple lytic lesions throughout the skeleton. The two dyscrasias differ on certain points however. First, plasma cells in murine plasmacytoma are found (and usually stay) inside the peritoneum where they develop rapidly whereas in

myeloma, plasma cells tend to disseminate slowly inside the marrow cavities. Second, plasma cells exhibit a more mature phenotype in multiple myeloma. Third, myelomas are spontaneously occurring cancers, not induced by any known carcinogenic agent, and not related to any chromosomal translocation. However, the plasma cell diseases are similar in the sense that they both involve the proliferation of mature cells, with variable degrees of atypia. It was also shown that both the human and mouse plasma cell dyscrasias have a step-wise evolution, i.e., from granulomas, to foci, to free cell production, to tumors and metastases (Potter, 1986).

Echinacea

Since the aim of this study was to evaluate the possible role of Echinacea purpurea as a new treatment for plasmacytomas, its definition will be elaborated on herein at some length. Echinacea is a plant member of the Compositae family. Echinacea purpurea, Echinacea pallida, and Echinacea angustifolia are the three species of most common medicinal interest. Anecdotal utilizations of those herbs included medication for eye infections, bites, wounds, infectious diseases, and skin conditions (Weiss, 1988; Hill et al., 1996). Echinacea is thought to be useful for infections with viruses, bacteria, protozoa, and fungi (Stimpel et al., 1984; Tragni et al., 1985; Roesler et al., 1991a, b; Lersch et al., 1992; Steinmuller et al., 1993). Other indications include immunity stimulation, inflammation decrease, local anaesthesia, and free radical protection (possible

chemopreventive action) (Stoley et al., 2001; Ernst, 2002). Echinacea has an excellent safety profile and no apparent side effects, apart from rare allergic reactions (Weiss, 1988; Mengs et al., 1991; Ernst, 2002). Echinacea is mainly found in humid regions of United States where it grows wild, although it is now cultivated internationally. The root of this herb (as well as other parts to a lesser extent) is used to extract the main active components: high molecular weight polysaccharides (arabinogalactan, inulin, heteroxylan), essential oils (vanillin, germacrene, humulene, borneol), alkylamides (echinacein), caffeoyl conjugates, isobutylamides, polyacetylene, vitamin C, tannins, and flavonoids (Leuttig et al., 1989; Wagner and Jurcic, 1991; Muller-Jakic et al., 1994; Stoley et al., 2001). The two main active components appear to stimulate NK cells via two different but complementary pathways. Arabinogalactan acts on monocytes-macrophages directly in order to increase the production of cytokines (TNF-alpha, IFN-beta2, IL-1) useful to NK cell development and functions (Leuttig et al., 1989). Further, alkamides inhibit the 5-lipoxygenase and cyclooxygenase enzymes (Wagner et al., 1989; Muller-Jakic et al., 1994), thus inhibiting the production of prostaglandins, which suppress NK cell activity. A decrease in prostaglandins also affects the production of IL-1 and its receptor (increased expression) by the monocyte-macrophage lineage. Finally, Currier and Miller (2001; 2002) have already demonstrated the potential of Echinacea as a powerful anti-leukemia treatment.

Natural Killer cells

Echinacea acts primarily, although indirectly, on natural killer cells. NK cells are part of the non-specific innate resistance system, the first line of defence. Immunosurveillance of virally/bacterially-infected cells as well as tumor cells is their main function. NK cells do not need "priming", i.e., prior exposure to antigen, to accomplish their lytic functions; they are not MHC-restricted but they require the presence of macrophages (via cytokine production) for complete activation (Miller and Poirier, 1988). They will spontaneously bind to and lyse target cells via cell-cell contact. Targets for NK cells do not express the same amount of carbohydrates or MHC-I molecules as normal self-cells do (Cerwenka and Lanier, 2001). Abnormal or infected cells may therefore be recognized easily by these killer cells. Lectin-like receptors on NK cells recognize the presence or absence of sugar moieties whereas MHC molecules of type I are detected by Ly-49 receptors (Biassoni *et al.*, 2001). NK cells exhibit a lymphoid morphology and are usually granular. They are short-lived, circulating for 2 to 4 days prior to being replaced (Miller, 1982). Contrary to T and B lymphocytes, they do not have any specialized receptors (TCR or BCR), they are radioresistant, and they do not have "memory" components (Seaman *et al.*, 1978; Zoller *et al.*, 1982; Miller, 1982). NK cells have Fc receptors on their surfaces that permit them to kill antibody-coated cells in a process called antibody-dependent cell-mediated cytotoxicity (ADCC) (Attallah and Folks, 1979). The killing mechanism of NK cells is a similar process to that of cytotoxic T cells. When NK cells contact the targets, they degranulate and kill the tumor cells with perforins and granzymes

(Velotti *et al.*, 1989). Perforins insert into the target cell membranes, form pores to destabilize the cellular integrity and to ensure granzymes get access to the cell cytoplasm (Liu *et al.*, 1986; Young *et al.*, 1986). Granzymes are serine proteases and they activate the apoptotic pathway (caspase-nuclease pathway). Identified markers of NK cells in rodents include ASGM-1, NK1.1, DX5, Ly-49, Mac-1, and CD2 (Biassoni *et al.*, 2001). NK cells also express the CD3-zeta marker whereas a specific subset of NK cells possess the CD3 marker, a hallmark of T-killer cells, and are therefore named NK T cells. NK cells originate in the bone marrow from the pluripotent stem cells (Haller and Wigzell, 1977; Hackett *et al.*, 1986; Pollack and Rosse, 1987) and they migrate via the bloodstream to reach their predominant site of function, i.e., the spleen (Miller, 1982). This trafficking is unidirectional (Miller, 1982). NK cell ontogenesis is independent of the thymus. Pro-NK cells differentiate into NK 1.1- pre-NK cells and then into NK 1.1+ pre-NK cells, followed by lytic primary NK cells. Those cells will be exported to the periphery (spleen) where they become mature NK cells that will be active once stimulated by appropriate cytokines. Positive regulators of NK cells include IL-1, 2, 5, 6, 7, 12, 13, 15 as well as IFN-alpha, IFN-beta and TNF-alpha (Minato *et al.*, 1980; Djeu *et al.*, 1981; Brunda and Gately, 1994; Puzanov *et al.*, 1996; Condiotti and Nagler, 1998). IL-4 and TGF-beta are inhibitors of NK cells (Robertson, 2002). Upon activation, NK cells produce IL-1, 8, 10, GM-CSF, IFN-gamma, TNF-alpha, and TGF-beta (Gallily *et al.*, 1985; Warren and Ralph, 1986; Kovacs *et al.*, 1988; Verma and Mazumder, 1995; Ye *et al.*, 1995; Kundu and Fulton, 1997). Cytokines produced by NK cells are important regulators of immune

responses to pathogens and cancers. There is a well-defined age and organ distribution of NK cells. NK cells are most plentiful during the reproductive age, and in the spleen (Haller *et al.*, 1978). Overall, natural killer cells play a major surveillance role in the maintenance and protection of organisms against a variety of pathogenic aggressions.

Material and Methods

Animals

One hundred and twenty (120) male BALB/cByJ mice, 5 weeks old, were purchased, staggered in time, from Jackson Laboratories (Bar Harbor, Maine, USA), and comprised the entirety of this study. Upon arrival, the mice were housed three per cage and remained with the same cagemates unless dominance problems appear to affect the comfort of the mice. In such cases, mice were housed individually. Animals were evenly distributed into control and experimental groups. All mice were housed under specifically pathogen-free conditions, in autoclaved micro-isolator cages (7½ inches wide x 11½ inches deep x 5 inches high) with wire bar lids (Allentown Caging, N.J., USA). Animals were maintained in an Animal Care Facility of McGill University with room temperature between 20 ± 2°C and 50 ± 10% relative humidity during a 12-hour day/night cycle. The presence of sentinel mice demonstrated the consistent absence of common rodent pathogens.

Mice were given *ad libitum* autoclaved food pellets and water, and remained unmanipulated for at least 10 days prior to the initiation of the experiment. All manipulations (injections, blood extraction, paracentesis, weighings, feedings) were done under specific pathogen-free conditions.

Pristane injection

Sixty (60) mice were injected with pristane (2, 6, 10, 14-tetramethylpentadecane, 98% purity, Sigma Chemical Co., St-Louis, Mo.) and 60 mice received autoclaved physiological saline (0.9% NaCl; pH 7,4). The injection regimen consisted of 4 injections, one each on days 0, 21, 42, 100, to establish the tumor. The mice were physically restrained, although not immobilized, for injections. Each animal was injected with either pristane (0.75mL) or saline intraperitoneally (i.p.) in the region of the caudal left quadrant of the ventral abdomen, using a 26-gauge 1/2-in needle and a sterile syringe.

Plasmacytoma diagnosis

Blood smears

Blood smears were performed every 14 days, for the first 100 days, i.e., up to and following the final injection at day 100, by nicking the mouse tail with a razor blade, collecting the emerging drop of blood directly onto the test slide and doing the smear immediately. Three smears were prepared for each mouse (pristane-injected and saline-control) and then stained with Wright-Giemsa hematologic differential stain. The microscopic analysis was qualitative, looking primarily at the relative amounts of precursor cells and mature cells. In other words, by "reading" the total number of cells per slide, an assessment of each of the 4 different cell categories (lymphoid, myeloid, erythroid, and monocytoid) was established.

Paracentesis

Paracentesis was performed every 14 days on the animals after their third injection of pristane or saline (day 42) on the basis of the detection of an abnormal blood smear or the appearance of abdominal distention (weight over 40 grams). Mice were manually restricted for the procedure (less than a minute), which included the insertion of a 26-gauge 1/2-in needle attached to a syringe into the left portion of the ventral abdomen. Intraperitoneal fluid was collected by aspiration (volume less than 0.1mL) and a small drop was smeared onto a glass slide. As with blood smears, the slides were stained using Wright-Giemsa. The purpose of this procedure was to record the relative numbers of plasma cells and nucleated cells per smear in order to assess the degree of disease state, i.e., plasmacytoma. A total of 10 plasma cells per 1000 nucleated cells per smear of peritoneal fluid was indicative of frank (fulminating) plasmacytoma. Plasma cells appeared as relatively large cells, round to ovoid, with a heterochromatic nucleus, a basophilic cytoplasm, and a negatively stained perinuclear Golgi apparatus.

Weights

Animals were weighed and clinically evaluated every 14 days (along with the blood smear procedure above). See Appendix Figure 1. The baseline for plasmacytoma development was established as a continuous and stable weight gain (a gain of approximatively 1 gram per week) up to a maximum of 50.0 grams

for total animal weight. Clinical evaluation consisted of assessing subjectively: general appearance, coat texture, posture, level of activity, and joint inflammation. The presence of joint inflammation was determined by the degree of redness and swelling of the ankles of the pristane-injected animals. See Appendix Figure 2.

No significant differences in body weight were noted between pristane-injected mice fed with either diet (regular or *Echinacea*-enriched) and between saline-injected mice fed with either diet (Appendix A: Fig. 16), indicating that *Echinacea* feeding does not mediate changes in body weight. Significant increases in weight were however identified between the pristane-injected and the saline-injected treatment groups. Those differences can be attributed to the major increase with time in ascites fluid and the presence of tumor masses in the peritoneal cavity of plasmacytoma-bearing animals. Morbidity-associated events (dehydration, rough coat, breathing problems, and hunched posture) were rare, even in the pristane-injected populations, even when the mice had developed frank plasmacytomas. Finally, there were no differences in the onset of plasmacytoma ± arthritis, as diagnosed by the methods just described, in the pristane-injected mice consuming *Echinacea* enriched diet and those consuming normal food (Appendix B: Fig. 17).

Echinacea

A commercially available extract of *Echinacea purpurea* root was purchased from Phyto Adrien Gagnon (Sante Naturelle Ltee, La Prairie, Quebec). Via dose-response studies previously done in our laboratory, this agent, in the dose used in the present experiments, has been proven successful as a NK stimulant (Sun *et al.*, 1999; Currier and Miller, 2001; 2002).

Echinacea administration

Control mice were given food pellets and water *ad libitum* for the duration of the project. Experimental mice received a known quantity of ground rodent chow (LabChow, Agribrands, Canada) containing *Echinacea* as well as water *ad libitum*. *Echinacea* feeding started 7 days after the third pristane (or saline) injection, i.e., day 49 of the experiment, and continued until sacrifice. Stock containers of experimental food (1.5kg or 2.25kg) were prepared in advance of feeding times. The food was transferred into small glass jars from which mice can easily feed, and was administered in a 1mg *Echinacea* : 3g ground chow ratio, each mouse consuming 6 grams total food chow per day. The average daily consumption of the herb per mouse was therefore 2 mg, a dose which is NK stimulating yet still well below the maximal dose tested (Weiss, 1988; Mengs *et al.*, 1991; Ernst, 2002) to establish its toxicity. Since this is a long-term study, any minor variations in consumption of food among mice in any one group, or

between the control and experimental groups, would be cancelled or averaged over time.

There are 4 animal treatment groups under investigation in this research project :

1. Pristane-injected mice fed with regular chow (PR)
2. Pristane-injected mice fed with *Echinacea*-treated food (PE)
3. Saline-injected mice fed with regular chow (SR), and
4. Saline-injected mice fed with *Echinacea*-treated food (SE).

Mouse sacrifice

Animals were sacrificed by asphyxiation in a CO_2 chamber based on the diagnosis of frank pristane-derived plasmacytoma. Once diagnosed with plasmacytoma by paracentesis, mice were kept for another 5 to 12 days and then sacrificed. However, pristane-injected mice, from which a positive plasmacytoma diagnosis was not established via a peritoneal fluid smear, i.e., some plasma cells were present in the abdominal cavity but their absolute numbers were insufficient to diagnose plasma cell tumors, were allowed to gain weight up to 50.0 grams, at which they were killed and their organs taken for subsequent assessment of disease. Therefore, animal sacrifice was performed after 1) establishment of frank disease, or 2) where plasmacytoma was presumed to cause the weight increase (Appendix Fig. 1). Each pristane-injected mouse was killed with its age-matched saline-injected mate, both animals having been fed the same diet.

Survival studies were conducted to investigate the role of *Echinacea purpurea* (vs normal chow) in the long-term survival of mice bearing frank plasmacytoma. Once a positive diagnosis had been made, some mice were isolated and left untouched, except for the routine evaluation procedure, until natural death occurred. The causes of death were assumed to be plasmacytoma and associated solid tumors, arthritis, lymphatics obstruction, intestinal adhesion and blockage, as well as diaphragm immobilization (Potter and Wax, 1983; Potter, 1986). Times/dates of death were graphed for both *Echinacea purpurea*-

treated and control tumor-bearing mice. If the animal condition was such that we could predict its death in the following 12 hours, the mouse was sacrificed by CO_2 inhalation. Animals dying without a positive paracentesis result were also recorded in treated and control groups as being part of the survival studies.

Preparation of spleen and bone marrow cell suspensions

Dissection was performed on sacrificed mice and organs were removed for further investigation (spleen, femurs, and ankles). Organs were placed in ice-cold pH 7.4 Minimal Essential Medium (MEM) containing 10% Millipore-filtered Fetal Bovine Serum (FBS) (both products from Gibco Invitrogen Corporation, Burlington, Ontario). Spleens were gently crushed through a stainless steel screen mesh using a pestle and MEM. Femurs were cleaned from all external tissues, i.e., muscle, connective tissue, etc., with gauze. This was done to avoid contamination of bone marrow, upon its expulsion from the bone. The femurs were then trimmed at both ends to provide access to the bone marrow cavity, which was flushed with a 23-gauge 1-in needle attached to a 1cc syringe filled with MEM. Cells from spleen and marrow were expelled into a 15 mL centrifuge tube containing MEM and 10% FBS and the cells were pipetted up and down to form single cell suspensions. Underlying the suspensions with Newborn Calf Serum (NCS, denser that MEM; Gibco Invitrogen Corporation, Burlington, Ontario) for 7 minutes provided a gradient density through which non-cellular debris settled. The resulting supernatant, i.e., MEM, 10% FBS, and free cells, was centrifuged at 1100 rpm for 7 minutes at 4 degrees Celsius to form a dense

cellular pellet. Cells were resuspended in a fixed MEM volume (2mL for spleen cells and 0.5mL for marrow cells) before calculating the Total numbers of Nucleated Cells, i.e., TNC.

Total Nucleated Cell Count

The total numbers of nucleated cells were obtained via the hemacytometer and the trypan blue exclusion technique (0.04% in PBS pH 7.2, Gibco Invitrogen Corporation, Burlington, Ontario) assessed viability of such cells. The nucleated cells were counted and the suspensions were diluted with MEM to get a 40×10^6 cells/mL concentration, to facilitate subsequent immuno-labelling of natural killer (NK) cells.

