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Natural Resistance: Methods and Mechanisms of Enhancement in Normal and Tumor-Bearing Mice

by

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Short Title: Enhancement of Natural Resistance in Leukemic Mice.

Abstract

This project examined methods of boosting innate resistance, enhancing those cells responsible for mediating "immune surveillance", i.e., Natural Killer (NK) cells. NK cells represent the first line of defense against neoplastic cells, to which they are spontaneously cytolytic, without having had prior exposure. This work aimed, firstly, to determine the effects on NK cells of administration of the interferon inducer, polyinosinic-polycytidylic acid (poly I:C) in irradiated leukemic mice given a bone marrow transplant, with or without the administration of the NK enhancer, tumor necrosis factor-alpha. The results demonstrated NK cell enhancement and prolonged life span of the poly I:C-treated, leukemic hosts. Secondly, this work aimed to study the hormone, melatonin (MLT), as well as a commercially prepared extract from the herb Echinacea *purpurea,* on NK cells. The role of these agents was assessed on NK cells in both normal and leukemic young adult mice. The results showed that both MLT and E. *purpurea* are NK enhancers when administered separately, suggesting prophylactic and therapeutic roles for these agents. The third aim of this project was to determine the effects of an E. *purpurea* treatment protocol on the numbers and function of NK cells in elderly mice. The age-related decline in NK cell numbers and function in mice is well documented in laboratory animals and corresponds with the increased frequency of tumors in these animals. This project has shown the effectiveness of the above treatments, especially those utilizing the herb extract from E. *purpurea* in stimulating NK cell numbers and cytolytic function in healthy and leukemic mice. In summary, the entire project has shown the NK enhancing

effects and life span prolongation in leukemic mice by using cytokines, hormones and herbal extracts. The major contribution of this thesis has been to demonstrate that non-toxic, readily available and relatively inexpensive phytocompounds (herbal products) are not only as effective but even more effective in leukemia amelioration than are conventional chemotherapeutic methods – the latter being toxic, expensive, and usually devastating to quality of life.

Résumé

Le but de ce project fut d'examiner les méthodes d'exaltation de la résistance naturelle, et de la facilitation des cellules responsables pour la médiation de la "surveillance immunologique", c.-à-d. les cellules tueuses naturelles (NK). Les cellules NK représentent la première défense contre les cellules néoplastiques, auxquelles elles éprouvent spontanément une réaction cytolytique, sans y avoir eu une exposition antérieure. Le premier but de cet ouvrage était de déterminer les effets de l'administration sur les cellules NK, de l'induction d'interféron, d'acide polyinosinic-polycytidylic (poly I:C) chez les souris leucémiques irradiées ayant reçu une greffe de moelle osseuse, avec ou sans l'administration du facilitant NK, le facteur onconécrosant-alpha. Les résultats ont démontré une accéleration des cellules NK et une prolongation de la survie des souris leucémiques traitées avec poly I:C. Le second but était d'étudier l'hormone, mélatonine (MLT), ainsi que l'extrait commercialement préparé de la plante Echinacea *purpurea,* sur les cellules NK. Le rôle de ces deux agents fut évalué sur les cellules NK des souris adultes normales et celles qui étaient leucémiques. Les résultats ont indiqué que la MLT ainsi que l'E. *purpurea* servent tous les deux à accélérer les cellules NK lorsqu'elles sont administrées individuellement, nous proposant qu'elles jouent un rôle à la fois prophylatique et thérapeutique. Le troisième but de ce projet fut de déterminer les effets d'un protocole de traitement de E. *purpurea* sur le nombre et les fonctions des cellules NK chez les souris âgées. La diminution des cellules NK attribuée à l'âge élevée des souris, est bien documentée chez les animaux d'expérience et

correspond à la fréquence élevée des tumeurs dans ces animaux. Ce projet a démontré l'efficacité des traitements ci-dessus, particulièrement ceux utilisant l'extrait de la plante E. *purpurea* pour stimuler le nombre de cellules NK et la fonction cytolytique chez les souris saines et leucémiques. En récapitulation, le projet entier a démontré les effets des cytokines, des hormones et des extraits de plantes sur l'augmentation des cellules NK et le prolongement de la survie chez les souris leucémiques. De première importance, ce thèse a démontré que les phytocompositions (produits végétaux) non-toxiques, s'obtenant facilement et de façon assez économique, sont non seulement aussi efficaces mais même plus efficaces pour l'amélioration de la leucémie, que les autres méthodes traditionnelles chimiothérapiques – ces dernières méthodes étant toxiques, coûteuses, et souvent dévastatrices à la qualité de la vie.

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Preface (Format of the Thesis)

This thesis is prepared in accordance with the guidelines dictated by the Faculty of Graduate Studies and Research at McGill University for manuscript-based theses, which include the statement "As an alternative to the traditional thesis format, the dissertation can consist of a collection of papers of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research."

Published, or "in press" works appearing in this thesis may have been modified from their original published content, with respect to text and/or figures and tables, for the purpose of maintaining relevance of the work within the thesis proper. As well, to maintain the cohesiveness of the thesis, each manuscript chapter is preceded by one page of linking text, to provide logical progression from one chapter to the next. Permission has been received from all publishers for previously published material.

Materials and Methods sections from all works, with accompanying reference list, have been merged and edited for similarity in content, to enhance ease of reference for the reader and are included in a separate section preceding Chapter 1.

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INTRODUCTION (OBJECTIVES & RATIONALE)

Natural resistance is mediated by natural killer (NK) cells, which kill their targets on first contact, without prior exposure (Herberman et al., 1975a, b; Karre et al., 1980; Kasai et al., 1980; Biron & Welsh, 1982b; Hanna, 1985; Trinchieri et al., 1989), and in an MHC-independent manner (Karre et al., 1986; Trinchieri et al., 1989; Ljunggren & Karre, 1990). For these reasons they are the first line of defense against neoplastic or virally infected cells. A paucity of literature exists concerning the *in vivo* effects on NK cells of chemicals/compounds shown to be immunostimulants *in vitro*.

The initial objective of this project was to investigate the effect of a common therapeutic technique for the amelioration of leukemia, involving irradiation and bone marrow transplant (BMT), coupled with cytokine administration, on host NK cell numbers and life span. I utilized the interferon inducer, polyinosinic-polycytidylic acid (poly I:C), as well as the cytokine, tumor necrosis factor alpha (TNF-a), both of which are known powerful NK cell enhancers (Gresser & Tovey, 1978; Djeu et al., 1979; Senik et al., 1979; Degliantoni et al., 1985; Yang et al., 1990; Chan et al., 1991; D'Andrea et al., 1992). Nothing was known of the effects of poly I:C on young adult, irradiated, leukemic mice given a bone marrow transplant. It was hypothesized that administration of poly I:C both pre- and post-BMT would provide a large contingent of NK cells to prevent tumor regrowth following irradiation and BMT. The results demonstrate that leukemic mice receiving poly I:C + BMT have

significantly greater NK cell numbers and life span than control mice receiving the BMT protocol alone. The postulated addition of TNF-a to this therapeutic protocol further aided in post-BMT NK cell enhancement, the results demonstrating that such "double-treated" (poly I:C + TNF-a), leukemic, irradiated, transplanted mice did indeed have increased NK cell numbers compared to mice receiving only poly I:C and BMT.

It was the secondary objective of this project to investigate the *in vivo* effects of protocols involving immunostimulants, for which relatively abundant *in vitro* literature is established, i.e., the neurohormone melatonin (MLT). MLT acts as a chronomodulator regulating organisms with the photoperiod. Evidence is growing, establishing an axis for the relationship between the neuroendocrine and immune systems in mammals, yet nothing is known of the *in vivo* effects of MLT on those cells mediating natural resistance, NK cells. T helper cells produce several NK cell enhancing cytokines (Senik et al., 1979; Hinuma et al., 1986; Christopher et al., 1991; Dussault & Miller, 1993; Cho et al., 1996; Wu et al., 1996) and have been shown to have membrane receptors for MLT, and perhaps nuclear receptors as well (Cutando & Silvestre, 1995; Gonzalez-Haba et al., 1995; Garcia-Maurino et al., 1997; Lissoni et al., 1998; Maestroni, 1998a,b,c; Rafii-el-Idrissi et al., 1998). It was the hypothesis that administration of MLT would drive T helper cells to upregulate production of their profile of NK cell enhancing cytokines, which, in turn, would drive the production of increased numbers of NK cells. The results demonstrated that MLT does increase NK cells after one or two weeks of oral administration to healthy young adult mice. Although suggesting a prophylactic potential, this neurohormone is beset with

side effects paralleling those observed when cytokines and growth factors are exogenously administered.

A third objective was the need to determine the effect on NK cells, of phytocompounds, i.e., plant products, for which there only exists anecdotal or circumstantial evidence purporting *in vivo* beneficial/medicinal values. One such agent, for which such anecdotal evidence exists, alleging its activity as an immunostimulating compound, is a commercially available extract from the plant Echinacea *purpurea* (Stimpel et al., 1984; Tragni et al., 1985; Lersch et al., 1990; 1992; Roesler et al., 1991a, b; Steinmuller et al., 1993; Hill et al., 1996). Early herbal medicines took advantage of the apparent anti-inflammatory properties of the herb and abundant evidence exists for its topical use to treat infection and sores (Tragni et al., 1985; Hill et al., 1996). However, nothing is known of the *in vivo* effects of Echinacea *purpurea* on any immune cells, especially those cells mediating natural resistance, NK cells. It was the hypothesis that administration of the plant product would boost NK cell numbers through action of the purported repertoire of NK cell stimulants, known to be contained within the whole herb extract. The results of this objective demonstrate that NK cell numbers were significantly increased in the bone marrow and spleen of healthy, young adult mice, following one and two weeks of oral administration of the plant product, suggesting a prophylactic role for this phytocompound. It appeared thus, that not only was this plant product able to enhance the natural resistance mechanism, i.e., that mediated by NK cells, but no clinical signs of toxicity were observed at any dose, and above all, every other hemopoietic cell lineage remained unaffected by short or longer term presence *in vivo* of this phytocompound.