Viability Test

The standard viability test (above) was performed with trypan blue and hemacytometer. Viable cells were identified as cells unable to take up trypan blue whereas non-viable cells passively absorb the dye. This visual distinction permitted calculation of the overall viability as being the number of viable cells divided by the total number of nucleated cells (viable and non-viable), i.e., percentage of living cells after organ extraction.

Immunoperoxidase labelling of Natural Killer cells

All mature and maturing natural killer (NK) cells possess the ASGM-1 surface molecule. Although T blasts also bear the ASGM-1 molecule, those cells can easily be differentiated from NK cells by commonly used hematologic staining protocols along with known distinct morphology of T blasts vs NK cells. In order to assess the presence of NK cells in the bone marrow and spleen suspensions, NK cells were labelled using an indirect assay.

In the indirect assay for immunolabelling, one hundred (100) μL of each cell suspension (standardized at 40 x 10^6 cells/mL) were incubated with the primary antibody in a 96 round bottom multi-well plate (Sarstedt Inc., Newton, USA) for 30 minutes on ice. The primary antibody was the rabbit anti-asialo GM1 (Wako Pure Chemicals, Texas, USA) at a dilution of 1:40 with MEM. The cells (spleen or bone marrow) were centrifuged at 1100 rpm for 7 minutes followed by two consecutive washes with MEM, at the same centrifugation speed and time. The second incubation was performed with the biotinylated anti-rabbit IgG antibody (Sigma Chemicals Co., St-Louis, USA) diluted 1:100 with MEM. The same conditions were applied as with the first antibody incubation. The final pellets were resuspended in cytospotting medium (0.009% NaCl, 0.001% EDTA, and 0.05% bovine serum albumin in distilled water, pH 7.4). Cytospot preparations were made on slides and the centrifuged cells were dried quickly to preserve the original morphology.

All slides were fixed in pure methanol (Fisher Scientific, Ontario, Canada) for 30 minutes and then slowly rehydrated by a graded series of methanol and Phosphate Bovine Saline (PBS) (Gibco Invitrogen, Burlington, Ontario) solution of pH 7.2. The slides were allowed to stand in pure PBS for 5 minutes before being incubated for 10 minutes with a solution of 3.0% peroxide (Fisher Scientific, Ontario, Canada). This step was important to block the endogenous peroxidase activity of granulocytes and phagocytic cells. The slides were washed again in PBS for 10 minutes before being subjected to the immunoperoxidase labelling. The cytospots were incubated with 100μL of ABC solution, i.e., avidin-biotin horseradish peroxidase complex (Dako Diagnostics, Ontario, Canada), for 45 minutes in 100% humidity chambers. The residual chemicals were removed by a 10-minute PBS wash. The slides were immersed in a 3-3'-diaminobenzidine (DAB) solution (0.125g DAB in 250mL of PBS pH 7.6, 66.6μL of 30% hydrogen peroxide) for 13 minutes with two subsequent washes of 10 minutes each in PBS pH 7.2. The slides were finally blotted dry using bibulous paper before counter-staining with Wright-Giemsa stain.

Differential analysis of hemopoietic and lymphoid cells

The Wright-Giemsa differential staining permitted the relative categorization of hemopoietic and lymphoid cells. Along with the immunoperoxidase labelling, the stain allowed visualization of NK cells as being small to medium lymphoid cells, surrounded by a pale brown rim of marked ASGM-1 surface molecules. The other cells were identified by long established techniques (Miller and Osmond, 1973; 1974), still in continuous use in our laboratory.

The absolute numbers of each cell population (lymphoid, myeloid, erythroid, monocytoid) were obtained by differential counts on 1000 (spleen) or on 2000 (bone marrow) cells/cytospot/tissue/treated or control mouse and then multiplying those percentages by the Total Nucleated Cell numbers previously obtained with the hemacytometer.

Statistical analysis

Means and standard errors of each experiment were calculated and compared via a 2-tailed Student's t-test. Survival results were analysed by Kaplan-Meier survival analysis statistics and Mann-Whitney U-test. Differences were considered significant with p values less than 0.05 (5%).

Results

Effect of pristane on hemopoietic and lymphoid cell populations (SR vs PR)

The results indicate significant and contrasting influences of pristane on the various hemopoietic and lymphoid cells in BALBc/ByJ mice, balancing to produce no differences in the total cellularity of either the spleen or the bone marrow of pristane-injected vs saline-injected animals (Figs. 1, and 8, respectively). Pristane injection produced a significant decrease in non-NK lymphocytes of both organs (Figs. 2, and 9, respectively) as well as in NK cells in the spleen and bone marrow (Figs. 3, and 10, respectively). The absolute numbers of nucleated erythroid cells were significantly elevated in the spleen (Fig. 4) but significantly decreased in the bone marrow of pristane-injected animals (Fig. 11). By contrast, there was a very significant increase in granulocytes and their precursors in both organs of pristane-injected mice vs saline-injected (control) animals (Figs. 5 and 6; 12 and 13). The absolute number of monocytes was increased 2,5-fold by pristane in the spleen (Fig. 7) but unchanged in the bone marrow (Fig. 14). The results presented in Figure 15 demonstrated that pristane-injected mice began dying much sooner in life than did saline-injected mice and moreover, at the conclusion of the experiment (approximatively 395 days of age), control mice still showed at 45% survival rate whereas pristane-injected mice had only 20% of their "starting" numbers still surviving.

Effect of *Echinacea purpurea* in the diet of pristane-injected mice (PR vs PE)

Long-term Echinacea feeding (refer to Material and Methods) was responsible for a variety of changes in the spleen and bone marrow of animals exposed to pristane. The total numbers of nucleated cells of the spleen of both pristane-injected populations (PR vs PE) was not significantly different (Fig. 1). The total nucleated cell number in the bone marrow was significantly increased in those pristane-injected mice that were fed Echinacea (Fig. 8). The pristane-injected animals exposed to the herb had a significantly elevated absolute number of splenic NK cells (Fig. 3), a significantly decreased absolute number of splenic monocytes (Fig.7), whereas all other cellular components of the spleen remained unchanged (Figs. 2, 4-6). In the bone marrow, the total numbers of lymphoid cells, as well as monocytes were not altered by the presence of Echinacea in the pristane-injected mice (Figs. 9, 14). However, Echinacea feeding resulted in a significant increase in all other cell types (Figs. 10-13). No significant differences were noted in the survival of both plasmacytoma-bearing mice consuming normal diet and those consuming an Echinacea-treated diet. On the other hand, when all mice were almost a year old, the group of plasmacytoma-bearing mice which had not consumed Echinacea in their diet had more than twice as many survivors as did the group of plasmacytoma-bearing mice which had consumed Echinacea (Fig. 15).

Effect of *Echinacea purpurea* in the diet of pristane-injected mice: a comparison with saline-injected mice fed regular food (SR vs PE)

The splenic total cellularity of pristane-injected mice fed a diet containing Echinacea was significantly decreased compared to the control (saline-injected mice fed untreated chow) numbers (Fig. 1). However, in the bone marrow, the absolute numbers of nucleated cells were elevated in the pristane-injected animals fed Echinacea (Fig. 8) vs completely normal animals on a normal diet. In the spleen of pristane-injected mice (PE), the numbers of NK cells (Fig. 3), nucleated erythroid cells (Fig. 4), and monocytes (Fig. 7) were not significantly different from the control (SR) levels. The non-NK lymphocytes (presumptive T and B cells) were significantly decreased and the mature and immature granulocytic cells were elevated in the spleen (Figs. 2, 5, and 6) and bone marrow (Figs. 9, 12, and 13) of pristane-injected mice fed Echinacea vs vehicle-injected mice fed regular chow. The numbers of nucleated erythroid cells and NK cells in the bone marrow of pristane-injected mice (PE) were not different from the control (SR) numbers (Figs. 10, 11). Finally, the monocytes were significantly boosted by dietary Echinacea in the bone marrow of pristane-injected mice vs saline-injected animals fed with untreated chow (Fig. 14).

Effect of *Echinacea purpurea* in the diet of normal mice (SR vs SE)

Normal mice were greatly affected by their long-term diet containing *Echinacea* (refer to Material and Methods). The total cellularity of the spleen was significantly reduced in mice fed *Echinacea* whereas the cellularity of the bone marrow remained at normal levels (Figs. 1, and 8, respectively). Results in Figures 2-4 and 7 demonstrated that specific spleen populations (lymphocytes, NK cells, erythroid cells, and monocytes) were very sensitive to dietary *Echinacea*. Those non-NK lymphoid, nucleated erythroid, and monocytoid populations were significantly decreased following their exposure to the herb. In fact, only splenic NK cells (Fig. 3) were significantly increased. Moreover, in the spleen, presence of *Echinacea* did not result in changes in absolute numbers of mature and immature granulocytes (Figs. 5, 6). Total lymphoid cells, predominantly B cells (Fig. 9), granulocytic cells (Figs. 12, 13), and monocytes (Fig. 14) in the bone marrow remained at virtually control levels during exposure to *Echinacea*. NK cells (Fig. 10) and nucleated erythroid cells (Fig. 11) were specifically and significantly increased in the bone marrow by dietary *Echinacea*. Figure 15 revealed that mice consuming *Echinacea* throughout their youth and adulthood showed a later onset of mortality, i.e., greater survival throughout middle age, and the number of mice that reached one year of age was almost twice that of mice consuming a normal diet.

Figure 1. Total number (mean ± SE) of nucleated cells in the spleen of the 4 animal treatment groups.

SR : Saline-injected mice fed with regular diet
PR : Pristane-injected mice fed with regular diet
SE : Saline-injected mice fed with *Echinacea*-treated diet
PE : Pristane-injected mice fed with *Echinacea*-treated diet
Echinacea-treated diet was given to animals starting day 49 until sacrifice (refer to Material and Methods).
( ) = number of mice

SR vs PR p = 0,4
PR vs PE p = 0,1
SR vs PE p = 0,006
SR vs SE p = 0,002

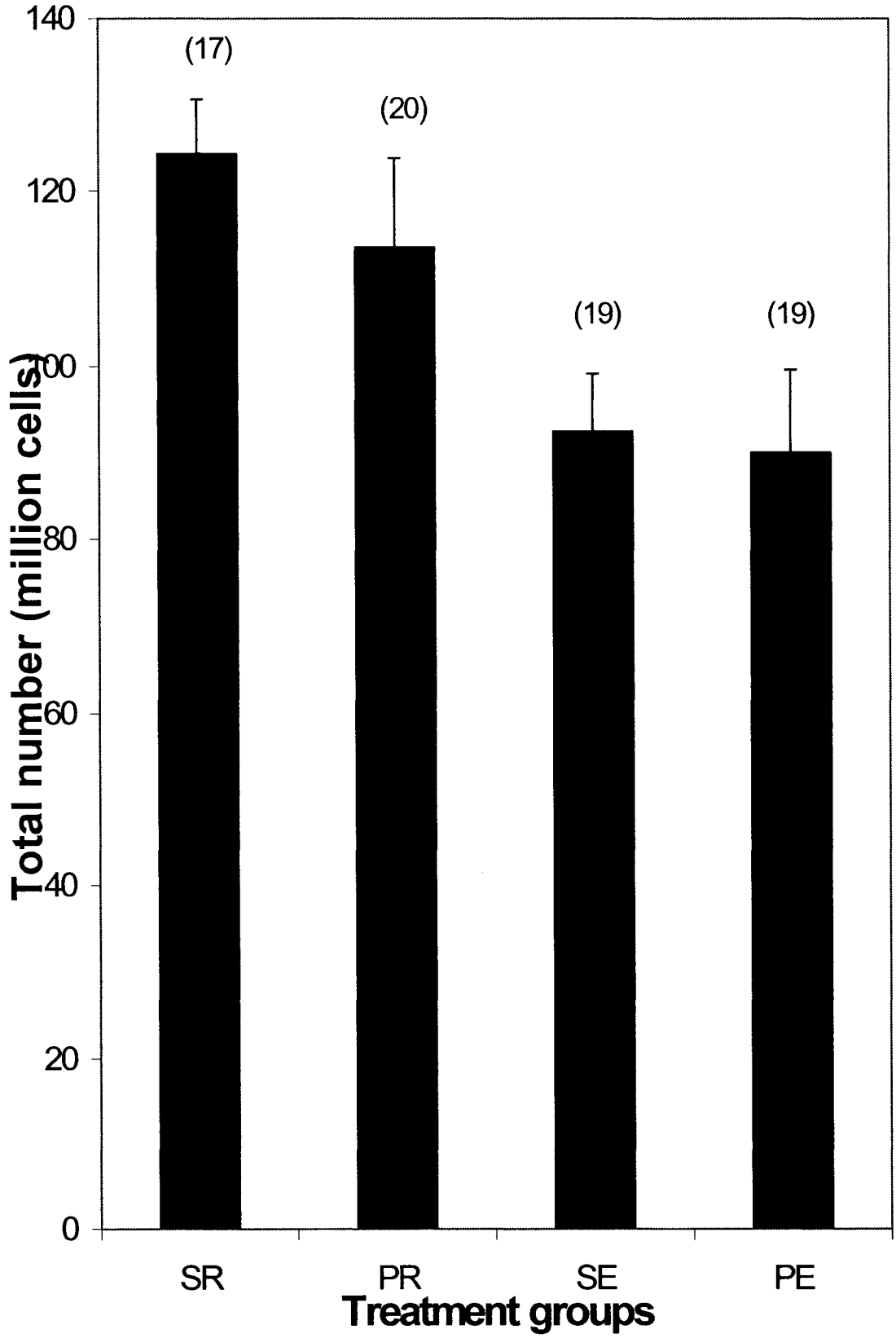

140
120
100
80
60
40
20
0
(17)
(20)
(19)
(19)
Total number (million cells)
SR
PR
SE
PE
Treatment groups

Figure 2. Total number (mean ± SE) of lymphocytes (small, medium, and large lymphocytes) in the spleen of the 4 animal treatment groups.

SR : Saline-injected mice fed with regular diet
PR : Pristane-Injected mice fed with regular diet
SE : Saline-injected mice fed with *Echinacea*-treated diet
PE : Pristane-injected mice fed with *Echinacea*-treated diet
Echinacea-treated diet was given to animals starting day 49 until sacrifice (refer to Material and Methods).
( ) = number of mice

SR vs PR $p < 0,0001$
PR vs PE $p = 0,09$
SR vs PE $p < 0,0001$
SR vs SE $p = 0,001$

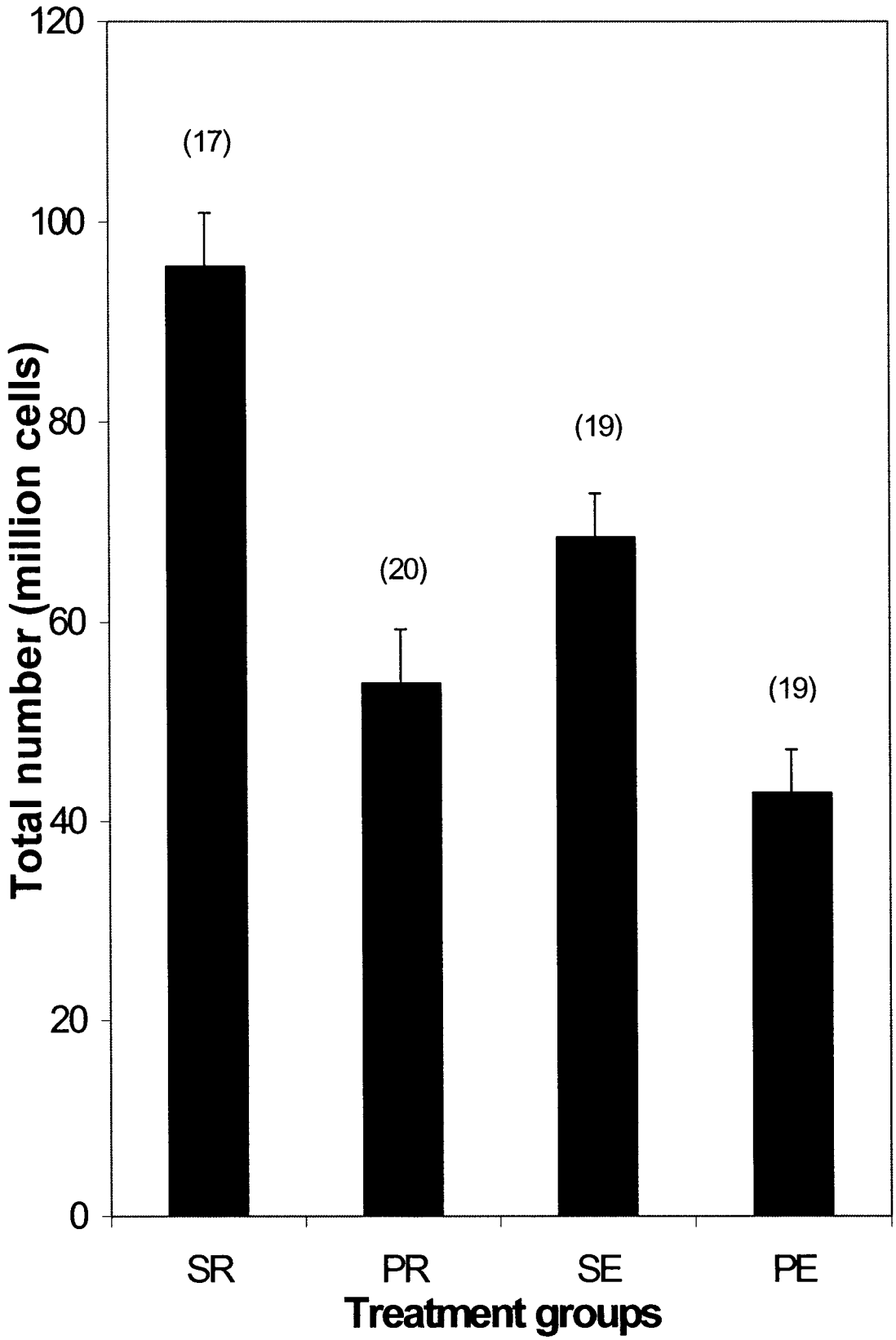

120
100
80
60
40
20
0
Total number (million cells)
(17)
(20)
(19)
(19)
SR
PR
SE
PE
Treatment groups

Figure 3. Total number (mean ± SE) of Natural Killer (ASGM-1 +) cells in the spleen of the 4 animal treatment groups.