In fact, the literature contains an extensive number of studies that have dissected out the separate components contained within Echinacea *purpurea*, and among them, several chemicals that could be responsible for the immunostimulating properties of the herb (Roesler et al., 1991a, b; Steinmuller et al., 1993; Muller-Jakic et al., 1994; Bauer et al., 1996). The complex carbohydrate arabinogalactan, for example, has been implicated in stimulating cells of the monocyte/macrophage lineage *in vitro* (Stimpel et al., 1984; Luettig et al., 1989) and *in vivo* (Rininger et al., 2000). Cells of the monocyte/macrophage lineage are known to produce many powerful NK stimulants (Hauer & Anderer, 1993; Luettig et al., 1989; Kelly, 1999; Stein et al., 1999; Rininger et al., 2000) and therefore it was the hypothesis that administration of arabinogalactan *in vivo* may enhance NK cells. In fact my results demonstrate that administration of arabinogalactan for two weeks significantly increased splenic NK cell numbers.

A fourth objective of this study was to determine the effects of administration of MLT and E. *purpurea* on leukemic mice, the hypothesis being that these 2 NK enhancing agents would be instrumental in tumor abatement. It was the aim of these studies to attempt to augment NK cell numbers with these NK enhancing agents and ultimately to expand the life span of these leukemic mice. Considerable evidence has established that increased NK cell numbers correlates well with increased NK tumor-lytic function (Keissling et al., 1975; Kasai et al., 1981; Biron & Welsh, 1982a; Itoh et al., 1982; Hefeneider et al., 1983; Koo et al., 1986; Lotzova et al., 1986; Kalland, 1987). Therefore, enhancing NK cell numbers would correspondingly increase the functionally active NK cell armament available for tumor cell combat. It was the hypothesis

that administration of E. *purpurea* and/or MLT would increase the number of NK cells in these leukemic mice, as seen with the results of their administration in healthy, young adult mice, and these increased NK cell numbers would be available to help eliminate the leukemic tumor. The results from this study demonstrate that indeed life span of leukemic hosts is significantly increased by exogenous administration of either MLT or E. *purpurea*, but the greatest survival advantage is observed with administration of both agents *together*.

The final objective of the work in this thesis was to determine the effect of E. *purpurea* administration on NK cell numbers and function in *elderly* mice. The age related decline of NK cell numbers and function is well documented in laboratory mice (Albright and Albright, 1983; Ghoneum et al., 1991). Previous work in our lab has shown that the cytokine IL-2 as well as the drug indomethacin were unable to restore NK cell numbers or function in these elderly mice (Dussault and Miller, 1994; 1995). Given the potent NK enhancing capacity of E. *purpurea* in young adult mice, based on our previous results, we hypothesized that E. *purpurea* may effect the same success in aged animals and that administration of E. *purpurea* to these elderly mice would increase NK cell numbers. Thus I gave E. *purpurea* to aged mice with this expectation. The results of this study indeed show that elderly mice administered E. *purpurea* for two weeks, did have restored NK cell numbers *and* functional activity, comparable to levels found in healthy, young adult mice. E. *purpurea* contains specific compounds, perhaps uniquely capable of stimulating natural resistance in the elderly.

In conclusion, this project evaluates the administration of several immunostimulants on NK cell numbers in healthy, young adult and elderly mice, as well as to leukemic, young adult mice. The effects of poly I:C, TNF-a, MLT and E. *purpurea* on NK cells have been investigated using different administration protocols. However, cytokines and hormones, such as those used here, do have tumor abatement potential, yet are so beset with harmful side effects that their use is limited for long-term, or even short-term cancer therapy. I have, for the first time, demonstrated that phytocompounds, such as those derived from E. *purpurea*, have led not only to enhanced NK cells in leukemic mice and normal mice, but also have significantly enhanced the life span of leukemia afflicted hosts. Such potential for cancer therapy in the broader sense, i.e., with solid tumors, remains to be seen.

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REVIEW OF THE LITERATURE

The Immune System and Natural Resistance

The immune system is comprised of an intricate array of cell types, their molecular signals/receptors, and various other molecular regulators. This complex network making up the body's self defense mechanism, and the interplay of its parts, allows explanation for the success of this protective adaptation to varied immune challenges and attacks throughout an organism's lifespan.

The immune system can be organized into that which is specific, and that which mediates natural resistance. The specific arm of the immune system is made up of cells, including T and B cells for example, which are selective and become 'primed' for action towards a chosen target, while in certain cases may be inhibited and not kill or destroy their targets. The cells and elements responsible for natural resistance, on the other hand, are somewhat arbitrary in their choice of target and kill their targets spontaneously, on first contact. This natural resistance includes cell lineages such as monocytes/macrophages and natural killer (NK) cells. NK cells are the primary effectors, or cytotoxic elements, of natural resistance, and are the core focus of this thesis within the murine immune system.

Inherently the term "immune system" implies adaptability and a 'learning' capability that technically can be considered to not include the natural resistance arm of immunity, as cells mediating natural resistance have no memory

component as other cells, i.e. T and B, may have. The discussion from this point on will respect this fact and avoid any implication that natural resistance, or what has been termed "innate immunity", could be considered a "true" type of immunity.

NK Cell Function

NK cells, the primary effectors of natural resistance, are innate, spontaneous killers of virus-infected (Welsh et al., 1979; Welsh, 1986), neoplastic (Herberman et al., 1975a, b; Kiessling et al., 1975a, b; Karre et al., 1980b; Talmadge et al., 1980; Hanna & Burton, 1981) or xenogeneic cells (Kiessling et al., 1977; Trentin & Bennett, 1977; Miller & Poirier, 1988). They play the role of an "immune surveillance" towards aberrant cells and are the first line of defense towards virally infected or tumor cells (Kasai et al., 1980; Biron & Welsh, 1982; Hanna, 1985; Lanier, 1998). This fact is underscored by their ability to kill on first contact, without enhancement by prior immunization to the relevant target (Herberman et al., 1975a, b; Capo et al., 1979; Riccardi et al., 1981). NK cells also have no long-lived, recirculating "memory" component, as does the specific arm of the immune system (Seaman et al., 1978; Greenberg & Greene, 1976; Trinchieri et al., 1979; Miller, 1982; Zoller et al., 1982). This innate cytolytic capacity is also coupled with the NK cells ability to secrete cytokines, i.e., tumor necrosis factor alpha (TNF-a) (Murphy et al., 1992; Jewett & Bonavida, 1993; Bemelmans et al., 1996).

The notion of NK "surveillance" originates from the discovery over twenty years ago (Horwitz & Lobo, 1975) that there existed a cell type that was

identified as neither T nor B (Sanchez et al., 1994), and killed in a major histocompatibility complex (MHC) independent manner (Herberman, 1982; Karre et al., 1986; Trinchieri, 1994). Evidence shows that cells lacking MHC class I expression are more susceptible to NK cell lysis than cells which do express MHC class I (Bix et al., 1991; Liao et al., 1991) and is reversible with the transfection of MHC class I into these cells (Shimizu and DeMars, 1989; Storkus et al., 1989).

NK cells do not rearrange B cell surface immunoglobulins (Ig) (Tutt et al., 1986) or T cell receptor (TCR) chains (Biron et al., 1987), as do B and T cells, respectively. It can be said that they do not possess antigen-specific receptors at their surface as T and B cells do, although the heterogeneous activating NK receptors do present limited structural and functional homology with T cell- and B cell-antigen receptors (Yokoyama et al., 1991; Hofer et al., 1992; Ryan & Seaman, 1997).

NK cells also have the capability of killing immunoglobulin G (IgG) antibody coated targets using antibody-dependent-cellular cytotoxicity (ADCC). Membrane bound IgG on target cells is recognized by NK cells through the Fc fragment of IgG using a membrane bound Fc receptor (FcgammaR) (Froland & Natvig, 1973; Herberman et al, 1977; Nagler et al., 1989; Ravetch & Kinet, 1991).

NK Cell Morphology and Phenotype

NK cells are described as large granular lymphocytes (LGLs) (Timonen et al., 1979, 1981; Kumagai et al., 1982; Pollack and Rosse, 1987), and are readily

identifiable through the use of common NK cell surface markers. Under normal *in vivo* conditions they do not express any of the characteristic T cell markers (CD3), including the T cell receptor (TCR) alpha, beta, gamma, and delta chains (Ritz et al., 1985; Lanier et al., 1986a, b; Tutt et al., 1986, 1987; Biron et al., 1987; Loh et al., 1988). Moreover, they do not rearrange TCR beta and gamma and do not transcribe TCR alpha (Ritz et al., 1985; Lanier et al., 1986a, b; Tutt et al., 1986, 1987; Biron et al., 1987; Loh et al., 1988). However, some evidence demonstrates that human fetal NK cells may transcribe and express the delta and epsilon chains of the TCR (Phillips et al., 1992).

Murine NK cells express characteristic markers that differ between mouse strains as well depending on the level of NK cell differentiation. For example, a characteristic mature NK cell marker in DBA/2 strain mice is ASGM-1 (Kiessling et al., 1975a, b; Kasai et al., 1980, Young et al., 1980; Shimamura et al., 1982), while other strain backgrounds, such as C57, express the marker NK1.1 (Glimcher et al., 1977; Ballas & Rasmussen, 1990; Giorda et al., 1990; Yokoyama et al., 1991).

ASGM-1.

Asialo-ganglio-N-tetraosylceramide (ASGM-1) is a glycosphingolipid found on all mature and maturing NK cells (Kiessling et al., 1975a, b; Kasai et al., 1980, Young et al., 1980) as well as a subset of activated cytotoxic T lymphocytes (Stein-Douglas et al., 1976; Stein et al., 1978; Shimamura et al., 1982; Doherty & Allen, 1987; Parker et al., 1988) and naïve T cells (Lee et al., 1996). It has been shown that ASGM-1 is an activating receptor found on the surface of NK cells (Shimamura et al., 1982). Further, anti-ASGM-1 does not

affect T or B cell function and only inhibits the activity of NK cells (Beck et al., 1982; Shimamura et al., 1982).

NK 1.1.

NK1.1 is a murine NK receptor (NKR-P1 rat homologue) found on the surface of all NK cells and a small subset of T cells and is encoded within the NKR-P1 gene family on mouse chromosome 6, a region called the NK gene complex (NKC) (Glimcher et al., 1977; Ballas & Rasmussen, 1990; Giorda et al., 1990, 1992; Yokoyama et al., 1991). It is a type II integral membrane protein (39kDa) with extracellular calcium dependent (C-type) lectin domains existing as a disulphide-linked homodimer (Ryan et al., 1992; Ryan & Seaman, 1997). NK1.1 is involved in NK cell activation allowing the initiation of transmembrane signals, activating cytotoxicity (Sentman et al., 1989b; Giorda et al., 1990, Ryan & Seaman, 1997).

CD16 and CD56.

CD16 (FcgammaIIIR) is the human NK cell homologue of the murine FcgammaR receptor and is able to activate human NK cells to become cytotoxic to target cells upon binding the Fc fragment of target-bound IgG (Ra et al., 1989).

CD56 (NKH1) is another characteristic human NK cell marker, although it is also present on a small subset of T cells (Lanier et al., 1986c, 1989; Schmidt et al., 1986a, b). It is a 175-185kDa glycoprotein belonging to the Ig superfamily and was identified as an isoform of neural cell adhesion molecule (NCAM) (Lanier et al., 1991). CD56 is not expressed on murine NK cells (Lanier, 1995;

Lanier & Hemperly, 1995) and appears to have no cytolytic function in response to NCAM$^+$ targets (Lanier et al., 1991).

DX5.

A recently developed murine pan-NK marker is DX5, which is present on all NK cells regardless of strain, and a small subset of T cells (Ybarrondo et al., 1997). DX5 is a monoclonal antibody with a comparable tissue distribution to NK1.1, showing no significance expression variance across strain backgrounds (Ybarrondo et al., 1997). While the majority of NK cells express both NK1.1 and DX5, there are small populations of NK1.1$^+$DX5$^-$ and NK1.1$^-$DX5$^+$ cells detectable (Ybarrondo et al., 1997). Neither NK1.1 nor DX5 are expressed on B cells (Ybarrondo et al., 1997). The cell surface antigen for DX5 has not yet been identified but it does not appear to have any effect on NK cell proliferation, cytokine production, adhesion, or lysis of target cells (Ybarrondo et al., 1997).

Other Cell Surface Markers.

NK cells can also express the integrins CD11a (LFA-1), b (Mac-1), and c (Leu-M5) (Nishimura & Itoh, 1988; Timonen et al., 1988; Ramos et al., 1989; Allavena et al., 1991; Werfel et al., 1991; Migliorati et al., 1992), which are important in the adhesion and transmigration of NK cells from blood into tissue parenchyma, and the binding to target cells (Schmidt et al., 1985; Smith et al., 1989; Diamond et al., 1990, 1993; Somersalo et al., 1992; Ross & Vetvicka, 1993). CD11a-c belong to the leukocyte function antigen (LFA) family and are all associated with the same beta chain, CD18 (Nishimura & Itoh, 1988; Timonen et al., 1988; Ramos et al., 1989; Allavena et al., 1991; Werfel et al., 1991).

Although the CD11/CD18 complexes are important in NK cell target binding, anti-CD18 Abs do not completely block NK cytotoxic activity (Timonen et al., 1988).

The murine homing receptor Mel-14 (L-selectin), a cell surface molecule with lectin-like domains, is also found on NK cells and participates in their adhesion, migration and function (Ballas & Rasmussen, 1990; Butcher, 1991; James et al., 1991; Miller, 1994).

NK Cell Production and Differentiation

Under normal physiological conditions NK cells are BM derived, the majority of which migrate via the blood to the spleen where they become functional (Haller et al., 1977; Hackett et al., 1986a, b; Kalland, 1986a, b; Pollack et al., 1989). Their development is not dependent on the thymus as they develop normally in athymic (nude) mice (Klein et al., 1979, Minato et al., 1979), and, in fact, it has been shown that NK cells from athymic mice have greater NK activity than do euthymic control mice (Herberman et al., 1975a; Lotzova et al., 1985). However, in some cases, the normal production and migration of NK cells, in, and from organs other than the bone marrow, can be altered, and has been shown before (Biron et al., 1983; Miller & Shatz, 1991). NK cells develop in the liver in fetal life and subsequently in the bone marrow after seeding of the bone marrow with stem cells (Koo et al., 1982; Jaleco et al., 1997; Aiba & Ogawa, 1998).

The differentiation of NK cells proceeds through a sequence of stages of maturation, each stage identifiable by diagnostic surface marker expression (Pollack, 1993). Their normal development *in vivo* requires the participation of

an intact bone marrow environment (Haller & Wigzell, 1977), heavily relying on cooperation and involvement of BM stromal cells, which are capable of producing a wide variety of growth factors necessary for NK development (van den Brink et al., 1990; Pollack et al., 1992; Sitnicka & Hansson, 1992; Tsuji & Pollack, 1995). Once produced and fully differentiated to mature NK cells through their trafficking to the spleen, NK cells are known to have a relatively short lifespan compared to other cells of the immune system, surviving only 2-4 days (Miller, 1982; Biron et al., 1983; Pollack & Rosse, 1987).

Briefly, highlighting the literature review of Pollack (1993), the development of NK cells is suggested to be similar to that of B cells, the other major lymphocyte population produced in the bone marrow. NK cells begin their differentiation arising from pluripotent stem cells in the bone marrow to become a large proliferating cell type called a pro-NK cell. Pro-NK cells give rise, under the influence of stromal cells and/or growth factors to less rapidly proliferating NK precursor cells (pre-NK). These Pre-NK cells then divide and differentiate into phenotypically recognizable primary NK cells in the bone marrow. Primary NK cells are not in cell cycle, have low but measurable lytic activity, and express the phenotype of resting NK cells. They exit the bone marrow to populate peripheral sites where they undergo further maturation, become activated by such agents as interferon and may undergo a new cycle of proliferation in response to viral infections or other stimuli.

NK Gene Complex (NKC)

The receptor differentiation of NK cells is ultimately initiated and regulated by the natural killer cell gene complex (NKC) found on distal mouse chromosome

6 (Yokoyama et al., 1991). The NKC encodes multiple NK cell surface receptors including the NKR-P1 receptor family and Ly-49 isoforms (Yokoyama & Seaman, 1993).

NKT Cells.

The NK cell can be considered to be unique and distinct from the development of T cells, evolving much earlier than this cell type, although shown to arise from a common lymphoid progenitor cell (Herberman and Ortaldo, 1981; Herberman, 1986; Versteeg, 1992). However, evidence is growing for a cell type that appears to have selective combined characteristics of both T cells and NK cells and relies somewhat on the thymus for its normal differentiation, namely the NKT cell (Lanier & Phillips, 1996; Cui et al., 1997; Kawano et al., 1997). This cell expresses CD3 alpha/beta$^+$ as well as characteristic NK cell markers and is capable of secreting several cytokines, especially IL-4 and IFN gamma, modulating the development of Th1 or Th2 cells (Leite-de-Moraes and Dy, 1997). NKT cells may represent a novel evolutionary link or early bipotential progenitor between both cells, as evidenced by their shared receptor profile. For example, human NKT cells express the T cell CD3 marker as well as the characteristic human NK cell marker CD56 (Schmidt et al., 1986a, b).

Beige Mutation.

The murine beige mutation (Chediak-Higashi syndrome in humans) results in the production of normal numbers of NK cells that have no lytic activity (Roder, 1979; Roder et al., 1979; Karre et al., 1980a; Clark et al., 1981). This point mutation on mouse chromosome 13 results in the inaccurate transcription of essential genes involved in NK cytotoxicity and the blocking of some post-

recognition event in the lytic cycle (Barbosa et al., 1996; Kingsmore et al., 1996; Perou et al., 1996). As well, the beige mutation results in the production of one large cytotoxic granule whereas normal NK cells and TNK cells contain multiple, dispersed, perforin-containing granules (Bannai et al., 2000). The functional activity of NK cells has been found to be modulated by several point mutations associated with mouse coat color beyond the beige mutation, including satin, leaden, fuzzy and pale ears (McGarry et al., 1984).

SCID Mice.

Severe immuno-compromised (SCID) mice have defects in T and B cell differentiation while their NK cells have been shown to be unaffected in numbers or in function (Dorshkind et al., 1985). The SCID mutation on mouse chromosome 16 affects a cells ability to repair DNA and these cells are unable to rearrange their TCR and B cell Ig genes, resulting in the absence of functioning T and B cells (Dorshkind et al., 1985; Bosma et al., 1989; Phillips & Fulop, 1989; Weaver & Hendrickson, 1989; Fulop & Phillips, 1990).