SR : Saline-injected mice fed with regular diet
PR : Pristane-injected mice fed with regular diet
SE : Saline-injected mice fed with *Echinacea*-treated diet
PE : Pristane-injected mice fed with *Echinacea*-treated diet
Echinacea-treated diet was given to animals starting day 49 until sacrifice (refer to Material and Methods).
( ) = number of mice

SR vs PR p = 0,003
PR vs PE p = 0,005
SR vs PE p = 0,2
SR vs SE p = 0,01

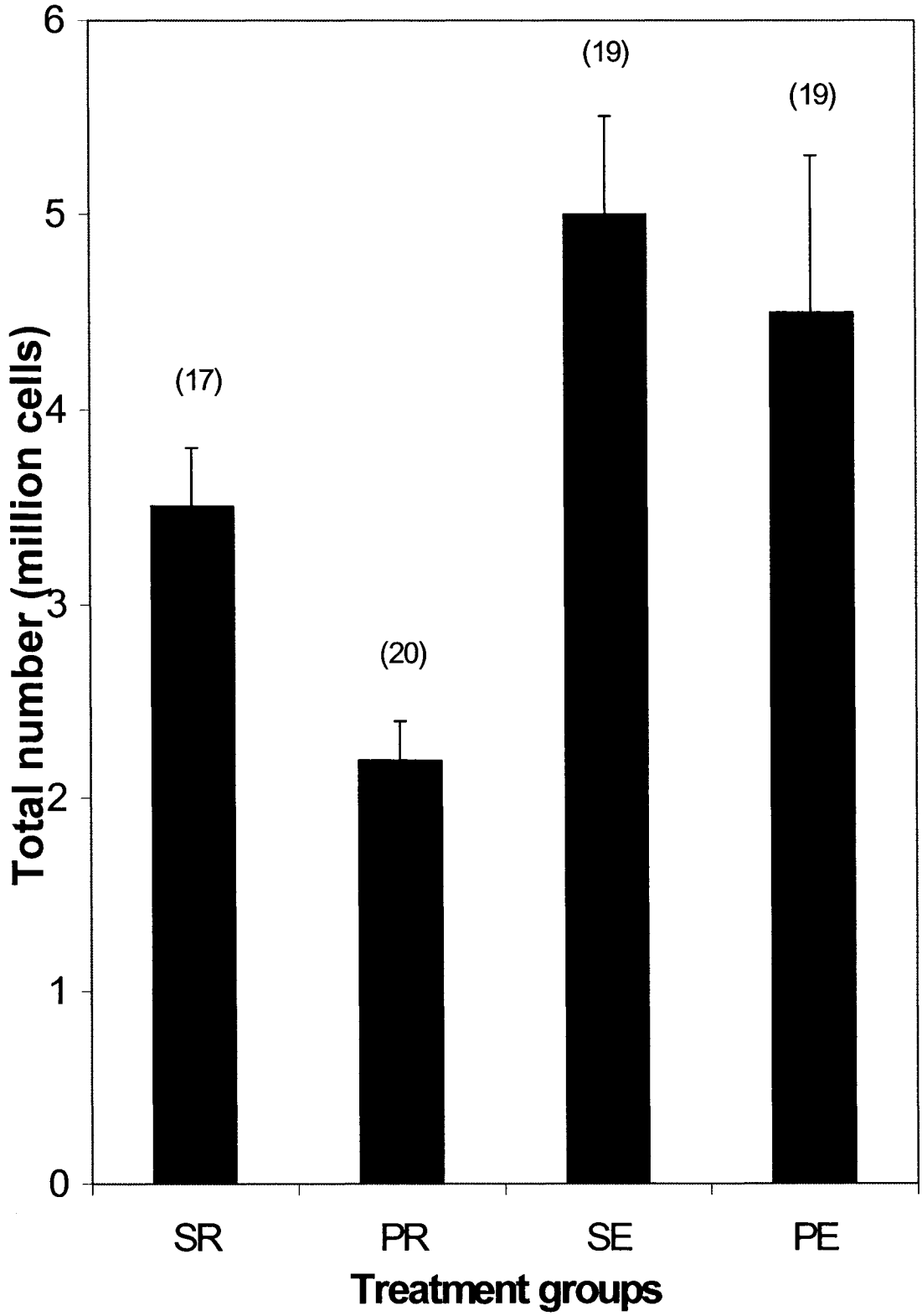

6
5
4
3
2
1
0
Total number (million cells)
(17)
(20)
(19)
(19)
SR
PR
SE
PE
Treatment groups

Figure 4. Total number (mean ± SE) of nucleated erythroid cells in the spleen of the 4 animal treatment groups.

SR : Saline-injected mice fed with regular diet
PR : Pristane-injected mice fed with regular diet
SE : Saline-injected mice fed with *Echinacea*-treated diet
PE : Pristane-injected mice fed with *Echinacea*-treated diet
Echinacea-treated diet was given to animals starting day 49 until sacrifice (refer to Material and Methods).
( ) = number of mice

SR vs PR p = 0,05
PR vs PE p = 0,08
SR vs PE p = 0,7
SR vs SE p = 0,007

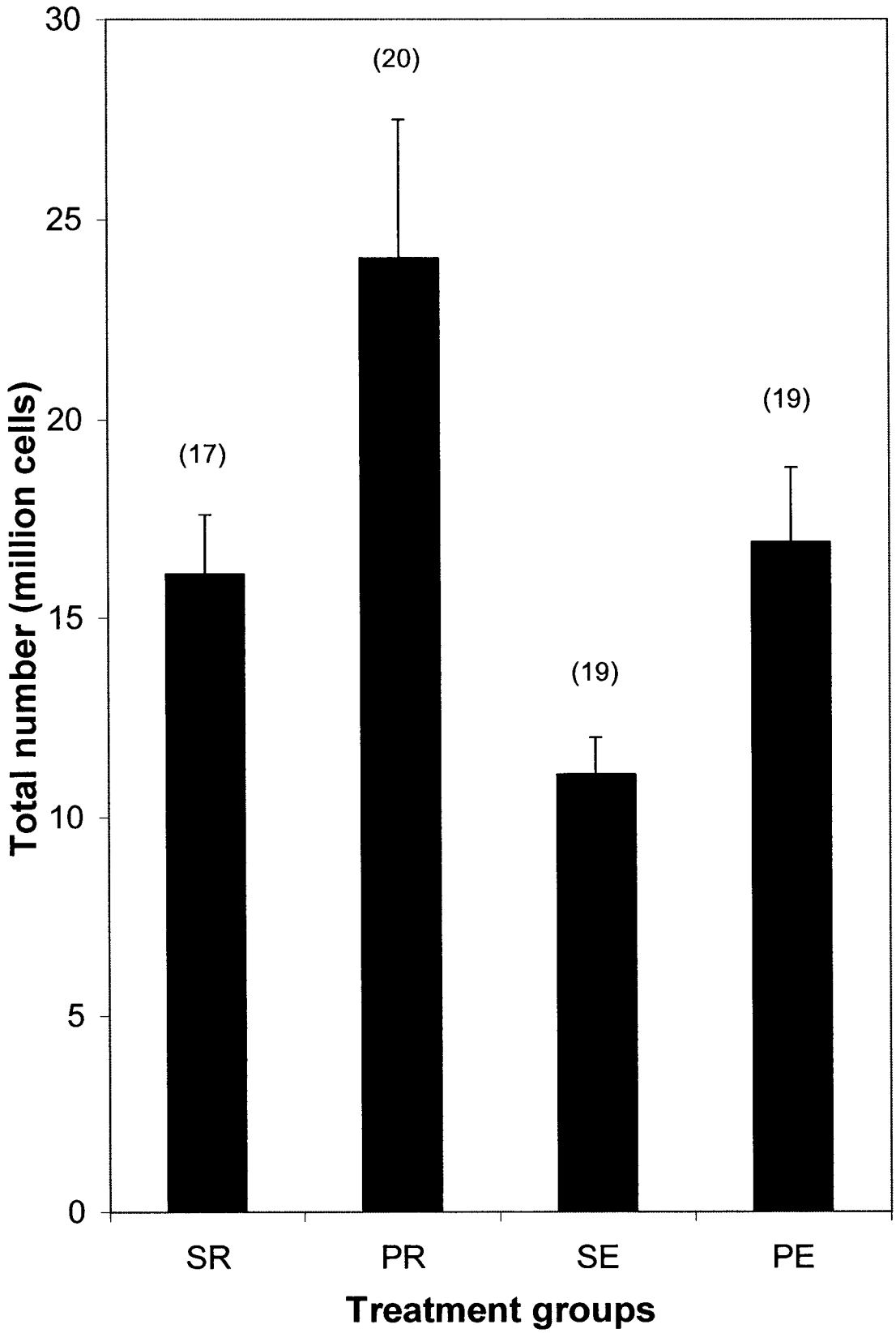

30
25
20
15
10
5
0
Total number (million cells)
(17)
(20)
(19)
(19)
SR
PR
SE
PE
Treatment groups

Figure 5. Total number (mean ± SE) of mature granulocytes in the spleen of the 4 animal treatment groups.

SR : Saline-injected mice fed with regular diet
PR : Pristane-injected mice fed with regular diet
SE : Saline-injected mice fed with *Echinacea*-treated diet
PE : Pristane-injected mice fed with *Echinacea*-treated diet
Echinacea-treated diet was given to animals starting day 49 until sacrifice (refer to Material and Methods).
( ) = number of mice

SR vs PR $p < 0,0001$
PR vs PE $p = 0,4$
SR vs PE $p < 0,0001$
SR vs SE $p = 0,4$

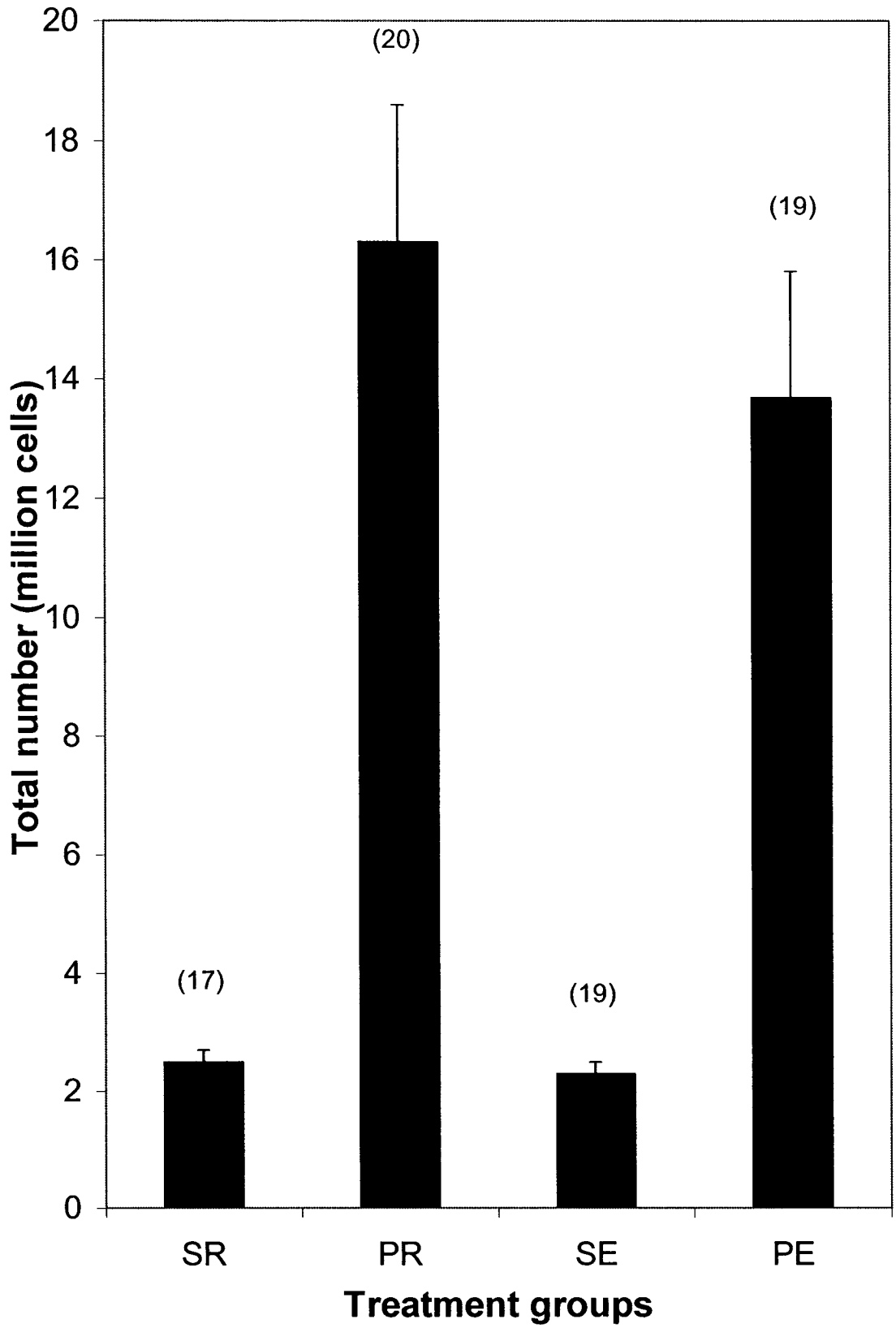

20
18
16
14
12
10
8
6
4
2
0
Total number (million cells)
(20)
(19)
(17)
(19)
SR
PR
SE
PE
Treatment groups

Figure 6. Total number (mean ± SE) of immature granulocytes in the spleen of the 4 animal treatment groups.