NK Cell Distribution and Migration

Under normal physiological conditions NK cells are found in their greatest numbers, and with greatest activity, in the spleen, decreasing in numbers in the blood, lymph nodes, bone marrow and thymus (Miller, 1982), and are virtually absent in the lymph nodes of healthy individuals (Westermann & Pabst, 1992). As previously mentioned, NK cells are produced by the bone marrow and migrate via the blood to the spleen where they become functional. This migration is unidirectional under normal physiological conditions; there is no

retrograde migration from the spleen back to the bone marrow environment (Miller, 1982).

NK cells will often have to execute transendothelial migrations during their movements to reach sites of defense and this requires the ability to cross the endothelial cell wall, basement membrane, intercellular matrix and the perivascular tissue space. Some of the receptors involved in NK cell migration and homing to the spleen have been identified and include MEL-14 (Ballas & Rasmussen, 1990; Kishimoto et al., 1990; Butcher, 1991; James et al., 1991; Miller, 1994), ICAM-1 (Smith et al., 1989; Diamond et al., 1990, 1993; Somersalo et al., 1992; Ross and Vetvicka, 1993), NK-LFA-1 ICAM1 found on endothelial cells (Springer et al., 1987) and perhaps involving the crucial NK receptor RGD sequence of fibronectin (Allavena et al., 1991; Somersalo et al., 1991).

Chemokines and cytokines have also been shown to be important in the adhesion and migration of NK cells, as well as enhancing their cytotoxicity. Some chemokines act as powerful NK chemoattractants *in vitro*, macrophage inflammatory protein (MIP-1) alpha, IFN-inducible protein-10 (IP-10) (Taub et al., 1995), or *in vivo*, monocyte chemotactic protein (MCP)-1, -2, and –3 (Allavena et al., 1994a), demonstrating their vital roles in NK response to chemotactic cellular responses. As well NK cells are able to produce chemotactic factors (Somersalo et al., 1994). Cytokines are also able to act as NK cell chemotactic factors, including IL-12 and IL-15 (Allavena et al., 1994b, 1997).

Influence of Age on NK Cells

NK cells are found in their greatest numbers in young adult mice with corresponding peak function (Herberman et al., 1975a, b; Kiessling et al., 1975a; Cudkowicz and Hochman, 1979). While NK cells are present in infant mice there is an associated lack of activity due to the action of NK cell suppressors in the spleen (Bassett et al., 1977; Argyris, 1981; Peeler et al., 1983a, b; Jadus & Peck, 1986). Spleen NK activity has been shown to be relatively absent in newborns and increases through to 3 weeks of age inversely correlated with diminishing suppressor activity (Calkins & Stutman, 1978; Cudkowicz & Hochman, 1979; Herberman et al., 1979; Argyris, 1981). In NK cells in fetal mice the splenic suppressing cells have been shown to involve T cells (Argyris, 1979, 1982; Murgita et al., 1981; Murgita & Wigzell, 1981), macrophages and mast cells (Peeler et al., 1983a, b).

NK cells are present as well in later stages of life in elderly mice, but evidence shows a progressive decline in the level of NK activity beyond the young adult stage, beginning at 10 weeks of age (Herberman et al., 1975; Kiessling et al., 1975; Roder & Kiessling, 1978a, b). It is well established that NK cells normally decline to low or absent levels, beginning at about 3 months (Herberman, 1977; Itoh et al., 1982; Albright & Albright, 1983). Whether this age related decline is due to the action of suppressor cells (Irimajiri et al., 1985; Riccardi et al., 1986a) or NK suppressor factors such as prostaglandin (Goodwin & Messner, 1979; Rosenstein & Strausser, 1980; Licastro & Walford, 1986) is unclear. There is evidence that these declines in NK activity are not associated with any decrease in NK cell numbers but perhaps related to an increased

inability of NK cell-target cell binding and lysis (Albright & Albright, 1985), while other evidence suggests that the decline is due to both decreased NK cell numbers and target binding capacity (Dussault & Miller, 1994). On the other hand, it has been shown that in these elderly mice, IL-2, a powerful NK cell enhancer in young adult mice, only partially restores NK activity (Dussault & Miller, 1995, 1996). This again suggests the impairment of NK cell activity rather than a decrease in overall NK cell numbers. Interestingly, the opposite result has been demonstrated in humans, with an increase in mature NK cell numbers, but impaired 'per-cell' activity, in elderly individuals (Solana et al., 1999; Solana & Mariani, 2000).

NK Cell Activity and Mouse Strain

There are differences in NK cell activity in relation to mouse strain. As previously mentioned the beige mutation affecting the NKC gene complex on mouse chromosome 6 allows NK cell production but results in inactive NK cells. Two mouse strains at opposing scales of relative NK activity are the A/J and C57 strains. A/J strain backgrounds have been shown to have relatively low NK cell numbers and activity (Herberman et al., 1975a; Haller et al., 1978; Kaminsky et al., 1983; Itoh et al., 1982; Whyte & Miller, 1998) and are therefore more susceptible to a wide variety of pathogens (Cheers & McKenzie, 1978; Pennington & Williams, 1979; Ahlstedt, 1980; Brown et al., 1982). On the other hand, it has been shown that C57 backgrounds have a very high NK cell activity (Nesbitt & Skamene, 1984; Yoshida et al., 1995) as evidenced in the low frequency of melanomas and neoplasms in these mice, compared to A/J mice.

Again, the SCID phenotype, resulting in inactive T cells, does allow for normal production and differentiation of NK cells. Evidence demonstrates that NK activity is increased in these mice perhaps correcting for the absence of T cell cytotoxicity (Peter et al., 1983; Pierce et al., 1986; Murphy et al., 1987; Chang et al., 1989; Hasui et al., 1989). Nude (athymic) mice also develop normal numbers and cytolytically active NK cell populations, demonstrating that NK cells develop independently of the thymus (Hasui et al., 1989).

NK Cell Activation + Inhibition = Whole Signal

NK cell activation or inhibition is the result of what can be referred to as a "whole signal" response, as an NK cell may receive multiple activating and inhibitory signals to regulate its activation or inhibition towards a specific target. The activation of NK cells may be initiated by the triggering of multiple adhesion or costimulatory molecules, and can be counterbalanced by inhibitory signals induced by receptors for class I MHC. This "whole signal" determines whether a particular NK cell will respond to the respective target or become inhibited and perhaps undergo programmed cell death (apoptosis) (Brumbaugh et al., 1998; Bakker et al., 2000; Moretta et al., 2000; Tomasello et al., 2000).

As mentioned previously, NK cells are not MHC restricted in the classical T cell sense that requires the recognition of MHC with associated peptide to become activated or inhibited (Swain, 1980; Altman & Katz, 1981; Pimlott & Miller, 1986; Fairchild, 1998; Garcia & Teyton, 1998; Mazza et al., 1998). This first line of defense, imparted by NK cells, functions to kill cells that for some reason have an irregular, or downregulated expression of MHC class I (Karre et

al., 1986; Jonges et al., 2000) termed the "Missing Self Hypothesis" (Ohlen et al., 1989; Ljunggren and Karre, 1990). This differs from the mode of action of T cells which through their T cell receptor (TCR) become inhibited by the recognition of appropriate cell surface MHC class I or "self" (Merkenschlager et al., 1994; Nakajima et al., 1995).

MHC class I acts to allow immune system components to distinguish self from non-self, thereby avoiding the disastrous effects of having the body's immune system attack itself, as seen in autoimmune disorders (Singer et al., 1997; Durinovic-Bello, 1998; Ridgway & Fathman, 1999). Normal cells escape NK lysis because their MHC class I is recognized by NK cells resulting in the NK cell's cytotoxic inhibition (Erikkson et al., 1999; George et al., 1999). As well, evidence shows that MHC class I antigens regulate the survival of activated NK cells as NK cells lacking recognition of MHC class I are shown to be more likely to undergo anergy through mechanisms involving TNFα, fas and the FcgammaIII receptor (Jewett and Bonavida, 1995, 1996, 2000).

MHC is a cell surface molecule, encoded on mouse chromosome 17 and found on the surface of most murine cells. It exists in two forms, class I and class II, the latter being involved in B cell recognition of bound antigenic peptide from antigen presenting cells (APC) to produce clonal selection and antibody production. MHC class I polymorphisms, like TCR polymorphisms, allow for exquisite diversity in immune systems.

NK Cell Receptors and Ligands

To date extensive progress has been made regarding the negative regulation of NK cell function, while knowledge of the activating receptor complexes is still relatively poor.

Ly49.

The Ly49 receptor family is encoded on the NK gene complex of mouse chromosome 6 (Yokoyama et al., 1990). Its members bind specific MHC class I motifs expressed on target cell surfaces (Wong et al., 1991; Brennan et al., 1994; Smith et al., 1994). Different specificities of Ly49 receptors are known to respond to different repertoires of class I MHC molecules (Brennan et al., 1994). For example, the inhibitory receptor Ly49C, has specificity for H-2k, b, d, k+s (Sentman et al., 1989; Brennan et al., 1994; Salcedo et al., 1998)

Ly49 members are C-type lectin-like, type II integral membrane proteins with a disulfide-linked homodimeric structure (Yokoyama et al., 1989; Chambers et al., 1993; Drickamer, 1993). There have been shown to be at least 9 members in the Ly49 family, rising in expression from null levels in infant mice to maximal levels at 6-8 weeks of age, coinciding with NK cell activity maturity (Dorfman and Raulet, 1998). To date, the Ly49 molecule family is known to contain the subclasses A-I (Wong et al., 1991; Brennan et al., 1994; Smith et al., 1994) and each may contain allelic forms, some with limited homology to NK1.1 (Yokoyama et al., 1991). Ly49A, Ly49C and Ly49G are expressed on subsets of cells comprising 10-60% of NK cells, and the subsets only partially overlap (Wong et al., 1991; Mason et al., 1995; Brennan et al., 1996).

Some of the members of the Ly49 family of receptors, including Ly49A, Ly49C, Ly49G2, and Ly49I, contain a cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) that is phosphorylated upon receptor cross-linking, resulting in the recruitment of a SHP-1 phosphatase. This phosphatase dephosphorylates intermediates in the activating kinase cascade and thus target cell lysis is inhibited (D'Ambrosio et al., 1995; Olcese et al., 1996; Mason et al., 1997; Nakamura et al., 1997). It has been demonstrated that Ly49A transmits inhibitory signals from H2-D^d and D^k, Ly49G2 from D^d and L^d, and Ly49C and Ly49I from K^b and D^k MHC class I molecules (Davis and Bjorkman, 1988; Karlhofer et al., 1992; Daniels et al., 1994; Kane, 1994; Mason et al., 1995; Stoneman et al., 1995; Tormo et al., 1999). Alternatively, another Ly49 family member, Ly49D, lacks a cytoplasmic ITIM, and cross-linking of Ly49D activates intracellular kinase activity, calcium mobilization, and redirected lysis of FcR^+ targets (Mason et al., 1996, 1997, 1998; Raziuddin et al., 1998).

Thus, F_1 hybrids reject parental bone marrow cells, and MHC class I normal hosts reject MHC class I-deficient bone marrow cells (Cudkowicz & Bennett, 1971, Bennett, 1987; Bix et al., 1991). H2-D^d transgenic D8 mice reject nontransgenic (but otherwise syngeneic) B6 bone marrow cell grafts in an NK-mediated fashion, demonstrating that lack of a single MHC class I molecule expressed by the host, but not by the target cells can result in susceptibility to rejection (Hoglund et al., 1988; Ohlen et al., 1989; Johansson et al. 1997). *In vitro* studies demonstrate that Ly49C/I-expressing F_1 ($H2^{d/b}$) NK cells lyse BALB/c ($H2^d$) lymphoblasts, but lyse B6 ($H2^b$) lymphoblasts only if the interaction between Ly49C/I and H2K^b is interrupted by antibodies against either of these

two molecules (Yu et al., 1996). This suggests that the inability of a target cell class I to interact with inhibitory Ly49 receptors expressed on a particular NK subset can account for rejection on the basis of the missing self hypothesis.

Evidence suggests that as NK cells develop, their potential to initiate expression of specific Ly49 receptors becomes restricted, and once they initiate expression of a specific Ly49 receptor they are less likely to initiate expression of a different Ly49 receptor (Roth et al., 2000). However, further evidence suggests that specific Ly49 levels are not fixed (Williams et al., 2000) but can be changed in mature NK cells when they are exposed to a changed class I MHC environment, allowing an adaptation of MHC class I products (Kase et al., 1998). These characteristics of the relationship between Ly49 and MHC allow for the shaping of the NK cell repertoire and the maintenance of self-tolerance. Auto-aggressive NK cells not recognizing MHC class I are believed to be silenced, undergoing anergy, ensuring that each NK cell expresses at least one self-specific Ly49 receptor (Raulet, 1999).

FcgammaR.

As previously mentioned, an activating NK cell membrane receptor is the F_c gamma receptor (FcgammaR) involved in ADCC (Eischen et al., 1996). FcgammaR exists in a single form in mice (FcgammaIIIR) and humans (CD16) but has been shown to exist in several isoforms in rats (Farber & Sears, 1991).

NK 1.1.

NK1.1 (NKR-P1A, B, C), one of the three mouse homologues of the rat NKR-P1 receptor family, is another activating receptor encoded by the NKC on distal mouse chromosome 6 (Giorda et al., 1990, 1992; Yokoyama et al., 1991).

fas/fasL.

NK cells are also able to kill target cells via fas/fasL interactions (Arase et al., 1995; Montel et al., 1995a, b; Berke, 1997) independent of traditional cell-mediated cytotoxicity mechanisms (Montel et al., 1995a, b), as well as through TNF related apoptosis inducing ligand (TRAIL) (French & Tschopp, 1999). Fas antigen is a cell surface signal molecule that induces nuclear condensation and DNA cleavage upon binding its ligand (fasL) (Kagi et al., 1994b; Lowin et al., 1994b; Oshimi et al., 1996) and whose expression has been demonstrated to be upregulated after FcR activation (Eischen et al., 1996). FasL has been identified as a member of the tumor necrosis family (Lynch et al., 1994).

It has been shown that tumor cells may induce cell death in activated NK cells but not in non-activated NK cells (Taga et al., 1996). This presents a novel mechanism by which potential target cells may elude NK cell mediated killing by expressing functional fasL and inducing apoptosis in the attacking, activated NK cell (Griffith & Ferguson, 1997).

NK Receptor Signaling Pathways

Several intracellular mechanisms are believed to mediate the effect of NK receptors, be they activating or inhibitory. Recent evidence suggests a direct association with a Ras-independent mitogen-activated protein kinase (MAPK) signal pathway (Wei et al., 2000).

ITIMs & ITAMs.

The family of immunoreceptor tyrosine-based inhibitory motifs (ITIMs), along with corresponding immunoreceptor tyrosine-based activating motifs

(ITAMs), have been shown to play important roles in the intracellular signaling upon NK cell receptors binding their ligands. Respectively, these responses mediate inhibitory or activating effects on NK cell cytotoxicity (Isakov, 1997; Lanier, 1998; Bakker et al., 2000).

ITIMs, upon binding their ligands, are known to mediate the recruitment of SH2 domain-bearing tyrosine phosphatases-1 (SHP-1) in their cytoplasmic domains, with the tyrosine residue being critical for this interaction (Motoda et al., 2000). A common pathway of activatory signaling is mediated by immunoreceptor tyrosine-based activating receptors (ITAMs) (Cambier, 1995; Flaswinkel et al., 1995; Yao et al., 1995).

KIRs & KARs.

A family of killer-cell-inhibitory receptors (KIRs) has been identified in humans, mediating intracellular signaling pathways through ITIMs. Killer cell inhibitory receptors (KIR) are type I membrane glycoproteins of the Ig superfamily expressed on the surface of natural killer (NK) cells and some T lymphocytes (Moretta et al., 1996; Lanier, 1997). KIR recognize HLA class I major histocompatibility complex (MHC) molecules on target cells and antigen-presenting cells and in so doing inhibit NK and T cell-mediated target cell lysis and T cell cytokine production (Moretta et al., 1996). In humans killer cell activatory receptors (KARs) have also been identified which activate NK cells upon binding MHC class I through associated KAR-associated polypeptides (KARAPs) that are phosphorylated on tyrosine and serine residues (Blery et al., 2000).

NK Cell Cytotoxic Mechanism

The NK cell mechanism of killing is a perforin dependent cytotoxicity involving the release of perforin-containing intracellular granules from the NK cell acting to kill the target cell (Podack et al., 1991; Berke, 1994; van den Broek et al., 1995, 1996; Kagi et al., 1996). These elements are necessary for the crucial NK cell control of tumor metastasis (Smyth et al., 1999). The exocytosed intercellular NK cytotoxic granules have been shown to contain many factors important in the cytolytic capability of NK cells, among them perforin (Podack & Konigsberg, 1984; Criado et al., 1985; Massonn & Tschopp, 1985; Podack et al., 1985), granzymes (Krahenbuhl et al., 1988; Jenne et al., 1988; Jenne & Tschopp, 1988; Krahenbuhl & Tschopp, 1990), and calreticulin (Dupuis et al., 1993; Andrin et al., 1998; Fraser et al., 1998, 2000).

Perforin.

Perforin compromises the integrity of the target cell membrane acting as a pore-forming protein, mediating effects similar to the cell membrane compromising adenovirus (Masson & Tschopp, 1985; Young et al., 1986a, b; Lowin et al., 1994a; Walsh et al., 1994; Kagi et al., 1995). Evidence shows that purified perforin, in contrast to whole NK granules, does not by itself mediate target cell cytotoxicity involving DNA damage (Duke et al., 1989; Felzen et al., 1994), but may be singly responsible for an induced target cell necrosis (Podack et al., 1991; Henkart, 1994; Liu et al., 1995; Kagi et al., 1996). However, DNA degradation is only seen when targets are pretreated with detergent or perforin, suggesting perforin, or other pore-forming agents, are essential to allow target cell DNA damage and cytotoxicity (Jans et al., 1996; Moretta, 1997; Pinkoski et

al., 1998; Trapani et al., 1998b; Blink et al., 1999; Browne et al., 1999). In fact, induction of target cell death by NK cytotoxic granules can be blocked completely by anti-perforin antibodies, indicating that perforin is essentially involved in this process (Lehmann et al., 2000). This evidence suggests a novel escape mechanism for target cells, not by impairing NK cell activation, but by being resistant to perforin and other molecules within the cytotoxic granules of NK cells.

Granzymes.