SR : Saline-injected mice fed with regular diet
PR : Pristane-injected mice fed with regular diet
SE : Saline-injected mice fed with *Echinacea*-treated diet
PE : Pristane-injected mice fed with *Echinacea*-treated diet
Echinacea-treated diet was given to animals starting day 49 until sacrifice (refer to Material and Methods).
( ) = number of mice

SR vs PR $p < 0,0001$
PR vs PE $p = 0,09$
SR vs PE $p = 0,003$
SR vs SE $p = 0,4$

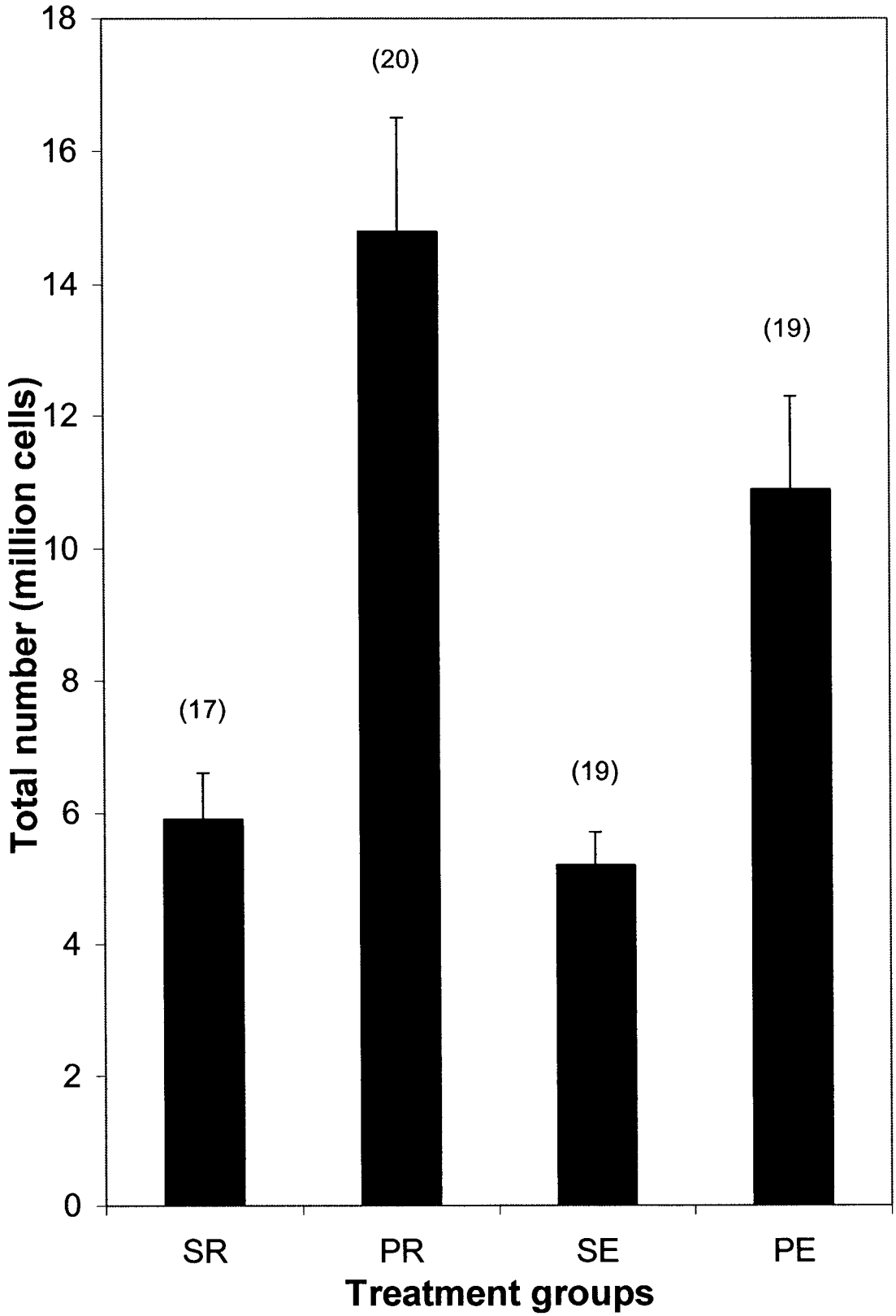

18
16
14
12
10
8
6
4
2
0
Total number (million cells)
(17)
(20)
(19)
(19)
SR
PR
SE
PE
Treatment groups

Figure 7. Total number (mean ± SE) of monocytes in the spleen of the 4 animal treatment groups.

SR : Saline-injected mice fed with regular diet
PR : Pristane-injected mice fed with regular diet
SE : Saline-injected mice fed with *Echinacea*-treated diet
PE : Pristane-injected mice fed with *Echinacea*-treated diet
Echinacea-treated diet was given to animals starting day 49 until sacrifice (refer to Material and Methods).
( ) = number of mice

SR vs PR p = 0,003
PR vs PE p = 0,01
SR vs PE p = 0,2
SR vs SE p = 0,004

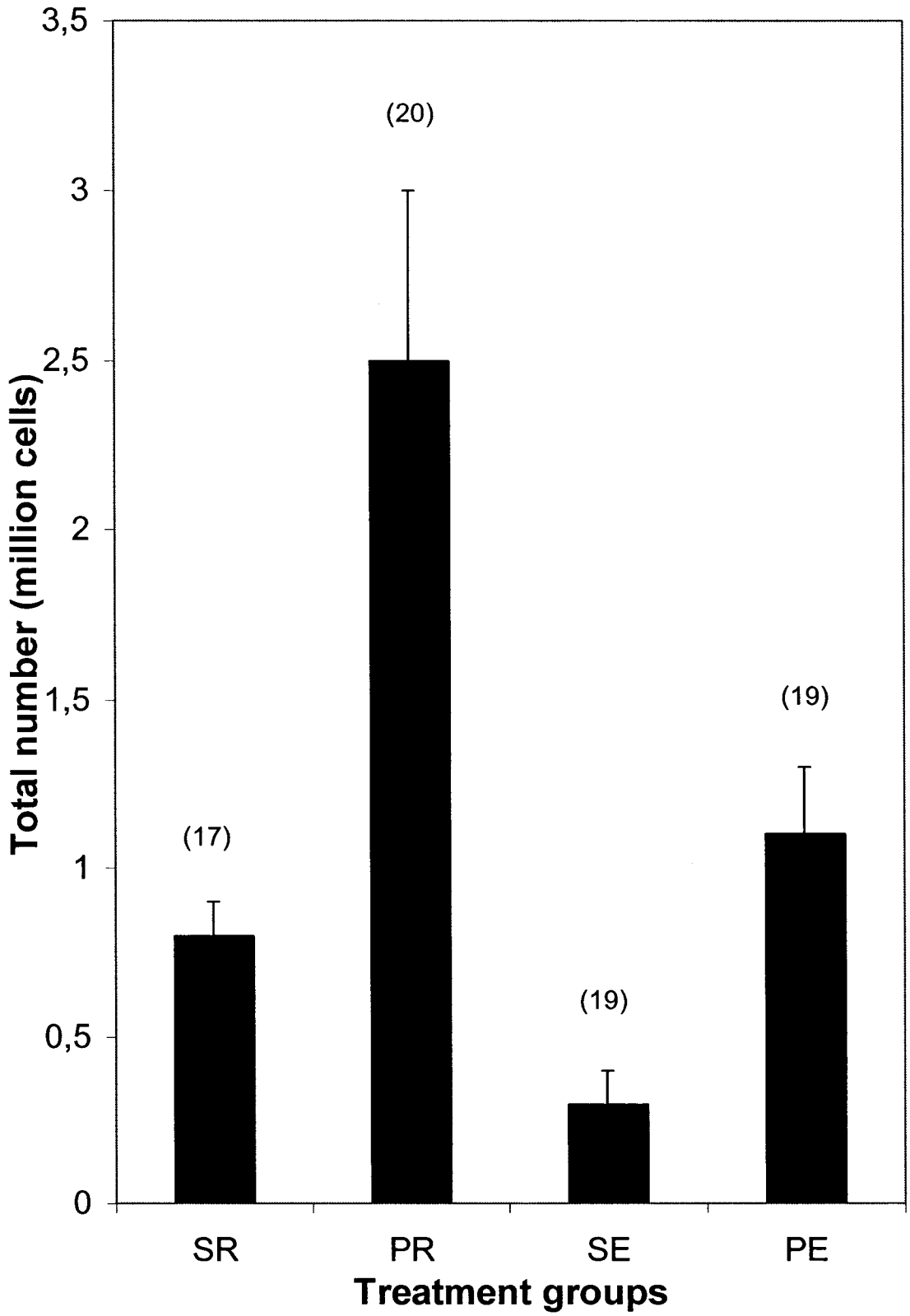

3,5
3
2,5
2
1,5
1
0,5
0
Total number (million cells)
(17)
(20)
(19)
(19)
SR
PR
SE
PE
Treatment groups

Figure 8. Total number (mean ± SE) of nucleated cells in the bone marrow (2 femurs) of the 4 animal treatment groups.

SR : Saline-injected mice fed with regular diet
PR : Pristane-injected mice fed with regular diet
SE : Saline-injected mice fed with *Echinacea*-treated diet
PE : Pristane-injected mice fed with *Echinacea*-treated diet
Echinacea-treated diet was given to animals starting day 49 until sacrifice (refer to Material and Methods).
( ) = number of mice

SR vs PR p = 0,2
PR vs PE p = 0,001
SR vs PE p = 0,0003
SR vs SE p = 0,1

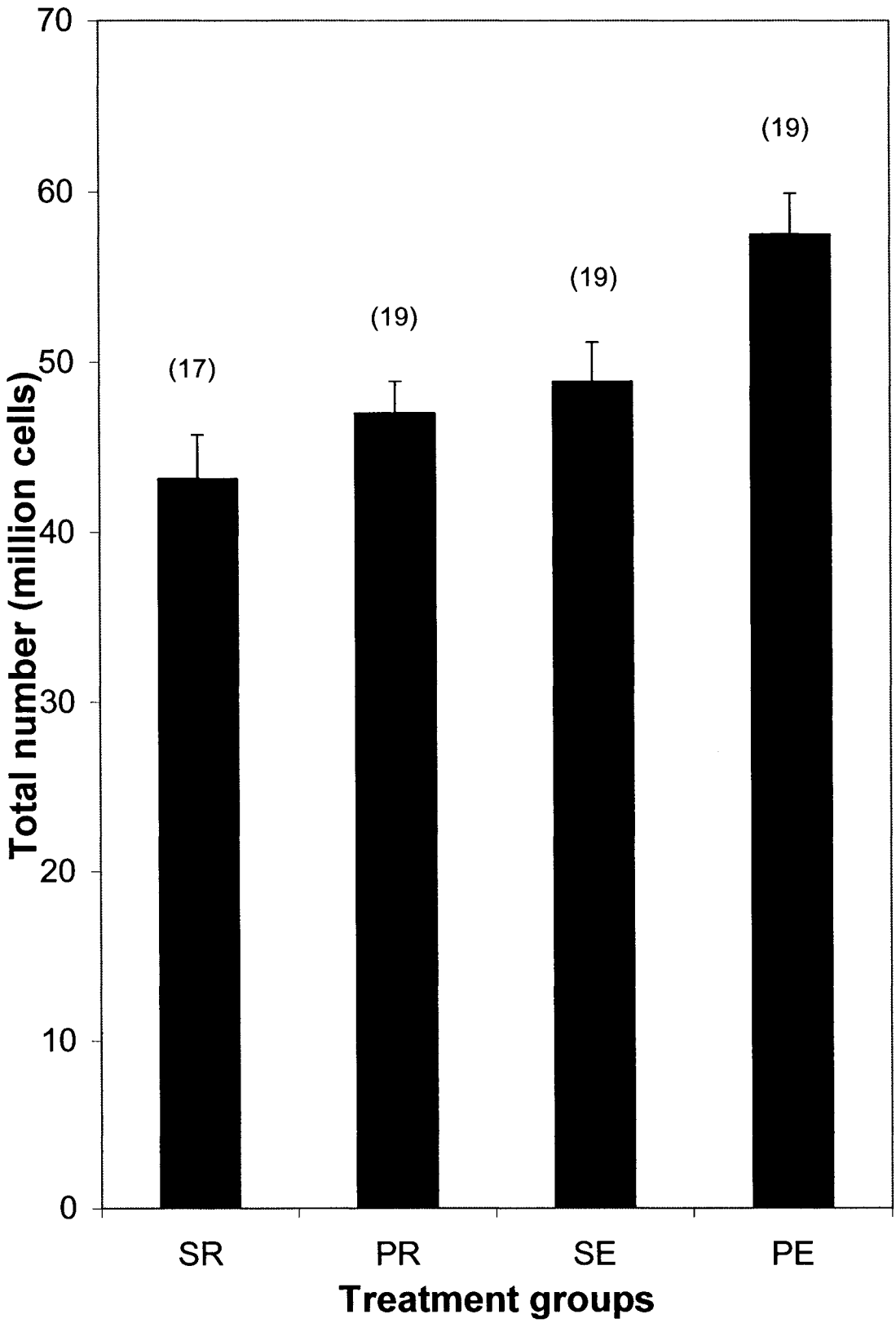

70
60
50
40
30
20
10
0
Total number (million cells)
(17)
(19)
(19)
(19)
SR
PR
SE
PE
Treatment groups

Figure 9. Total number (mean ± SE) of lymphocytes (small, medium, and large lymphocytes) in the bone marrow (2 femurs) of the 4 animal treatment groups.

SR : Saline-injected mice fed with regular diet
PR : Pristane-injected mice fed with regular diet
SE : Saline-injected mice fed with *Echinacea*-treated diet
PE : Pristane-injected mice fed with *Echinacea*-treated diet
Echinacea-treated diet was given to animals starting day 49 until sacrifice (refer to Material and Methods).
( ) = number of mice

SR vs PR	$p < 0,0001$
PR vs PE	$p = 1,0$
SR vs PE	$p < 0,0001$
SR vs SE	$p = 0,6$

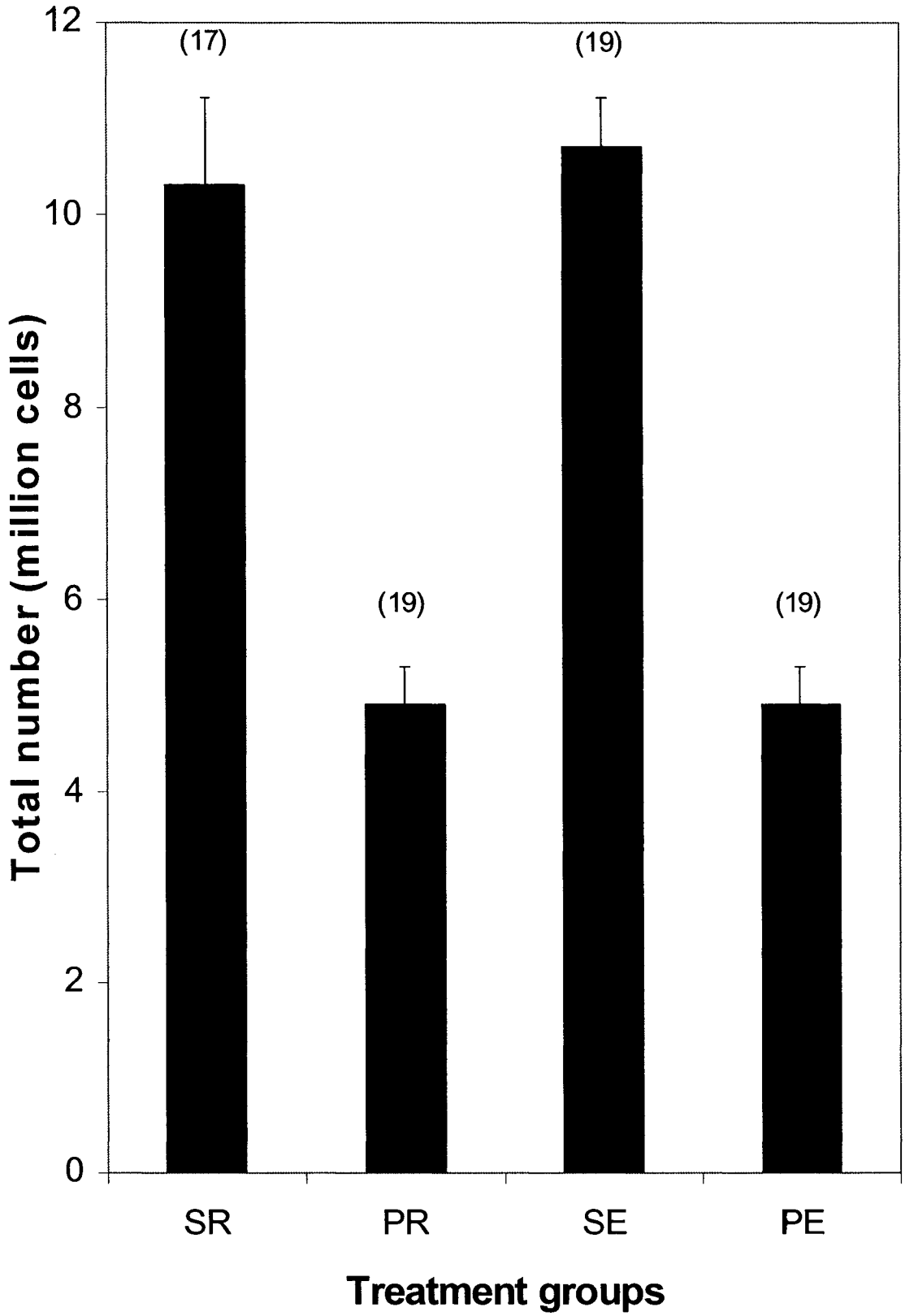

12
10
8
6
4
2
0
(17)
(19)
(19)
(19)
Total number (million cells)
SR
PR
SE
PE
Treatment groups

Figure 10. Total number (mean ± SE) of Natural Killer (ASGM-1 +) cells in the bone marrow (2 femurs) of the 4 animal treatment groups.