At least 11 members of the granzyme family of proteins have been discovered to date (Kam et al., 2000). NK cells have been shown to constitutively express granzyme A and B (Velotti et al., 1992). Granzyme B (GrB) is the most thoroughly investigated to date and believed to be the primary molecular apoptotic mediator of cytotoxic T cell and NK cells (Masson & Tschopp, 1987; Simon et al., 1997; Pinkoski et al., 1998; Trapani et al., 1998a; Blink et al., 1999; Shi et al., 2000). GrB is a unique aspartic acid-cleaving serine protease, involved in the activation of procaspases to their functional form (Andrade et al., 1998; Kam et al., 2000). Activated caspases do indeed induce apoptosis within cells and act to effect fatal DNA damage (Duriez & Shah, 1997), although some evidence demonstrates that caspase activation is not essential for target cell killing in response to perforin and granzyme B (Trapani et al., 1998a). Granzymes, notably GrB, thus activate the target cells programmed cell death machinery promoting DNA fragmentation (Heusel et al., 1994; Shresta et al., 1995; Andrade et al., 1998; Trapani et al., 1998a). The search continues for natural substrates for the other granzymes using synthetic substrates and their

known primary substrate specificities, tryptase, aspase, metase or chymase (Kam et al., 2000).

Calreticulin.

As mentioned, the cytotoxic granules released by NK cells have also been shown to contain calreticulin. Calreticulin is a chaperone protein of the endoplasmic reticulum (ER) and is the only resident ER protein to be found in the cytotoxic granules (Dupuis et al., 1993; Andrin et al., 1998). Its role appears to be one of controlling osmotic lysis mediated by perforin (Dupuis et al., 1993; Andrin et al., 1998; Fraser et al., 1998, 2000). In fact, at appropriate concentrations, calreticulin can completely block perforin-mediated lysis (Fraser et al., 2000). Calreticulin stabilizes membranes to prevent polyperforin pore formation (Dupuis et al., 1993; Andrin et al., 1998; Fraser et al., 1998, 2000).

PAF.

The choline phosphate-containing lysolipid platelet activating factor (PAF) also appears to be important in perforin-induced target cell membrane damage (Gebhardt et al., 1988; Mandi et al., 1989). PAF binds to perforin and agonistically interacts with phospholipids containing a choline phosphate headgroup, as well as target cell membrane PAF receptors (PAFr) (Honda et al., 1991; Nakamura et al., 1991; Kunz et al., 1992), to induce membrane target cell damage. In fact evidence exists suggesting that IFNγ release by activated NK cells, will upregulate target cell expression of PAFr, and render target cells more sensitive to NK perforin lysis (Berthou et al., 2000). Cells able to downregulate PAFr expression thus present another novel escape mechanism from NK lysis (Berthou et al., 2000).

The above events of perforin release and granule exocytosis have been shown to rely exclusively on the successful accomplishment of NK cell-target cell binding (Roder et al., 1978a, b; Carpen et al., 1981, 1982; Trinchieri et al., 1981a, b; Hiserodt et al., 1982; Timonen et al., 1982; Carpen, 1985). This fact is underscored by evidence showing that in elderly individuals it is a decreased ability of NK cells to bind successfully to their target that inhibits proper and successful NK cytotoxicity (Albright & Albright, 1985; Dussault & Miller, 1994).

NK binding to targets is energy independent (can occur at 0°C) (Roder et al., 1978b) and cytotoxicity requires Ca^{2+} and Mg^{2+}, both necessary for conjugation (Carpen et al., 1981; Hiserodt et al., 1982; Carpen, 1985). Upon binding, the Golgi, microtubule organizing center and dense cytoplasmic granules move towards the target cell interface and the granules are released into the intercellular space (Carpen et al., 1982; Geiger et al., 1982; Henkart & Henkart, 1982; Hiserodt & Beals, 1985; Kupfer et al., 1986; Atkinson & Bleackley, 1995). Ca^{2+} mobilization and phosphoinositide turnover are also induced upon target binding (Cassatella et al., 1989; Hommel-Berrey et al., 1991). Further, IL-2R gene transcription begins, as well as genes for IFNγ and TNFα production (Anegon et al., 1988; Ben-Aribia et al., 1989; Ortaldo et al., 1990; Trotta et al., 1996).

Thus, induction of target cell apoptosis has been shown to be the main effective mechanism of killing by activated NK cells. Target cell death is characterized by rapid condensation of chromatin, nuclear membrane blebbing and rapid DNA breakdown.

In whole, it is believed, and has been shown, that it is the net effect of the numerous activating and inhibitory signals acting on NK cells, upon binding their respective ligands, that determines their activation and corresponding cytotoxicity, or inhibition, perhaps including anergy.

Cytokines and Chemokines and NK cells

NK cells produce and respond to many different factors *in vivo* and *in vitro*. Cytokines and chemokines are families of proteins that mediate their effects on NK cell development and function, either directly, through NK cell surface receptors, or indirectly, through the production of NK enhancers released by other cell types upon recognition of the cytokines or chemokines. It has been shown that NK cells are influenced by the cytokines produced by macrophages, for example, because they produce several of the cytokines that activate NK cells (Chantry et al., 1990; Liesveld et al., 1991; Thomassen et al., 1991; Collins & Bancroft, 1992; Mazzarella et al., 1998). In fact, work has shown that blocking macrophage activity will inhibit NK cell activity, both *in vivo* and *in vitro* (Djeu et al., 1979; Moore et al., 1993; Xu et al., 1998a). As well, NK cells have the ability to produce a wide profile of cytokines when activated, including TNF-a (Peters et al., 1986; Cuturi et al., 1987), IL-2 (Kasahara et al., 1983; Ortaldo, 1986) and IFN-g (Handa et al., 1983; Kasahara et al., 1983).

The cytokines are a heterogeneous group of proteins that regulate immune and inflammatory reactions, as well as hematopoiesis. This group includes the interleukins (IL), tumor necrosis factor (TNF), interferon (IFN), and colony-stimulating factors (CSF).

IL-2.

The members of the interleukin family of proteins are signaling peptides of which at least 18 are known to date. Many members of the IL family directly or indirectly influence NK cells and therefore affect NK cell development or function, either activatory or inhibitory. Perhaps the most crucial of the interleukin family of proteins involving the augmentation of NK cell development, numbers, and activity is IL-2.

IL-2 is a powerful NK enhancer, inducing increases in NK lytic activity (Henney et al., 1981; Lotzova et al., 1987) and proliferation (Suzuki et al., 1983; Ortaldo et al., 1984; Trinchieri et al., 1984; Lanier et al., 1985; Piguet et al., 1986; Riccardi et al., 1986b; Biron et al., 1992) through interaction with NK cell surface IL-2 receptors (IL-2R) (Shirakawa et al., 1986; Vitte-Mony et al., 1992). As well, IL-2 induces IFN-g production (Biron et al., 1992), increases target-binding ability (Uchiyama et al., 1991), enhances NK expression of ICAM-1 among other adhesion molecules (Triozzi et al., 1992), and induces the secretion of other cytokines besides IFN-g (Trinchieri et al., 1984; Phillips & Lanier, 1986; Cuturi, 1987; Cuturi et al., 1989; Trinchieri, 1989). The IL-2 receptor (IL-2R) consists of at least four parts; two alpha chains, one beta chain, and one gamma chain (Voss et al., 1992; Dmoszynska & Rolinski, 1995) and may exist as a soluble factor as well as in its membrane bound form (Wagner et al., 1990; Hanninen et al., 1991). NK cells possess cell surface receptors for IL-2 (Phillips et al., 1989; Voss et al., 1992; Dmoszynska & Rolinski, 1995).

IL-12.

IL-12 (NK stimulatory factor), produced by cells of the monocyte/macrophage lineage (Hayes et al., 1995; Fan et al., 1996), increases perforin and granzyme B gene transcription in NK cells and thus could be important in effective antineoplastic therapies (Salcedo et al., 1993; De Blaker-Hohe et al., 1995; Argentati et al., 2000). In fact, IL-12 has been shown to be important in activation of T cytotoxic, NKT and NK cells (Chehimi et al., 1992; Lauwerys et al., 2000; Smyth et al., 2000), inhibition of angiogenesis (Yao et al., 1999), as well as the induction of IFN-gamma production (Tannenbaum et al., 1996; Lee et al., 2000), and thus functions as a potent anti-metastatic agent (Kodama et al., 1999; Schultz et al., 1999). IL-10 inhibits IFN-g production by suppressing accessory cell IL-12 production (D'Andrea et al., 1993) and thus IL-10 is an indirect, potent suppressor of NK cells (D'Andrea et al., 1993; Moore et al., 1993). Evidence demonstrates that IL-12 induced NK and NKT cytotoxicity is mediated by adhesion to target cells through LFA-1 (CD11a/CD18) (Matsumoto et al., 2000). NK cells possess cell surface receptors for IL-12 (Desai et al., 1992; Gillessen et al., 1995; Wu et al., 1996).

IL-15.

IL-15 is a 14-15kDa member of the alpha-helix bundle family of cytokines that plays crucial roles in the development, survival, and function of NK cells (Carson et al., 1994, 1997; Waldmann and Tagaya, 1999), as well as determining the final fate of bipotential T/NK cells along with the participation of IL-2 (Leclerq et al., 1996). This is underscored by evidence showing that defects in, or blocking the activity of chains of the IL-2R results in the inhibition of IL-15

induced NK cell production (Carson et al., 1994; Ohteki et al., 1997; Suzuki et al., 1997). As well, mice deficient/defective for the alpha chain of IL-2R do not develop T or NK cells (Carson et al., 1997). IL-15R shares the beta and gamma chains of the IL-2R and they appear to be of the same family of molecules (Carson et al., 1994; Chae et al., 1996; Carson & Caligiuri, 1998). IL-15 also appears to stimulate expression of Ly49 (Puzanov et al., 1996; Williams et al., 1999).

IL-2 and IL-15 both require the beta and gamma chain of the IL-2R for binding and signal transduction, although they do not share complimentarity with respect to their recognition by their receptors (Chae et al., 1996). Only IL-2 is able to bind to IL-2R and IL-15 only to IL-15R (Chae et al., 1996). The gamma chain of IL-2R is shared by the IL-4R, IL-9R and IL-15R (Russell et al., 1993; 1994; Kondo et al., 1994; Schumann et al., 1996).

IL-18.

IL-18 is produced by activated macrophages (Okamura et al., 1995, 1998) and has only recently been identified (Ushio et al., 1996). Formerly named IFN gamma inducing factor (IGIF) (Nakamura et al., 1989), it has been shown to have structural and functional similarities to IL-1 (Dinarello, 1999b) including NK activation and T helper cell regulation (Adachi et al., 1998; Okamura et al., 1998; Dinarello, 1999a). Evidence shows that it plays an important role in augmenting T helper cell type 1 (Th1) activity, resulting in the ability to induce IFN gamma production in T cells and NK cells (Micallef et al., 1996; Ahn et al., 1997; Kohno et al., 1997) and thus mice deficient in IL-18 have suppressed IFN gamma

production (Takeda et al., 1998). In fact, Th1, but not Th2 cells, express receptors for IL-18 (Xu et al., 1998b; Yoshimoto et al., 1998).

IL-18 induces gene expression and synthesis of TNF (Puren et al., 1998; Netea et al., 2000), IL-1 (Puren et al., 1998; Netea et al., 2000), fas ligand (Dao et al., 1996; Tsutsui et al., 1996), and several chemokines (Fehniger et al., 1999; Wang et al., 1999; Reznikov et al., 2000) in numerous cell types through their IL-18 receptor complex (IL-18R). The IL-18 receptor is comprised of a binding chain (IL-18R alpha) and a signaling chain related to the IL-1R family (Parnet et al., 1996; Torigoe et al., 1997; Born et al., 1998).

Interferon (IFN).

The interferons are a class of cytokines profoundly affecting innate immune function and are in common use for the clinical treatment of viral diseases and cancer. IFNs, and their inducers, i.e., polyinosinic-polycytidylic acid, were among the first factors identified as being NK cell enhancers (Gidlund et al., 1978; Djeu et al., 1979; Herberman et al., 1979). The IFN family of proteins is comprised of two types, type I members (IFN-alpha, -beta, -omega and -tau) (Gross et al., 1981; Weissmann et al., 1982; Adolf, 1990; Li & Roberts, 1994) and a type II member (IFN-gamma) (Gray & Goeddel, 1983). NK cells mainly produce IFN-gamma upon activation (Arase et al., 1996; Rottenberg et al., 2000) but are also able to produce the type I IFNs as well (Nocera et al., 1985). Evidence demonstrates that mice with targeted mutations in the STAT1 gene, an essential mediator of IFN signaling, have decreased NK cell activity (Lee et al., 2000). In addition to their desired immune effects in therapeutic use,

IFNs have prominent toxic effects including neurotoxicity, when exogenously administered (Menkes et al., 2000).

TNF-alpha.

Tumor necrosis factor alpha (TNF-a), a member of a rapidly growing superfamily of cytokines, is the product of activated monocytes and macrophages (Bemelmans et al., 1996; Branch & Guilbert, 1996), among other cell types. Indeed, NK cells themselves can produce TNF-a (Murphy et al., 1992; Jewett & Bonavida, 1993; Bemelmans et al., 1996). This multi-functional cytokine mediates its activity by binding to cell membranes in a receptor-ligand fashion (Ding & Porteu, 1992; Grell et al., 1993) and directly stimulates/activates NK cells (Degliantoni et al., 1985; Yang et al., 1990; Chan et al., 1991; D'Andrea et al., 1992). As a pleiotropic regulator of hemopoiesis, TNF-a induces a variety of responses in cells other than NK cells, ranging from functional activation of the target cell, to proliferation, differentiation, and apoptosis (Slordal et al., 1989; Furmanski & Johnson, 1990; Sidhu & Bollon, 1993; Mayani et al., 1995; Selleri et al., 1995; Walczak et al., 1999). TNF-a alone, however, has a transient anti-tumor effect, with re-growth of the tumor soon resulting (Lejeune, 1995). However, it has been well established (Aggarwal et al., 1985; Fiers et al., 1986; Johnson et al., 1988; Sohumra, 1998) that profound tumor amelioration can occur in tumor-bearing humans and mice, if TNF-a is combined with interferon-gamma (IFN-g).

TGF-B.

Transforming growth factor-beta belongs to a family of polypeptide growth factors that have a fundamental role in cell growth and differentiation (Sporn et

al., 1987). Lymphocytes, as well as most mammalian cells, have the ability to produce this molecule and possess receptors for it (Harpel et al., 1992). TGF-beta inhibits the proliferation and differentiation of many immune cells, including NK cells (Kasid et al., 1988; Ortaldo et al., 1991; Su et al., 1991), while also inhibiting cytotoxicity and antibody production (Wahl, 1994). It has also been shown to induce the proliferation and differentiation of $CD8^+$ T cells in mice (Swain et al., 1991; Lee & Rich, 1993).

Colony Stimulating Factors (CSF).

Briefly, both macrophage-colony stimulating factor M-CSF (Misawa et al., 2000) and granulocyte/macrophage-colony stimulating factor GM-CSF (Sitnicka & Hansson, 1992; van den Bosch et al., 1995) have been found to increase the proliferation and activity of NK cells, while G-CSF has been found to be inhibitory (Rondelli et al., 1998).

Chemokines.

Chemokines, i.e., macrophage chemotactic protein (MCP), have been shown to be important in the adhesion and migration of NK cells as well as other leukocytic cell types into inflammatory sites (Allavena et al., 1991, 1994a, b, 1997; Taub et al., 1995; Loetscher et al., 1996). The family of chemokine molecules has at least 4 sub-families designated gamma (C), beta (CC), alpha (CXC) and delta (CX3C), depending on the presence of amino acids separating the cysteine residues in their N-terminal region. Chemokine induced killing by NK cells has been shown to require the binding of NK to target cell, and suggested to involve the release of granule-derived serine esterases (Taub et al., 1995).

NK Cell Enhancement

NK cells possess receptors for members of the cytokine family of proteins and have the ability to be directly enhanced by these factors. As well, for this reason, they are sensitive to the cytokine profile of other cell types, i.e., macrophages and T helper cells, which can produce these cytokines. The following section will focus on selected compounds utilized in this thesis that have the ability to directly enhance NK cells, or indirectly, through the cytokine profile released by other cell types, i.e., macrophages and T helper cells, when exogenously administered.

Melatonin.

The neuroendocrine-immune system axis is one that has gained considerable attention in recent years. Among the neuromodulators of the various components of immunity is the pineal hormone, melatonin, (N-acetyl-5 methoxytryptamine), a biogenic indoleamine long known to act as a chronomodulator in biological systems, acting to functionally synchronize eukaryotes with the photoperiod. Endogenous MLT production follows a strict circadian rhythm, being maximally produced during the dark periods of the 24 hr cycle, being broken down soon after production, having species-dependent (hamster, rat, dog, monkey, human), and even age-dependent half lives in the blood ranging from 7.5-59 minutes (Mallo et al., 1990; Pang et al., 1990; Cavallo & Ritschel, 1996; Yellon, 1996; Yeleswaram et al., 1997; Brown et al., 1997). The more recently recognized interactions of melatonin with the immune system primarily have dealt with its effects on cells mediating specific immunity, primarily

T helper lymphocytes (Cutando & Silvestre, 1995; Gonzalez-Haba et al., 1995; Garcia-Maurino et al., 1997; Lissoni et al., 1998; Maestroni, 1998a, b, c; Rafii-el-Idrissi et al., 1998), and functional T lymphocyte enhancement in the periphery (Pioli et al., 1993; Champney et al., 1997; Garcia-Maurino et al., 1997; Demas & Nelson, 1998). Evidence, however, is even more scant with respect to the interplay of melatonin and another prominent cell lineage mediating non-specific immunity, i.e., natural killer (NK) cells, which act in the first line of defense against tumorigeneic and virus-infected cells.

In the presence of MLT, in old mice, the thymus and T cell mediated immune functions in the periphery recovered to young adult levels (Mocchegiani et al., 1996). MLT has also been shown to have anti-apoptotic properties among normal (non-tumor) cells (Maestroni et al., 1994a, b; Maestroni, 1998a), while another advantage attributed to this neurohormone is its ability to counter the toxicity of conventional anti-cancer therapeutic agents (Maestroni et al., 1994a, b; Cutando & Silvestre, 1995; Lissoni et al., 1995, 1997 a, b, c). Moreover, MLT itself has no known side effects (Lissoni et al., 1996, 1998; Wichmann et al., 1996), and has no stimulatory influence on tumor cell growth (Maestroni et al., 1994a, b).

MLT, by itself, appears to act on sub-cellular events pertaining to cell function, having no *direct* role in stimulating proliferation of any hemopoietic or immune cell lineage studied to date (Provinciali et al., 1997; Rogers et al., 1997). Moreover, MLT has no direct affinity for B cells, the latter not possessing MLT receptors, while other evidence indicates that MLT does not enhance humoral immunity (Gonzalez-Haba et al., 1995, Champney et al., 1997; Demas &

Nelson, 1998). MLT has a role in general hemopoiesis stimulation as well (Maestroni & Conti, 1996; Maestroni, 1998 a, b, c). Receptors for MLT have been found on cells of the monocyte/macrophage lineage and the binding of MLT to these receptors results in increased production of GM-CSF, enhanced antigen presentation (spleen macrophages), and increased macrophage function (Pioli et al., 1993; Lissoni et al., 1994; Maestroni et al., 1994a, b; Garcia-Maurino et al., 1997; Williams et al., 1998).