SR : Saline-injected mice fed with regular diet
PR : Pristane-injected mice fed with regular diet
SE : Saline-injected mice fed with *Echinacea*-treated diet
PE : Pristane-injected mice fed with *Echinacea*-treated diet
Echinacea-treated diet was given to animals starting day 49 until sacrifice (refer to Material and Methods).
( ) = number of mice

SR vs PR	$p = 0,008$
PR vs PE	$p < 0,0001$
SR vs PE	$p = 0,4$
SR vs SE	$p = 0,01$

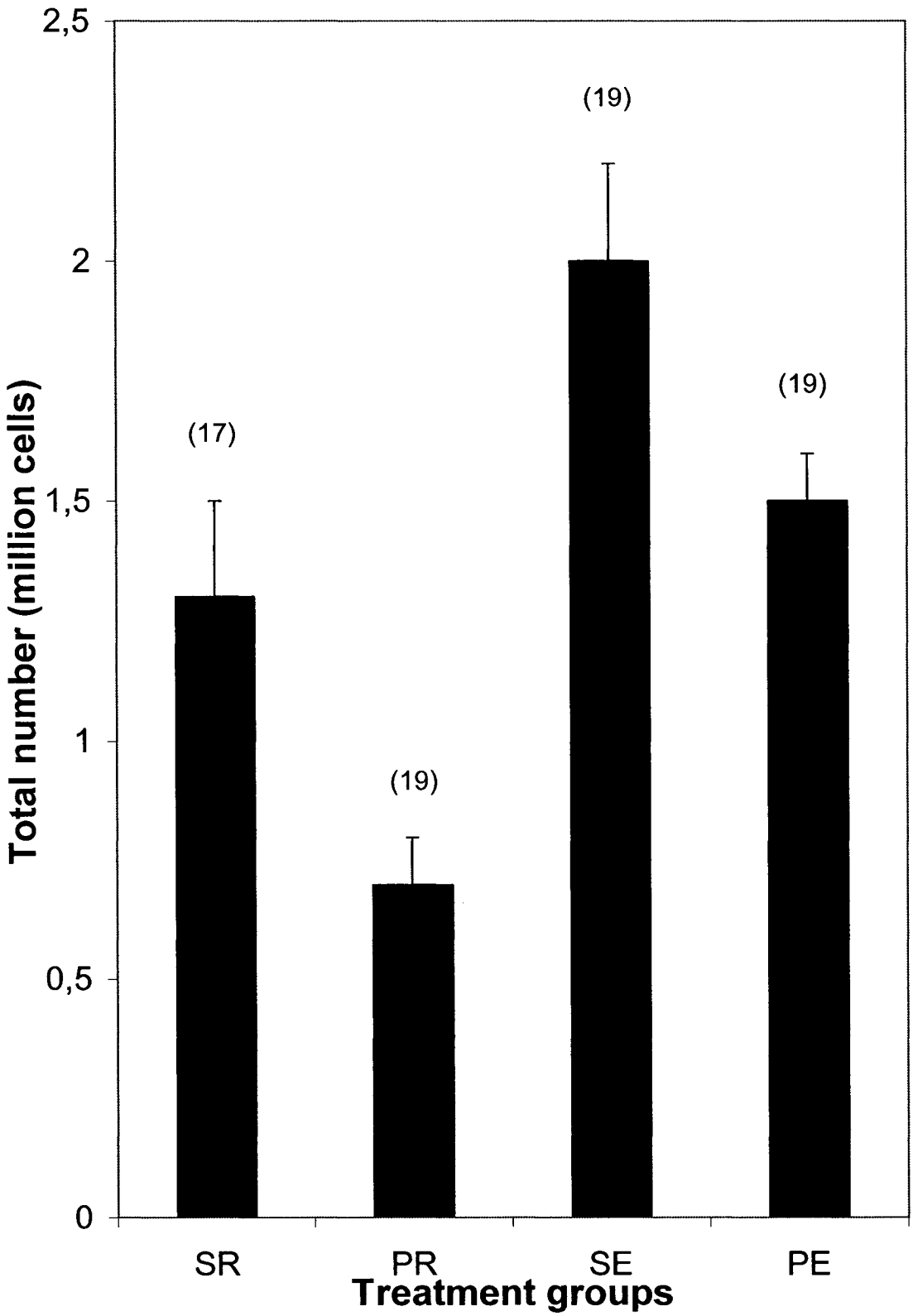

2,5
2
1,5
1
0,5
0
Total number (million cells)
(17)
(19)
(19)
(19)
SR
PR
SE
PE
Treatment groups

Figure 11. Total number (mean ± SE) of nucleated erythroid cells in the bone marrow (2 femurs) of the 4 animal treatment groups.

SR : Saline-injected mice fed with regular diet
PR : Pristane-injected mice fed with regular diet
SE : Saline-injected mice fed with *Echinacea*-treated diet
PE : Pristane-injected mice fed with *Echinacea*-treated diet
Echinacea-treated diet was given to animals starting day 49 until sacrifice (refer to Material and Methods).
( ) = number of mice

SR vs PR	p = 0,0006
PR vs PE	p = 0,02
SR vs PE	p = 0,3
SR vs SE	p = 0,02

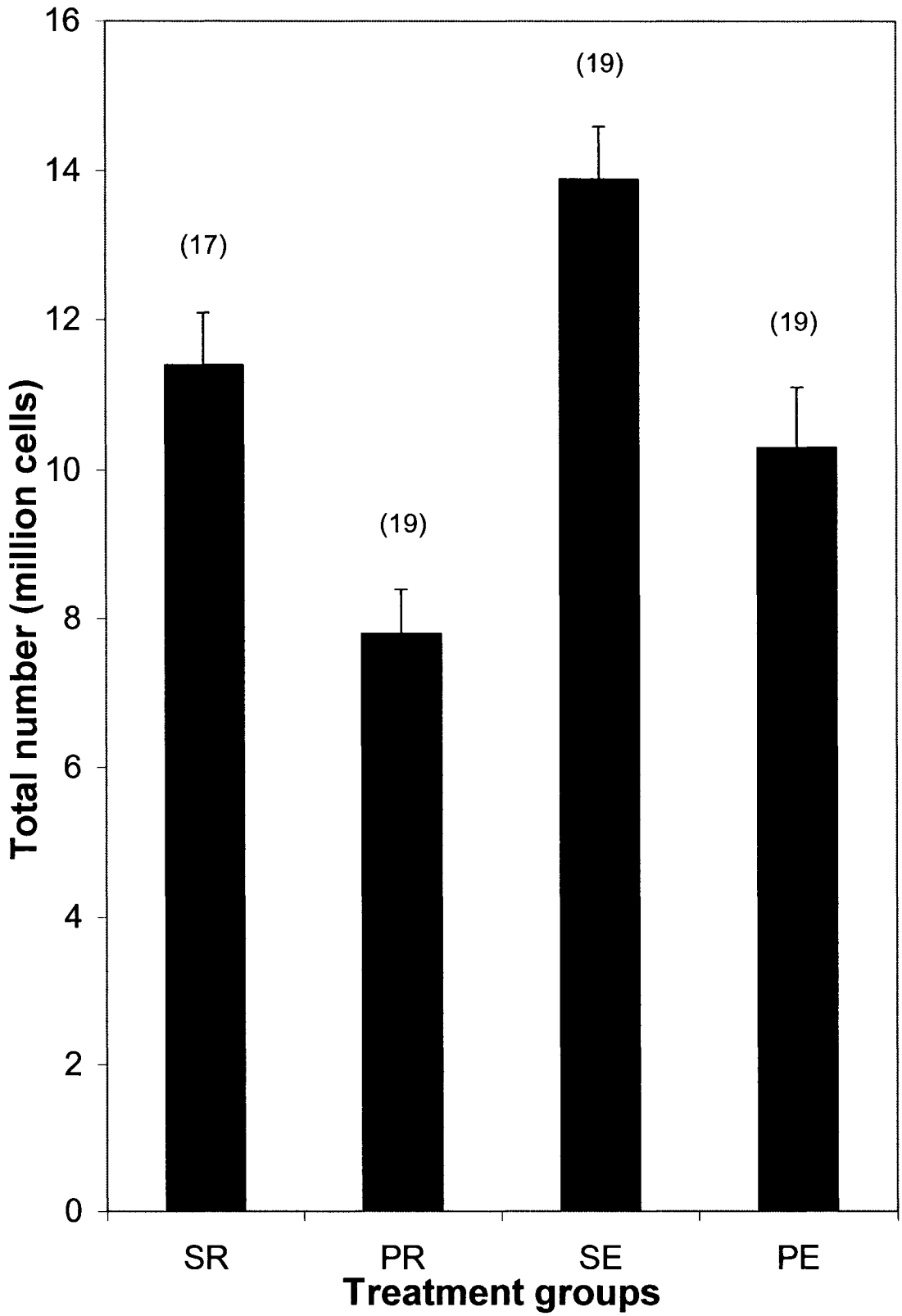

16
14
12
10
8
6
4
2
0
Total number (million cells)
(17)
(19)
(19)
(19)
SR
PR
SE
PE
Treatment groups

Figure 12. Total number (mean ± SE) of mature granulocytes in the bone marrow (2 femurs) of the 4 animal treatment groups.

SR : Saline-injected mice fed with regular diet
PR : Pristane-injected mice fed with regular diet
SE : Saline-injected mice fed with *Echinacea*-treated diet
PE : Pristane-injected mice fed with *Echinacea*-treated diet
Echinacea-treated diet was given to animals starting day 49 until sacrifice (refer to Material and Methods).
( ) = number of mice

SR vs PR	$p < 0,0001$
PR vs PE	$p = 0,02$
SR vs PE	$p < 0,0001$
SR vs SE	$p = 0,7$

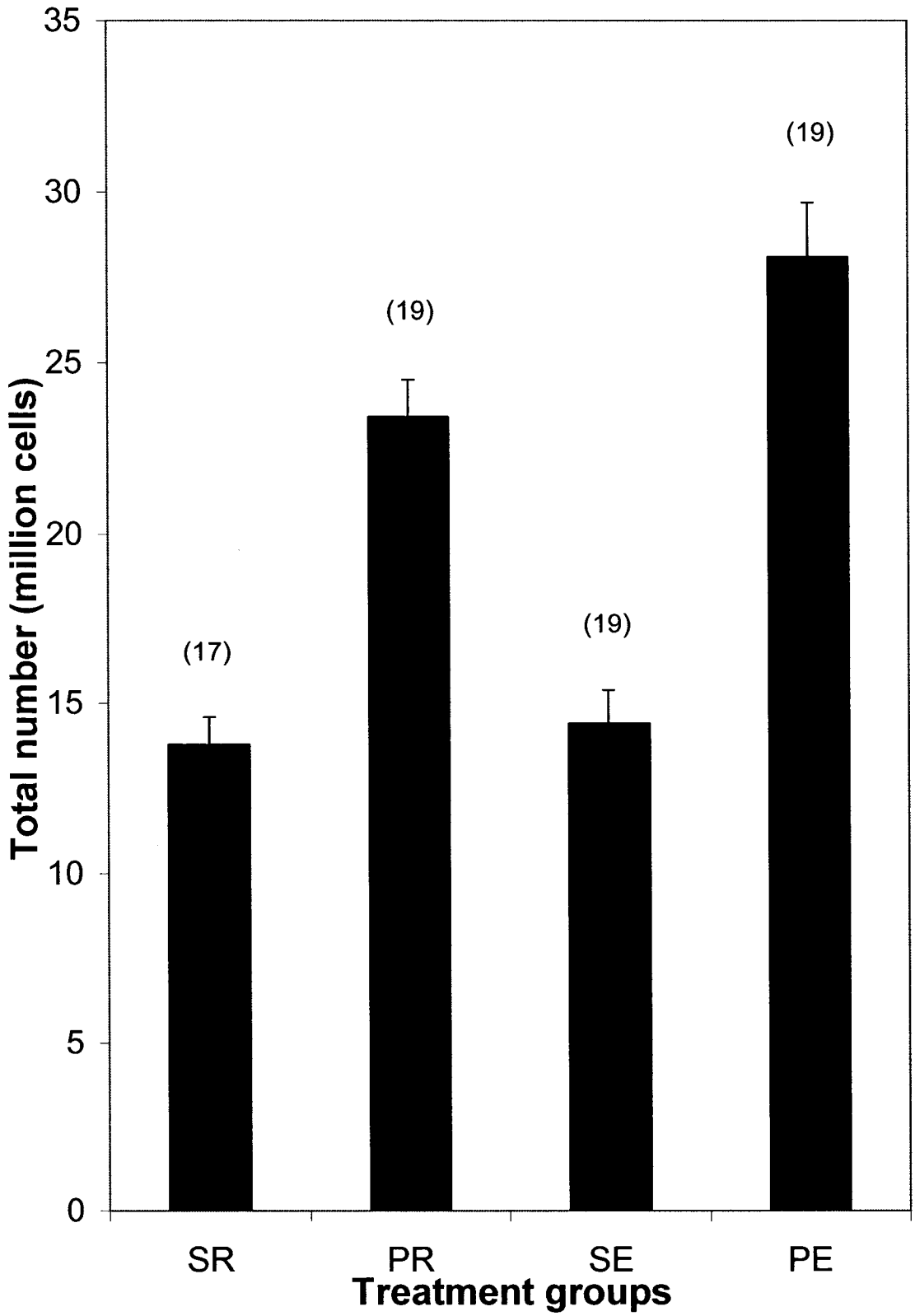

35
30
25
20
15
10
5
0
Total number (million cells)
(17)
(19)
(19)
(19)
SR
PR
SE
PE
Treatment groups

Figure 13. Total number (mean ± SE) of immature granulocytes in the bone marrow (2 femurs) of the 4 animal treatment groups.

SR : Saline-injected mice fed with regular diet
PR : Pristane-injected mice fed with regular diet
SE : Saline-injected mice fed with *Echinacea*-treated diet
PE : Pristane-injected mice fed with *Echinacea*-treated diet
Echinacea-treated diet was given to animals starting day 49 until sacrifice (refer to Material and Methods).
( ) = number of mice

SR vs PR	$p < 0,0001$
PR vs PE	$p = 0,009$
SR vs PE	$p < 0,0001$
SR vs SE	$p = 0,08$

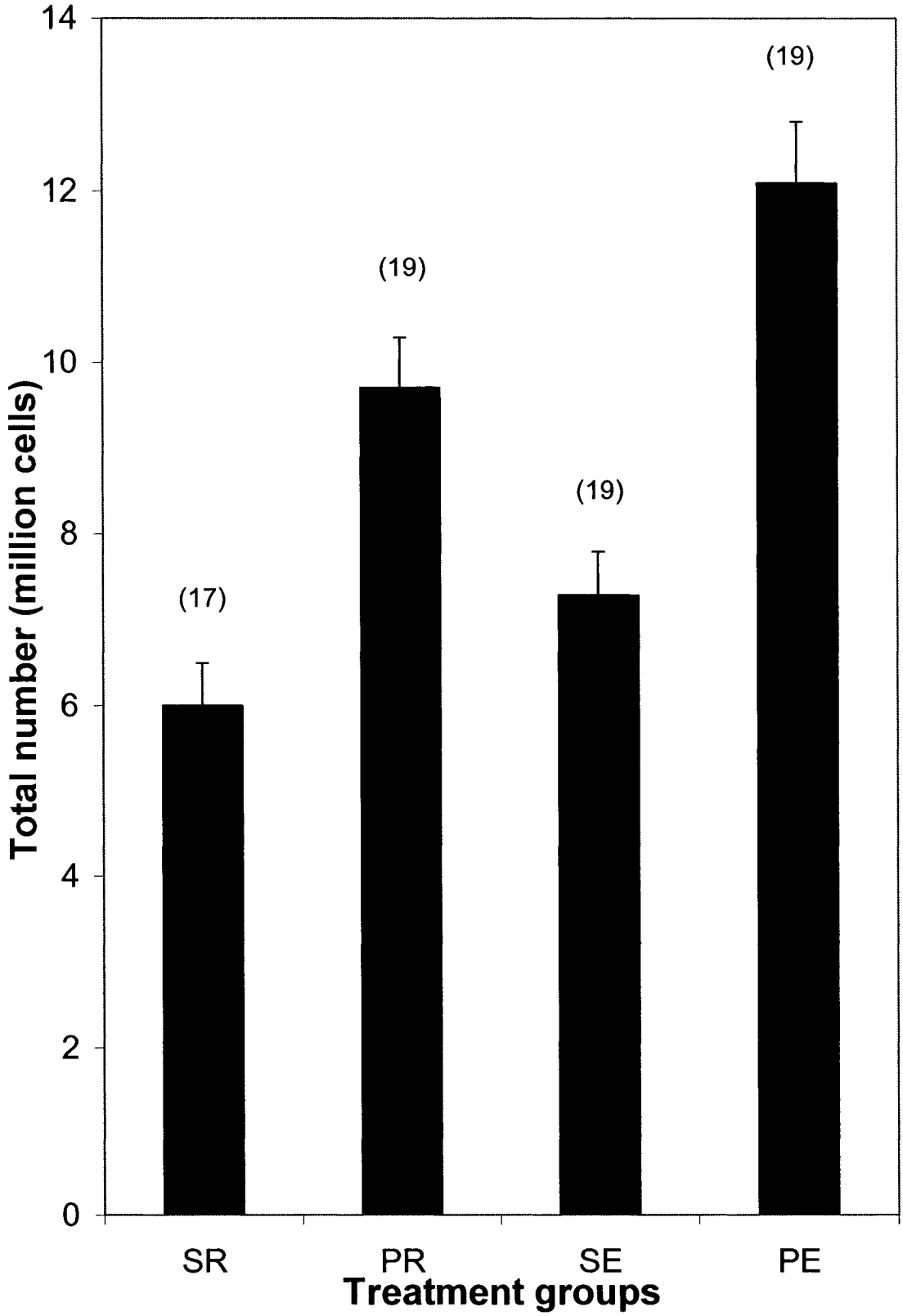

14
12
10
8
6
4
2
0
Total number (million cells)
(17)
(19)
(19)
(19)
SR
PR
SE
PE
Treatment groups

Figure 14. Total number (mean ± SE) of monocytes in the bone marrow (2 femurs) of the 4 animal treatment groups.

SR : Saline-injected mice fed with regular diet
PR : Pristane-injected mice fed with regular diet
SE : Saline-injected mice fed with *Echinacea*-treated diet
PE : Pristane-injected mice fed with *Echinacea*-treated diet
Echinacea-treated diet was given to animals starting day 49 until sacrifice (refer to Material and Methods).
( ) = number of mice

SR vs PR	p = 0,06
PR vs PE	p = 0,4
SR vs PE	p = 0,002
SR vs SE	p = 0,2

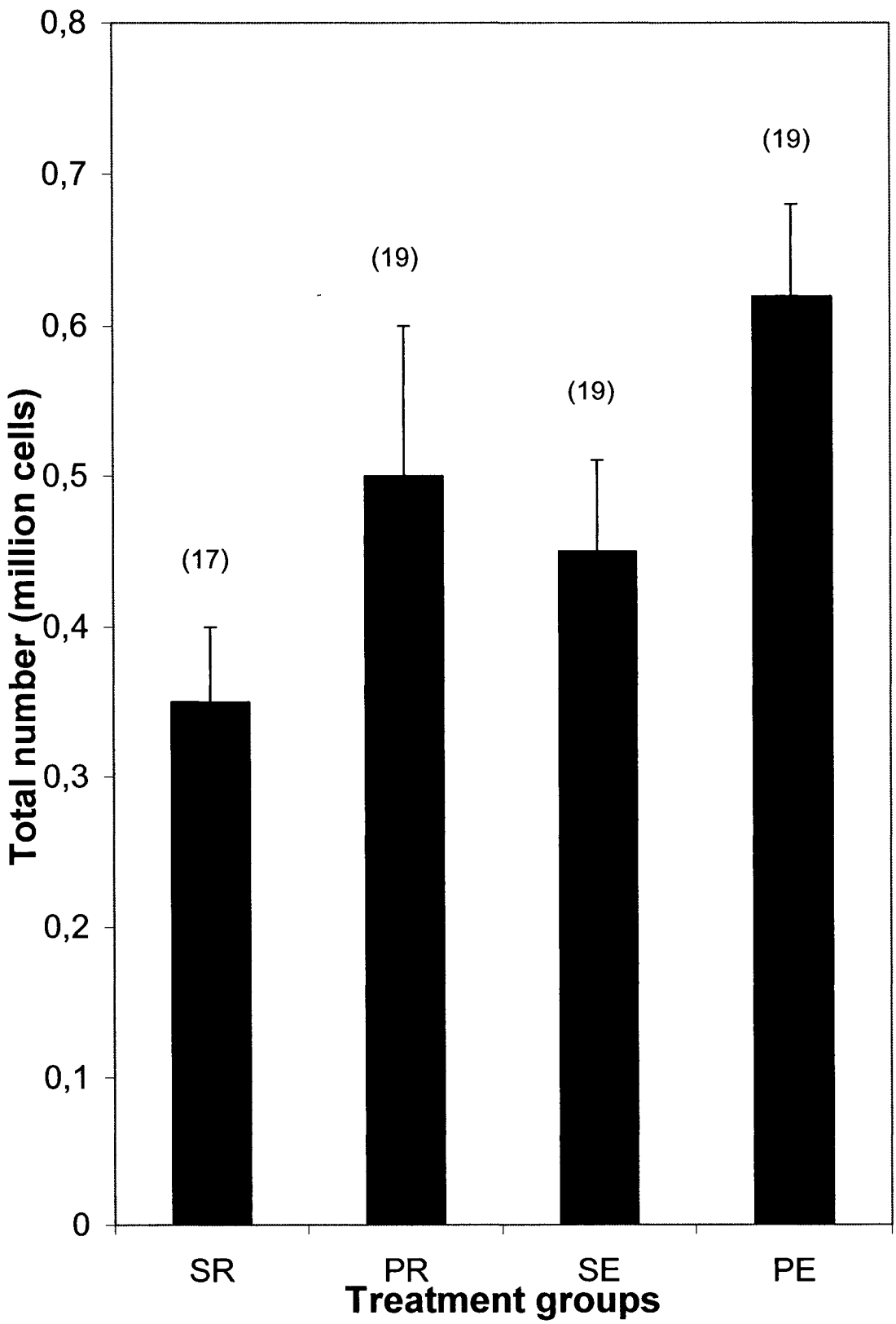
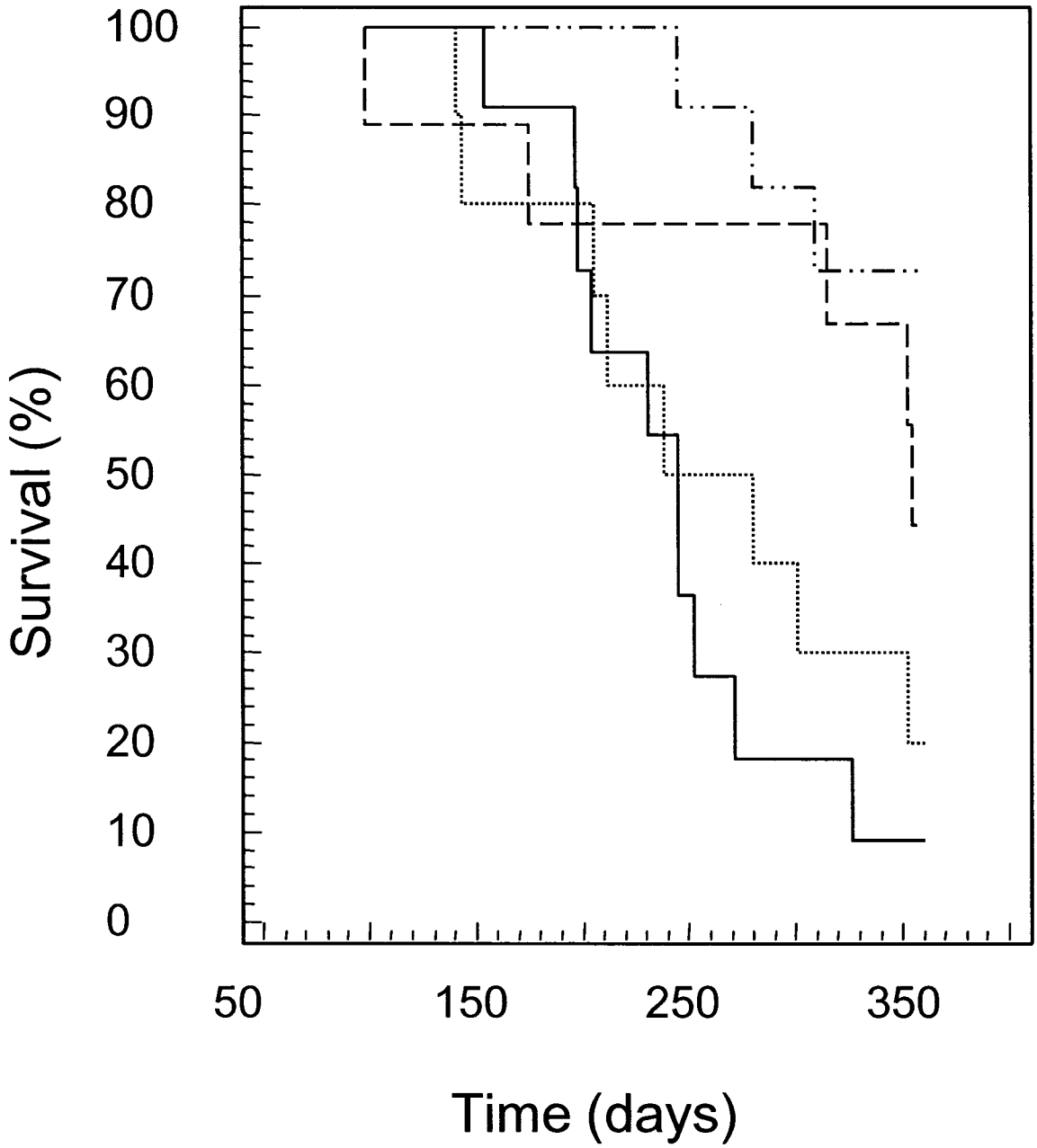

0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
Total number (million cells)
(17)
(19)
(19)
(19)
SR
PR
SE
PE
Treatment groups

Figure 15. Effects of *Echinacea*-treated diet on the lifespan of pristane-injected and saline-injected animals.

SR : Saline-injected mice fed with regular diet
Number of mice : 9
PR : Pristane-injected mice fed with regular diet
Number of mice : 10
SE : Saline-injected mice fed with *Echinacea*-treated diet
Number of mice : 11
PE : Pristane-injected mice fed with *Echinacea*-treated diet
Number of mice : 11
Echinacea-treated diet was given to animals starting day 49 until death (refer to Material and Methods).

SR vs PR p = 0,17
PR vs PE p = 0,44
SR vs PE p = 0,04
SR vs SE p = 0,25


100
90
80
70
60
50
40
30
20
10
0
Survival (%)
50
150
250
350
Time (days)
TREATMENT GROUPS
SR
SE
PR
PE

Discussion

Effect of pristane on hemopoietic and lymphoid cell populations (SR vs PR)

The results demonstrated that pristane, a pure alkane, did, indeed, induce a plasma cell dyscrasia in mice injected intraperitoneally with this specific chemical. The pristane-induced tumors were characterized by the proliferation of mature cells, i.e., plasma cells with various degrees of atypia, inside the peritoneal cavity. Plasma cells do not have the capacity to metastasize, and therefore, they remain inside the peritoneum. Moreover, the microscopic analysis of the spleen and femoral bone marrow confirmed the absence of plasmacytoma cells outside the peritoneal space as originally observed by Anderson and Potter (1969).

Production of hemopoietic and lymphoid cells inside the bone marrow was greatly influenced by pristane. The increased production of granulocytic cells in the bone marrow was a direct response to the injection of pristane. Oil droplets of pristane inside the peritoneal cavity had to be cleared by these bone marrow generated monocytes-macrophages and granulocytic cells. This observation corroborates a previous study by Anderson *et al.* (1985) and Potter (1986) where it was shown that there was a massive influx of phagocytic cells inside the oil granuloma following injection of pristane. Furthermore, because of the demand, phagocytic cells were produced not only in the bone marrow but in

extramedullary sites as well, such as the peritoneum and associated organs, i.e., diaphragm, gastrointestinal tract, etc (Anderson *et al.*, 1985; Potter *et al.*, 1985; Potter and Wax, 1986). Those granulocytic cells were then exported mostly to the spleen and the interior of the peritoneal cavity, where they attempted to neutralize the granuloma. The monocytoid population, by contrast to the granulocytic population, was produced, in the present study, in the bone marrow of pristane-injected animals to the same extent as in the marrow of saline-injected control animals. However, significant increases were seen in the absolute numbers of monocytes in the spleen of pristane-injected vs saline-injected mice. Monocytes apparently had been recruited from extramedullary sites as well as from the bone marrow into the spleen, and this is what may have caused increased absolute numbers of monocytes in the spleen of pristane-injected mice, even though the bone marrow absolute numbers of monocytes was the same in both pristane-injected and control (saline-injected) animals.

The results demonstrated that there was a decrease in the absolute numbers of lymphocytes (mostly B cells), NK cells, as well as nucleated erythroid cells in the bone marrow of pristane-injected mice. Previous work (Pietrangeli and Osmond, 1985) showed that B cell percursors were increased in number in the bone marrow of pristane-exposed mice. It was further demonstrated that this increase in the proliferation of B cell precursors in response to pristane was accompanied by a significant loss of B lymphocytes (Rico-Vargas *et al.*, 1995) from the bone marrow. Thus, the increased production of B cell precursors

followed by an even greater loss of B cells could well explain the decrease in absolute number of mature lymphocytes in the bone marrow of pristane-injected animals.

The mechanisms causing the decrease in absolute numbers of NK cells may reflect increased migration out of the bone marrow, and/or, decreased NK cell production therein under the influence of pristane. With respect to the nucleated erythroid cells however, it is known that such cells never normally migrate out of their bone marrow birth site; hence decreased production of nucleated erythroid cells under the apparently toxic influence of pristane in the bone marrow appears to have resulted.

The spleen is one of the main sites of action for mature hemopoietic and lymphoid cells. The influences of pristane were analyzed on the cell lineages in this organ in order to observe any disturbances in the patterns of migration of those cells. A large, supernormal influx of monocytes and granulocytes was seen in the spleen and peritoneal cavity of pristane-injected mice vs saline-injected control animals, as previously indicated by McDonald and Degrassi (1993). This recruitment of cells resulted in the observed splenomegaly in agreement with that observed by Weissinger *et al.* (1990). Ruiz-Bravo *et al.* (1995) showed that was a consequence of disrupted cell traffic into that organ, and that those cells were retained in the spleen because of the high degree of acute and chronic inflammation present in pristane-injected animals. Moreover, Anderson and

Potter (1969) demonstrated that the spleen histological organization was also disrupted by pristane. In fact, the red pulp portion of the spleen was enlarged, resulting in a dramatic reduction of lymphoid nodules and germinal centers. The influx and *in situ* production of granulocytic and erythroid lineages in the spleen would result in splenomegaly and enlargement of splenic red pulp. Moreover, compensatory erythropoiesis (thus accounting for the increased absolute numbers of nucleated erythroid cells) in the spleen has been demonstrated in mice bearing other types of tumors, i.e., leukemia, lymphoma, and ascites tumor (De Gowin and Gibson, 1978; Ray and Chowdhury, 1984; Bacon *et al.*, 1985). The erythropoietic function of the spleen in these pristane-injected mice was apparently resumed (given that the spleen is an erythropoietic organ in fetal/infant life). This increase in the splenic red pulp could have been at the cost of the white pulp and be related, therefore, to the observed decrease in the area occupied by lymphoid cells as shown by Anderson and Potter (1969). In the present study, our observations of significantly reduced numbers of splenic lymphocytes in pristane-treated mice would correlate with that early histological observation.

The observation of a decrease in absolute numbers of splenic NK cells and other lymphocytes in pristane-treated mice could be explained by a decreased production of these cells in the bone marrow, and/or a reduction in the numbers of those cells reaching the spleen due to aging (superimposed upon the effects of pristane). These animals are 8-12 months old at the time of sacrifice,

and aging has been shown to reduce significantly the number of NK cells in secondary immune organs. Decrease in NK cell production and other lymphocytes as well as changes in immune cell-mediated functions (Sato *et al.*, 1986; Dussault and Miller, 1994) have been observed in animals of advancing and advanced age.

Responses of spleen localized T and B lymphocytes to mitogens, i.e., PHA and LPS, as well as NK cell lytic activity were diminished in the presence of pristane (Freund and Blair, 1982; Wilson and Munson, 1996). Moreover, NK cells exposed to pristane responded to poly I: C (a potent stimulator of NK function) by rising back to their levels of cytolysis seen in saline-injected control mice, indicating that the negative effects of pristane could be overridden (Freund and Blair, 1982). Freund and Blair concluded that the decreased NK cell mediated activity of the spleen in pristane-injected animals was caused by either 1) a decrease in the number of splenic NK cells in the spleen or 2) an increase in suppression by NK cell-sensitive negative factors, i.e., microenvironment signals or cytokines (Katzman, 1978). Based on the results obtained in this project, it appears that the decrease in NK cell numbers to the spleen is in part, at least, responsible for the decrease in spleen functions in pristane-injected mice. Such decreased splenic NK cell numbers may well be a consequence of reduced production of NK cells as observed in the bone marrow of these mice, as found in the present study.

The total numbers of lymphocytes were subnormal in both the spleen and bone marrow of pristane-injected animals. This could readily reflect the presence of ascitogenic toxic chemicals produced in the peritoneal cavity in the presence of pristane, and dispersed systemically. A series of immune dysfunctions were demonstrated and analyzed in pristane-exposed rodents. Those immune dysfunctions were not seen, however, for the first ten days following pristane injections (Ruiz-Bravo *et al.*, 1995). A decrease in antibody production, graft-vs-host reactivity, ability to reject tumors, and immunosurveillance by immune cells (Kripke and Weiss, 1970; Mandel and DeCosse, 1970; Potter, 1972; Anderson *et al.*, 1985) was characteristic of pristane exposure. Moreover, pristane injections resulted in (i) induction of B-cell autoimmunity to genomic constituents, (ii) polyclonal hypergammaglobulinemia, (iii) immune complex-induced glomerulonephritis, (iv) arthritis, (v) stimulation of suppressor factors and macrophages, and (vi) a dysregulation of cell-mediated immune responses (Katzman, 1978; Chen *et al.*, 1982; Ulrich and Zolla-Pazner, 1982; Richards *et al.*, 1999). The overall dysregulation in the lymphoid cells of pristane-exposed mice could explain the differences seen in the survival of pristane-injected mice vs saline-injected animals, i.e., a significantly reduced lifespan in pristane-injected mice after the latent period of minimum 120 days required for pristane to induce plasmacytoma (Potter, 1986). Indeed, after day 200, pristane-injected animals began dying very rapidly.

Effect of *Echinacea purpurea* in the diet of pristane-injected mice (PR vs PE)

Echinacea purpurea feeding in pristane-injected mice resulted in a significant increase in absolute numbers, in the bone marrow, of several lineages of hemopoietic and lymphoid cells. The increase in mature and immature (precursor) granulocytes can be explained, in part, by the necessity to produce inflammatory and phagocytic cells to degrade pristane oil droplets in the peritoneal cavity of pristane-injected animals. This possibly is the result of cytokine feedback to the bone marrow-based granulocyte precursors. Factors responsible for such a feedback mechanism include TNF-alpha, interferon-beta, IL-1, IL-6, and insulin growth factor (IGF)-1 (Platica *et al.*, 1982; Stimpel *et al.*, 1984; Leuttig *et al.*, 1989; Roesler *et al.*, 1991a; Arkins *et al.*, 1993; Potter *et al.*, 1997; Barak *et al.*, 2002).

Previous work in our laboratory (Sun *et al.*, 1999) demonstrated that *Echinacea purpurea* was an immunostimulant for NK cells in both the bone marrow and spleen of normal mice. The present results extend that finding in demonstrating that *Echinacea* significantly enhanced the numbers (production) of NK cells in the bone marrow of pristane-injected animals as well. By contrast, the number of lymphoid cells (predominantly B cells) in the bone marrow was insensitive to the presence of dietary *Echinacea purpurea* in pristane-injected animals. This result is in accordance with what was demonstrated *in vivo* in

normal DBA/2 mice by our laboratory (Sun *et al.*, 1999) and *in vitro* in normal mice by Roesler's group (Roesler *et al.*, 1991a, b).

Granulocytes and their precursors were unchanged in the spleen of pristane-injected mice consuming *Echinacea* in spite of the fact that mature granulocytes and their precursors were increased in number in the bone marrow of those mice. Absolute numbers of monocytes were unchanged by *Echinacea* in the bone marrow of pristane-exposed animals whereas the splenic monocytes were significantly decreased. This probably reflects the decreased recruitment of myeloid and monocytoid cells to the spleen and/or disrupted trafficking of these cells in response to the cascade of inflammatory cytokines. Another possibility resides in direct pristane-induced cell death. Roesler *et al.* (1991a) demonstrated, *in vitro*, that exposure of cells of normal mice to *Echinacea* resulted in a significant increase in polymorphonuclear cell adhesion to endothelial cells of blood vessels. Since the oil granuloma is a vascularized tissue (Potter, 1986), *Echinacea* would cause increased adhesion and diapedesis of mature and immature granulocytes as well as monocytes to the inflamed peritoneum. Those events would be promoted by activated monocytes-macrophages producing a host of chemokines, such as IL-8, important for phagocytosis of oil droplets inside the peritoneal cavity (Ebina and Murata, 1990).

The absolute number of nucleated erythroid cells in the spleen of pristane-injected animals remained unchanged after *Echinacea* consumption, even though

the erythroid cells were, by contrast, significantly elevated in the bone marrow of these mice. One plausible explanation resides in the fact that pristane injections often result in hemorrhagic ascites (De Deken *et al.*, 1994; Peterson, 2000). Our analysis of peritoneal fluid smears demonstrated that indeed pristane injections caused the presence of nucleated erythroid cells in the peritoneal cavity. It was demonstrated that inflammation of the peritoneum caused erosion of the blood vessels and leakage of hemopoietic and lymphoid cells inside oil granulomas (Potter and MacCardle, 1964). This movement of nucleated erythroid cells to the peritoneum, i.e., shift in trafficking due to the endogenous pathology, could have caused the observed result (unchanged number) in erythroid cells in the spleen of pristane-injected animals.

Previous studies have shown that indomethacin, an anti-inflammatory drug, stimulated NK cells in leukemic mice, resulting in absence of measurable disease and significant extension of lifespan (Christopher *et al.*, 1991; Dussault and Miller, 1993). Similar results were also obtained with dietary *Echinacea* (Currier and Miller, 2001; 2002) in leukemic mice. The increase in absolute number of splenic NK cells in the present work, i.e., in pristane-injected mice consuming *Echinacea* can be explained by 1) a major decrease of pristane-mediated prostaglandins in the presence of *Echinacea purpurea,* and/or 2) an increase in bone marrow production of NK cells due to *Echinacea*-associated activation of monocytes-macrophages which then secrete NK cell stimulants, i.e.,

interferon-alpha, IL-12, and IL-15 (Leuttig *et al.*, 1989; Brunda and Gately, 1994; Puzanov *et al.*, 1996; Condiotti and Nagler, 1998).

There were no significant differences in the survival of pristane-injected mice fed either with *Echinacea*-treated or regular diet. The results demonstrated that the enhancing properties of *Echinacea* on NK cells were not sufficient or even relevant for plasmacytoma combat. *Echinacea purpurea* may have been more toxic in pristane-stressed animals than in saline-injected mice. This is in direct contrast to the results found in leukemic mice. This may mean one (or both) of the two following phenomena existed: (1) plasmacytoma cells are not seen as targets for NK cells, possibly because they lack the retrovirus-mediated, NK-responsive surface receptors expressed by leukemic cells , or, (2) plasmacytoma cells are targets for T and B blasts, which clearly were not augmented in pristane-injected mice, in either the spleen or bone marrow.

Effect of *Echinacea purpurea* in the diet of pristane-injected mice: a comparison with saline-injected mice fed regular food (SR vs PE)

The hypothesis of this research project was that *Echinacea purpurea* would boost natural killer cells in pristane-injected animals and the increased numbers of NK cells would kill cancerous plasma cells, leading to abatement/cure of their plasmacytoma. The presence of *Echinacea* in the diet of pristane-injected mice resulted in the return of NK cell numbers to normal levels, i.e., levels seen in bone marrow and spleen of vehicle-injected animals. Dietary *Echinacea* therefore appears to have precisely cancelled the effects of pristane on the NK lineage of cells.

Apart from exposure to *Echinacea purpurea* and pristane, mice in this research project were moreover subjected to the natural age-associated changes which occur in all hemopoietic and lymphoid cell lineages. Many immune and hemopoietic cells are sensitive to the negative effects of aging (Goodwin, 1995; Burns and Goodwin, 1997). Previous investigations demonstrated that immune system cells of aged animals and humans were less responsive to microenvironment factors (Burns *et al.*, 1990; 1994), and that immune cells also undergo increased apoptosis (Pawelec *et al.*, 2002; Zhang, 2002). It was important to take aging into account in the comparison and contrast of the different treatment groups because plasmacytoma tumor exhibited a latent period of at least 120 days (Potter, 1986). By the time frank plasmacytoma developed

into pristane-injected mice, most animals were over 6 months in age. The efficacy of the immune system of those older mice was further subjected to the age-related decrease in thyroid hormone (Fabris *et al.*, 1982; 1986; Provinciali *et al.*, 1991).

NK cell numbers and functions decrease significantly with age in mammals (Albright and Albright, 1983; Ghoneum *et al.*, 1991; Krishnaraj, 1992; Kutza and Murasko, 1994). Our laboratory further demonstrated that this decrease in NK cell activities was caused by decreased production of NK cells in the bone marrow as well as decreased NK cell binding capacity to target tumor cells (Dussault and Miller, 1994). NK cells in aged mice do not respond to common drugs (indomethacin and IL-2) used to boost NK cells in younger adult animals (Dussault and Miller, 1995a; 1995b). By contrast, it was found that *Echinacea purpurea* extracts could significantly increase NK cell functions in older mice, and in fact, old mice fed *Echinacea* experienced a rejuvenation of their NK/other immune cells (Currier and Miller, 2000). The results mediated by pristane and *Echinacea* were therefore superimposed on the decrease in lymphoid cell numbers found in the present study, due to aging.

Besides decreased production in the bone marrow, and decreased affinity for target cells, immune cells of aged individuals were shown to be more sensitive to prostaglandins. As mentioned previously, prostaglandins are known suppressors of immune system genesis and function (Goodwin and Webb, 1980).

Previous work demonstrated that cells from elderly humans were more easily inhibited by prostaglandins compared to cells of middle-aged people (Goodwin, 1979; Goodwin and Messner, 1979). Thus, the production of an additional load of prostaglandins in the presence of pristane (Potter *et al.*, 1997; Potter, 1999) was yet another factor potentially responsible for the very low numbers of NK cells in older animals, in spite of the fact that *Echinacea* is a powerful NK-cell enhancer.

Effect of *Echinacea purpurea* in the diet of normal mice (SR vs SE)

Exposure to *Echinacea* through the diet significantly affected NK cells in the normal mice in this study, without interfering with the numbers/production of other cells (non-NK lymphocytes, granulocytes, and monocytes) in the bone marrow, in keeping with previous observations in another strain of mice, i.e., DBA/2 (Sun *et al.*, 1999; Currier and Miller, 2001; 2002). However, one study (Sun *et al.*, 1999) indicated that monocytes were also augmented in both the spleen and bone marrow under *Echinacea* influence, a finding that was not seen in the present project with BALB/c mice. The discrepancy can be explained readily by the duration of *Echinacea* exposure in the present study. The present results also demonstrated differences in cell numbers after long-term consumption of *Echinacea* through the diet. Non-NK lymphocytes, nucleated erythroid cells, and monocytes were significantly reduced in the spleen of *Echinacea*-consuming normal animals vs control diet mice. Thus, it appears that length of time of dietary exposure to this herb is of significance.

One action of *Echinacea* is to act at the level of prostaglandin synthases, cyclooxygenases and lipooxygenases, as an inhibitor of the production of prostaglandins (Wagner *et al.*, 1989; Muller-Jakic *et al.*, 1994; Raso *et al.*, 2002). It is well known that prostaglandins are inhibitors of NK cell production and function. A decrease in prostaglandins favors the IL-1-mediated autocrine and paracrine stimulation of monocytes-macrophages (Kendall and Targan, 1980;

Lala *et al.*, 1986), and those cells secrete, subsequently, interferons and other direct stimulants of NK cells. *Echinacea in vivo* results in a cascade of cytokines which favor NK cell generation (bone marrow) and lytic function (spleen). Those cytokines are TNF-alpha, interferon-beta, and IL-1 (Leuttig *et al.*, 1989; Roesler *et al.*, 1991a; Barak *et al*, 2002).

The absolute numbers of nucleated erythroid cells in the bone marrow of BALB/c mice fed *Echinacea*-containing diet was also increased in the present study, contrasting with the only known comparable study by Sun *et al.* (1999) where mice consumed *Echinacea* for a much shorter time. Nothing is known about the mechanisms by which *Echinacea* promotes erythropoiesis. Some laboratories have indicated that TNF-alpha, IL-1, and interferons, i.e., all cytokines produced by monocytes-macrophages following exposure to *Echinacea*, were inhibitory to erythropoiesis in the bone marrow (Feelders *et al.*, 1998; Goicoechea *et al.*, 1998; Allen *et al.*, 1999). On the contrary, some researchers indicated that those cytokines were stimulating the earliest erythropoiesis progenitors (BFU-E) but inhibiting the growth of later stages (CFU-E) (Trey and Kushner, 1995; Means, 1999; Barany, 2001). *Echinacea* may also have induced changes in the bone marrow microenvironment allowing proliferation and survival of certain erythropoiesis progenitors. By contrast, the numbers of nucleated erythroid cells in the spleen of mice consuming *Echinacea* was decreased compared to those animals consuming regular food. It appears that under the influence of the dietary herb, the bone marrow has reverted back

to being a primary erythropoiesis organ, at the cost of the splenic erythropoiesis which is, under normal conditions, low anyway.

Conclusion

In summary, the present work has revealed for the first time, the effects of long-term consumption of *Echinacea purpurea*, in normal animals. *Echinacea* resulted in the enhancement of natural killer (NK) cells in both the organ of production (bone marrow) and the organ of function (spleen). Moreover, the herb delayed the onset of old-age in normal mice compared to normal animals fed untreated diet. Secondly, this work has asssessed the various quantitative changes in the hemopoietic and lymphoid cell populations in plasmacytoma-bearing mice. Chronic consumption of *Echinacea* by plasmacytoma-bearing mice did not result in extention of lifespan but did indicate that such consumption for a long-term period led to the normalization of NK cells and nucleated erythroid cells, both of which were significantly sub-normal in plasmacytoma-bearing mice not consuming *Echinacea*.

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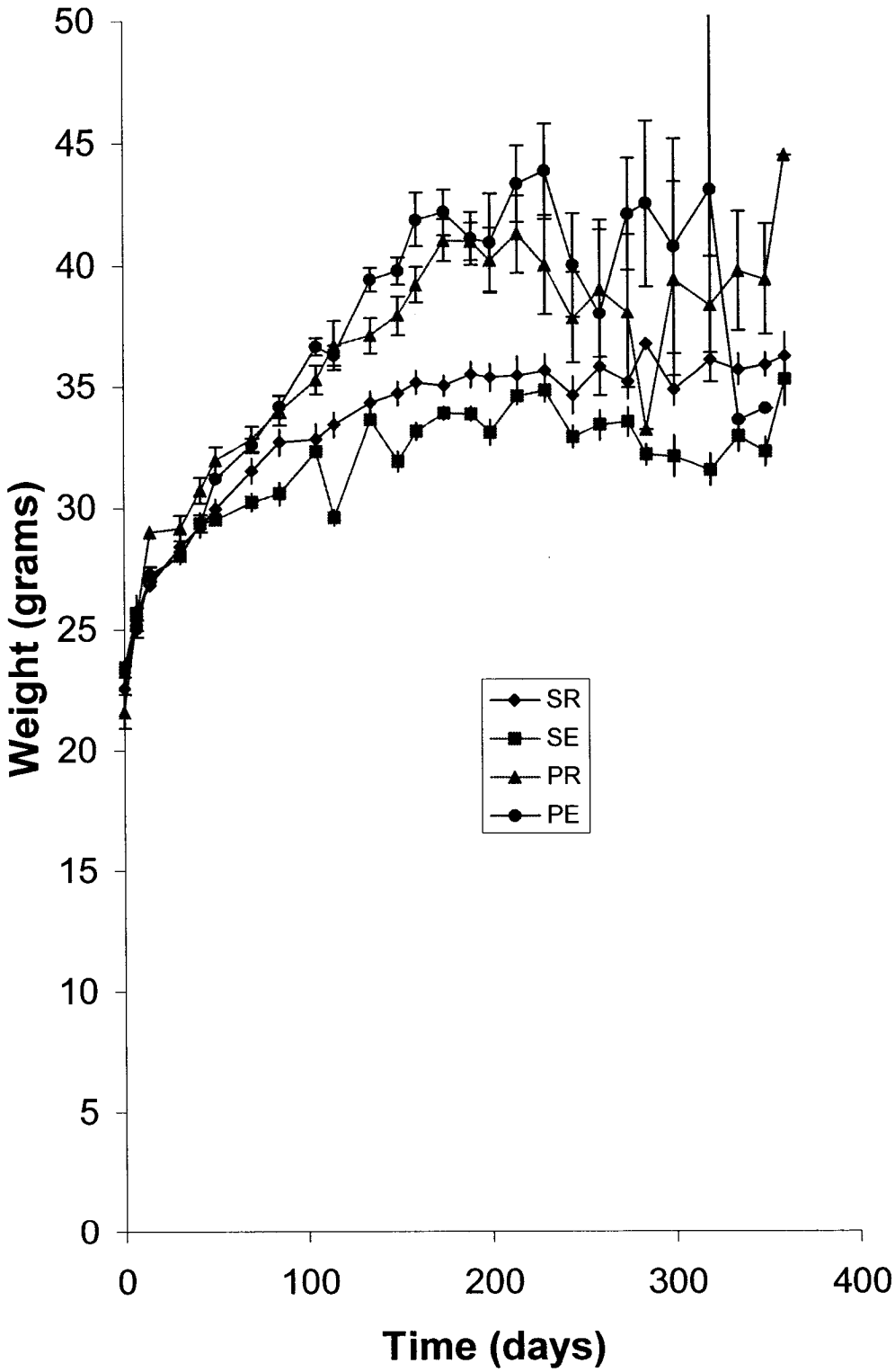
Appendix A. Body weight

Figure 16. Appendix A: Body weight (mean ± SE) of the 4 animal treatment groups for the duration of the study.

SR : Saline-injected mice fed with regular diet
PR : Pristane-injected mice fed with regular diet
SE : Saline-injected mice fed with *Echinacea*-treated diet
PE : Pristane-injected mice fed with *Echinacea*-treated diet

Significant differences ($p < 0,05$) were seen between SR and PR, and SR and PE.
No significant differences were seen between PR and PE, and SR and SE.

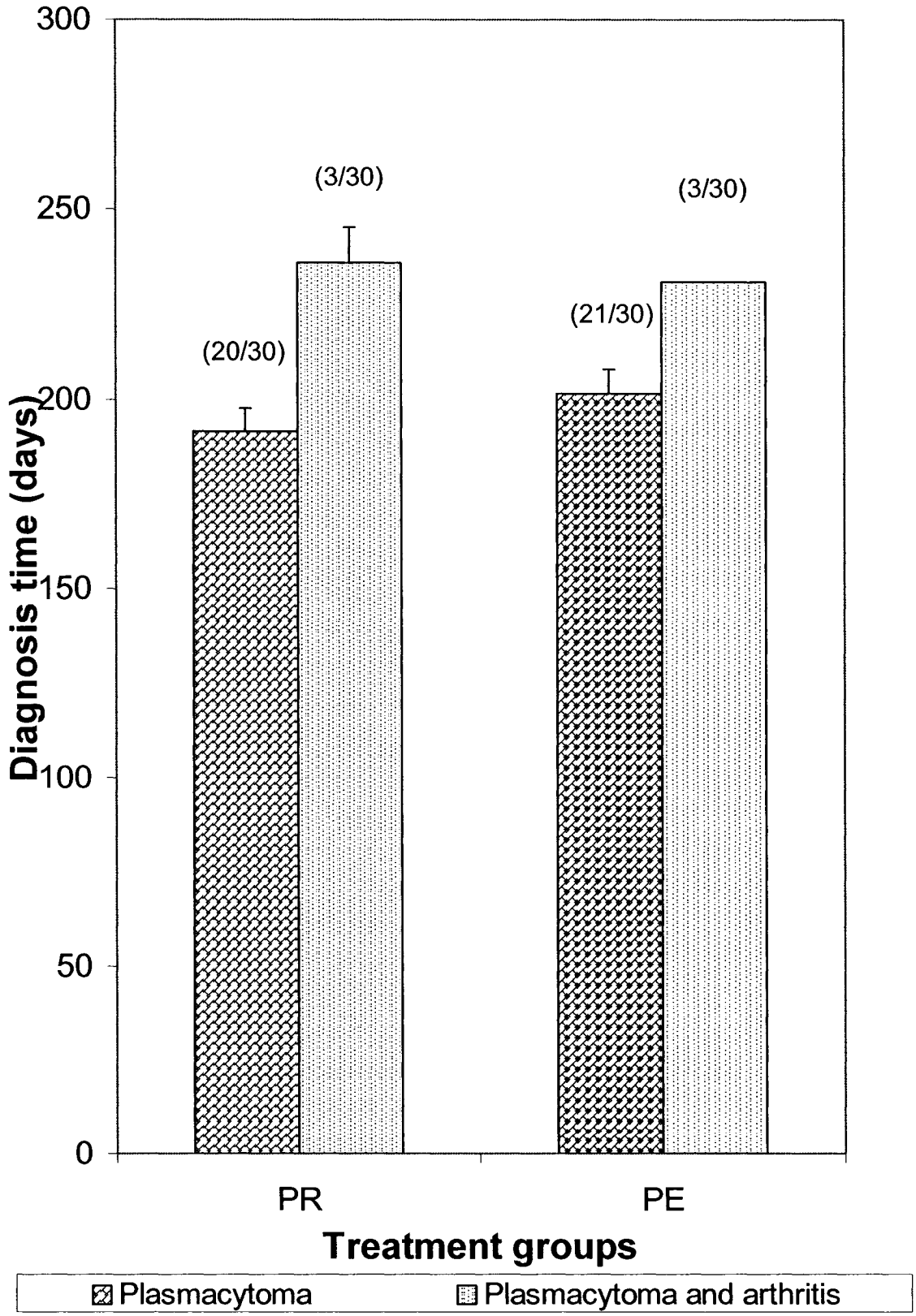

50
45
40
35
30
25
20
15
10
5
0
Weight (grams)
0
100
200
300
400
Time (days)
SR
SE
PR
PE

Appendix B. Time of diagnosis

Figure 17. Appendix B: Time of diagnosis (mean ± SE) of plasmacytoma and of plasmacytoma and arthritis in pristane-injected treatment groups.

PR : Pristane-injected mice fed with regular diet
PE : Pristane-injected mice fed with *Echinacea*-treated diet
( ) = number of cases over total number of "starting" mice

Significant differences were not seen between PR and PE.


300
250
200
150
100
50
0
Diagnosis time (days)
(3/30)
(3/30)
(20/30)
(21/30)
PR
PE
Treatment groups
Plasmacytoma
Plasmacytoma and arthritis

Appendix C. Ethics certificate for research with animal subjects

#needed at one time	20	10	10			
/ per cage	1-3	3	1-3			
TOTAL# /YEAR	400/yeard	180/year	180/year			

7 d) Justification of Animal Usage: BASED ON THE EXPERIMENTAL OBJECTIVES OF THE PROJECT, describe the number of animals required for one year. Include information on experimental and control groups, # per group, and failure rates. For breeding, specify how many adults are used, number of offspring produced, and how many offspring are used in experimental procedures. Use the table below when applicable. The arithmetic explaining how the total of animals for each column in the table above is calculated should be made clear. ***Space will expand as needed.***

Every "experiment" needs half the mice for controls alone divided as follows: for example, half the mice given tumor but no irradiation and normal diet; other mice given irradiation but no tumor and normal diet; others will be given tumor but no irradiation and dietary arbinogalactan; still others, tumor but no irradiation and dietary E.p. ; others, irradiation but no tumor and dietary arabinogalactan; others, tumor without irradiation and dietary E.p.; others, irradiation without tumor and dietary arabinogalactan. Other combinations include mice given tumor AND irradiation with and without all the various dietary combinations, samples at 7 and 14 days after tumor injection, for toal of mice, thus an annual total of 400/year for DBA/2 (main strain) mice. Baldb/c and NOD/LtJ mice will receive some but not all the above experiments.

The above numbers of mice are needed given (1) the numerous controls which must accompany each bone marrow transplanted mouse, and (2) the fact that in vivo studies necessarily involve inter-animal variations. Thus, to get adequate data with which to perform tests of statistical significance, the above projected numbers still represent a bare minimum.

The following table may help you explain the animal numbers listed in the 7c table:

(Table will expand as needed)	Sp/strain 1	Sp/strain 2	Sp/strain 3	Sp/strain 4	Sp/strain 5	Sp/strain 6
Test Agents or procedures	2	2	2			
# of animals per group	20	10	10			
Dosage and/or route of administration E.p.:food;0.45mg/day, ARAB:i.p.;500ug/day in 0.1 ml neutral PBS.	x 2 (ARAB E.p.)	x 2 (ARAB E.p.)	x 2 (ARAB E.p.)			
# of endpoints	x 2 (7, 14 days)	x 2 (7, 14 days)	x 2 (7, 14 days)			
Other variables (sex, genotypes...)	x 2 (Exp. Control)	x 2 (Exp. Control)	x 2 (Exp. Control)			
Total number of animals per year	= 320 successes + 80 "failures" = 400	= 160 successes + 20 "failures" = 180	= 160 successes + 20 "failures" = 180			

Exp. = E.p. or ARAB in the daily chow (or i.p.); Control = regular untreated chow, or simple saline (i.p.)

8. Animal Husbandry and Care

8 a) If projects involves non-standard cages, diet and/or handling, please specify

Special cages: Microisolators - before and after leukemia cell injection

8 b) Is there any component to the proposed procedures which will result in immunosuppression or decreased immune function (e.g. stress, radiation, steroids, chemotherapeutics, genetic modification of the immune system)?

NO ☐ YES ☒ **if yes, specify:** radiation

8 c) Indicate area(s) where animal use procedures will be conducted:

Building: Strathcona Bldg. **Room:** 2/26

Indicate area(s) all facilities where animals will be housed:

Building: AMI **Room:** 414

If animal housing and animal use are in different locations, briefly describe procedures for transporting animals:
Transport system to be arranged by McIntyre

9. Standard Operating Procedures (SOPs)

Complete this section if you plan to use any of the UACC SOPs listed below. IT IS UACC POLICY THAT THESE SOPS BE USED WHEN APPLICABLE. Any proposed variation of the SOPs must be described and justified. The Standard Operating Procedures can be found at the UACC website at www.mcgill.ca/rgo/animal . The completed and signed SOP from must be attached to the protocol.

Check all SOPs that will be used:

Blood Collection UACC#1	☐	Collection of Amphibian Oocytes UACC#9	☐
Anaesthesia in rodents UACC#2	☐	Rodent Survival Surgery UACC#10	☐
Analgesia in rodents UACC#3	☐	Anaesthesia & Analgesia Neonatal Rodents UACC#11	☐
Breeding transgenics/knockouts UACC#4	☐	Stereotaxic Survival Surgery in Rodents UACC#12	☐
Transgenic Generation UACC#5	☐	Euthanasia of Adult & Neonatal Rodents UACC#13	☐
Knockout/in Generation UACC#6	☐	Field Studies Form	☐
Production of Monoclonal Antibodies UACC#7	☐	Phenotype Disclosure Form	☐
Production of Polyclonal Antibodies UACC#8	☐	Other, specify:	☐

10. Description of Procedures

10 a) . IF A PROCEDURE IS COVERED BY AN SOP, WRITE "AS PER SOP", NO FURTHER DETAIL IS REQUIRED.

FOR EACH EXPERIMENTAL GROUP, DESCRIBE ALL PROCEDURES AND TECHNIQUES, WHICH ARE NOT PART OF THE SOPs, IN THE ORDER IN WHICH THEY WILL BE PERFORMED – surgical procedures, immunizations, behavioural tests, immobilization and restraint, food/water deprivation, requirements for post-operative care, sample collection, substance administration, special monitoring, etcAppendix 2 of the Guidelines (www.mcgill.ca/rgo/animal) provides a sample list of points that should be addressed in this section.

Gamma irradiation in 2 doses (450 R x 2) separated by 4 hr. This eliminates virtually all leukemic (but not 100%) and normal host hemopoietic blood-borne and bone marrow (vital) cells. No anaesthetic procedures are necessary during the irradiation since the mice roam freely on the floor of the irradiation chamber and the entire irradiation process is over in a very few minutes (repeated twice at 4 hr. intervals).

Bone marrow (adult) or fetal spleen transplantation is done via slow infusion of sterile, pure bone marrow/spleen cells which in the case of mice is delivered via the lateral tail vein while themouse is somewhat restricted in a small body cage, designed for the purpose, with its tail free for transfusion injection. The animal is not immobilized, has some free movement, its vision is uninterrupted and the entire transplant prodecure is over within 3-5 minutes.

Leukemia cells are delivered once to the mouse, via the tail vein as above, to give the animal leukemia from a precisely known onset point. The clinical progression of the tumor is well known and no animal even remotely apporaches the moribund stage. All animals are killed at early-tumor (9 days), or mid-tumor (14 days) development post injections of the tumor cells. All tumor-bearing animals subjected to tumor-erradication treatments will, if remaining clinically robust, be allowed to live on (survival analysis). The indications (at day 9 or day 14) which signal animal sacrifice are not clinical, but rather determined by sacrifice of parallel mice in every experiment to assess spleen enlargement. As stated above, no animal ever shows visible clinical signs of tumor-growth. This happens at day 22 of tumor-bearing only - a time well beyond the extent of this experiment. IN ANIMALS SHOWING NO CLINICAL SIGNS OF TUMOR DEVELOPMENT IN THE HERB/DRUG TREATED GROUPS, SOME OF THESE AS WELL AS PARALLEL SENTINEL MICE, AS ABOVE, WILL BE SACRIFICED EVERY 2 WKS UP TO 6 MOS TO MONITOR THE SPLEEN SIZE AND BONE MARROW HEMOPOIETIC STATUS. THE MAXIMUM TIME REQUIRED FOR COMPLETION OF THE ENTIRE EXPERIMENT IS THUS 6 MOS. ANIMALS SURVIVING FOR THIS LENGHT OF TIME ARE DEEMED TO HAVE BEEEN CURED.

E.p. or arabinogalactan are administered fresh daily, ground into the normal diet chow. See secton 7(b) for specifics.

*NOTE: THIS IS GM 360000 LINE OF ERYTHROLEUKEMIA-VIRUS-INDUCE CELLS FROM ATCC, OBTAINED ORIGINALLY BY US IN 1991, AND ... HAVE PUBLISHED 15 PAPERS, AFTER INVIV

	☐ Decapitation without anaesthesia * ☐ Decapitation with anesthesia, list agent/dose/route:
mouse	☒ Cervical dislocation without anaesthesia * ☐ Cervical dislocation with anaesthesia, list agent/dose/route:
mouse	☒ CO_2 chamber
	☐ Other, specify:
	☐ Not applicable, explain:

* For physical method of euthanasia without anaesthesia, please justify: Anesthetics dissolve in the lipid layer of cell membranes-since we use membrane surface markers,the lipid layers cannot be interfered with.

11. Category of Invasiveness: B ☐ C ☒ D ☐ E ☐

Categories of Invasiveness (from the CCAC *Categories of Invasiveness in Animal Experiments*). Please refer to this document for a more detailed description of categories.

Category A: Studies or experiments on most invertebrates or no entire living material.

Category B: Studies or experiments causing little or no discomfort or stress. *These might include holding animals captive, injection, percutaneous blood sampling, accepted euthanasia for tissue harvest, acute non-survival experiments in which the animals are completely anaesthetized.*

Category C: Studies or experiments involving minor stress or pain of short duration. *These might include cannulation or catheterizations of blood vessels or body cavities under anaesthesia, minor surgery under anaesthesia, such as biopsy; short periods of restraint, overnight food and/or water deprivation which exceed periods of abstinence in nature; behavioural experiments on conscious animals that involve short-term stressful restraint.*

Category D: Studies or experiments that involve moderate to severe distress or discomfort. *These might include major surgery under anaesthesia with subsequent recovery, prolonged (several hours or more) periods of physical restraint; induction of behavioural stresses, immunization with complete Freund's adjuvant, application of noxious stimuli, procedures that produce pain, production of transgenics (in accordance with University policy).*

Category E: Procedures that involve inflicting severe pain, near, at or above the pain threshold of unanaesthetized, conscious animals. *Not confined to but may include exposure to noxious stimuli or agents whose effects are unknown; exposure to drugs or chemicals at levels that (may) markedly impair physiological systems and which cause death, severe pain or extreme distress or physical trauma on unanaesthetized animals.* According to University policy, E level studies are not permitted.

12. Potential Hazards to Personnel and Animals It is the responsibility of the investigator to obtain the necessary Biohazard and/or Radiation Safety permits before this protocol is submitted for review.
A copy of these certificates must be attached, if applicable.

No hazardous materials will be used in this study: ☐

12 a) Indicate which of the following will be used in animals:

☐ Toxic chemicals ☐ Radioisotopes ☐ Carcinogens ☐ Infectious agents ☒ Transplantable tumours

12 b) Complete the following table for each agent to be used (use additional page as required):

Agent name	leukemia cells		
Dosage	3.0 x 10^6 cells		
Route of administration	i.v.		
Frequency of administration	once		
Duration of administration	9 - 14 days		
Number of animals involved	400/year		
Survival time after administration	5 wk or 6 mo		

12 c) After administration the animals will be housed in:

☒ the animal care facility ☐ laboratory under supervision of laboratory personnel

Please note that cages must be appropriately labeled at all times.

12 d) Describe potential health risk (s) to humans or animals:

Both the animal care technician and my own technician have taken the animal handling course offered by Animal Resources. The student named to work on this project (Melanie Brousseau) as well has taken the mouse workshop course.

All leukemic cells are handled in our P2 containment facility with which my technicans and myself are well familiar obviously, Moreover, ther is no cross transfer of this mouse leukemia into humans.

12 e) Describe measures that will be used to reduce risk to the environment and all project and animal facility personnel:

Spare (excess) leukemia cells are bleach killed for 1 hr, P-2 facility, trained technician.

13. Reviewer's Modifications (to be completed by ACC only): The Animal Care Committee has made the following modification(s) to this animal use procedure protocol during the review process. Please make these changes to your copy and comply with the recommended changes as a condition of approval.