With respect to any influence of MLT on NK cells, there is some evidence in mammals, which indicates that MLT *in vivo* produces enhanced peripheral (mature) NK cell-lytic function (Poon et al., 1994). Although, it is completely unknown whether this results from direct mechanisms, i.e., receptor dependent interactions between MLT and NK cells, or, indirect mechanisms, i.e., via T helper cell-enhanced IL-2 production, the latter being a well known, exquisite stimulant of NK cells (Christopher et al., 1991; Naume & Espevik, 1991; Biron et al., 1992).

MLT production is progressively reduced with advancing age (Pierpaoli, 1991; Pierpaoli et al., 1991; Cavallo & Ritschel, 1996; Lesnikov & Pierpaoli, 1996; Inserra et al., 1998), just as are the cells of the immune system. Thus, the potential clinical importance is apparent for maintaining prophylactically, youthful levels both of MLT and of cells mediating immunity, especially those which act in the first line of defense in tumor immunosurveillance, i.e., NK cells and monocytes.

Echinacea purpurea.

Variously prepared extracts of the roots and other parts of *Echinacea*

species *(E.angustifolia, E. purpurea)*, have gained considerable interest recently for their reported health benefits including amelioration of an assortment of pathologies such as inflammations, bacterial/viral infections, tumors and AIDS (Stimpel et al., 1984; Tragni et al., 1985; Lersch et al., 1990; 1992; Roesler et al., 1991a, b; Steinmuller et al., 1993; Hill et al., 1996). While all parts of Echinacea species plants appear to have medicinal value, the roots are most concentrated in medicinal properties. These species were originally used by the native Americans for the treatment of insect and animal bites and assorted skin irritations, given that at least part of the healing values of Echinacea species derives from its potent anti-inflammatory qualities (Tragni et al., 1985; Hill et al., 1996). Although herbal medicine was practiced by the American physicians in the 19th and early 20th centuries, Echinacea was never approved by the American Medical Association since rigorous experimental evidence of its medicinal efficacy did not exist, and, in fact, the healing properties of this herb were virtually forgotten with the development of antibiotics (Combest and Nemecz, 1997). Subsequently, however, with the debut of the science of immunology concomitant with an assortment of techniques for measuring the functional responses of different immune cells, at least in culture, herbs such as Echinacea were re-discovered, and immune-stimulation was advanced as a possible mechanism for their medicinal value in so many disease-defense processes. During the past 2 decades, much of the concentrated effort in studying the medicinally relevant herbs has been aimed at biochemically dissecting out the many and varied chemical compounds which, individually, may act uniquely on specific immune cells. These relatively exhaustive studies have

indicated that such compounds include: high molecular weight polysaccharides such as arabinogalactan, inulin, heteroxylan, essential oils such as germacrene, vanillin, humulene, borneol; the alkylamides such as echinacein, isobutylamides (penta- and hexa-decadienes); polyacetylene, tannins, Vitamin C and flavonoids.

It appears that at least one family of active ingredients in these plants is the alkamides, shown to inhibit 5-lipoxygenase and cyclooxygenase (Wagner et al., 1989; Muller-Jakic et al., 1994), key enzymes in the production of prostaglandin. Prostaglandins are inhibitory to natural killer (NK) cells. On the other hand, acid arabinogalactan, a 75,000 MW polysaccharide extractable from *Echinacea* species, was shown to be effective against *Leishmania, in vitro,* via direct stimulation of macrophages, cytotoxic for these microorganisms (Leuttig et al., 1989). While *Echinacea* species contain a host of other physiologically beneficial components (above), some of their most important immunoenhancing elements may be those that interfere with prostaglandin formation.

It has recently been found, that *in vivo* administration of inhibitors of prostaglandin such as indomethacin, significantly stimulated NK cells in leukemic mice, concomitant with cure and/or significantly longer life span (Christopher et al., 1991; Dussault & Miller, 1993). The literature is completely deficient in information which records and/or quantifies the behaviour of hemopoietic and immune cells, *in vivo,* during short or long term *Echinacea* exposure. Nothing is known of the population dynamics *in vivo* of monocytes or NK cells (first lines of defense in viral infections or tumor growth) in animals or humans given either whole plant or purified extracts of *Echinacea* species, under controlled conditions of exposure time and environment. To date, various extracts from these plants

have been assessed for their ability to stimulate, directly or indirectly, the function of macrophages (animal and human) *in vitro/in vivo* (Stimpel et al., 1984; Leuttig et al., 1989; Wagner & Jurcic, 1991; Fry et al., 1998). Similar assays show little or no change in animals or humans (peripheral blood), in the functional activity of T or B lymphocytes in the presence of *Echinacea* (Stimpel et al., 1984; Leuttig et al., 1989; Roesler et al., 1991b; Elsasser-Beile et al., 1996). There is some *in vitro* evidence that NK cells from animals, and from the blood of normal and AIDS-inflicted humans, can be augmented functionally by extracts from *Echinacea* species (Lersch et al., 1990; See et al. 1997).

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MATERIALS AND METHODS

Animals.

Mice. Male DBA/2 mice (Charles River Laboratories, St. Constant, QC, Canada) were used for all experiments. The data from each Chapter are obtained from separate groups of mice used exclusively for experiments in that Chapter. All experimental and control mice were housed under identical microisolator conditions, remaining unmanipulated for a period of at least 1 week (wk) before initiating experiments. Sentinel mice in the Facility regularly demonstrate the complete absence of all common mouse pathogens.

Young Adult Mice. Mice were obtained at 5 weeks of age, and housed under laminar flow conditions in the Animal Care Facilities of McGill University. For work in Chapter 7, all mice remained healthy until 10 wk of age at which time tumor was initiated.

Bone Marrow Donors and Recipients. For work in Chapters 1 and 2, young adult male DBA/2 mice served as tumor-bearing, irradiated bone marrow transplant recipients, as well as normal donors of syngeneic bone marrow.

Aged Mice. For work in Chapter 9, male DBA/2 mice (Charles River Laboratories, St. Constant, QC, Canada) were used at approximately 15-16 months (mo) of age. Mice of this strain and sex have a life span, in health, of 18-24 mo., with 50% surviving 4-6 mo. beyond this. Using an average life span of 21 mo., and an average 50% additional possibility of 5 mo., then our mice of 15-16 mo., represent mice aged in the last one-third to one-quarter of their lives.

Mice were purchased at 8 wk of age and maintained under constant conditions in the McGill University Animal Care Facility where they aged until use.

Tumor Cell Line.

Erythroleukemia Cells. Friend-virus-induced erythroleukemia cells (American Type Culture Collection, Camden, NJ) were maintained in vitro at 37°C, 100% humidity and 5% CO_2, in Basal Medium Eagle (BME), supplemented with 15% Cellect Gold Serum, 2% essential amino acids, 2% non-essential amino acids (Flow Laboratories, Mississauga, ON), L-glutamine (GIBCO BRL Life Technologies, Grand Island, NY), 3% sodium bicarbonate, 7.5M (Sigma Chemical Co., St. Louis, MO), and 1% HEPES (Boehringer Mannheim, Montreal, QC, Canada), at a concentration of 2M. These *in vitro*-maintained erythroleukemia cells served as the stock from which cells were extracted. Some tumor cells were lethally irradiated prior to injection for purposes of immunizing hosts, and other viable tumor cells were injected for the purpose of tumor initiation.

YAC Cell Line. The tumor cell line used as the *in vitro* target for Natural Killer cells in Chapter 9 was the Moloney virus-transformed A/Sn mouse strain-derived YAC-1 lymphoma cells. YAC-1 cells were maintained in logarithmic suspension culture under normal culture conditions.

Tumor Cell Irradiation.

For work in Chapter 8, three hundred million viable, FLV-induced erythroleukemia cells were irradiated under sterile conditions with 9.2 Gy; 115

rads/min for 8 minutes. Cells were then washed three times in phosphate buffered saline (PBS) at pH 7.2, by centrifuging at 250 g for 5 minutes. Subsequently, 3×10^6 irradiated (killed) tumor cells were injected in 0.1 ml sterile PBS via the lateral tail vein. Irradiated cells were replated and incubated for 14 days, under standard culture conditions (above) for maintaining viable erythroleukemia cells. Of the total cells plated, viability was below 0.5% at 14 days of incubation.

Tumor Cell Injection.

For the purposes of transplanting tumor, mice were aseptically injected with $3x10^6$ viable tumor cells in 0.1 ml phosphate-buffered saline (PBS: pH 7.2) via the tail vein. Tumor cells for all experiments were manipulated for injection during their log phase growth, showing a viability of 90% or more. For work in Chapter 8, five weeks after mice received killed tumor cells (above), they were injected with viable cells from the same stock, each mouse receiving $3x10^6$ cells in 0.1 ml sterile PBS via the lateral tail vein.

Poly I:C Administration.

For work in Chapter 1, Poly I:C (Sigma Chemical Co., MO), was administered intraperitoneally at a dose of 125µg/mouse (body wt: 25gm) once daily (early p.m.), beginning 5 days after tumor injection, for mice to be assayed 9 days after irradiation and bone marrow transplantation. This dose, in adult mice, has been well established to be non-toxic and to result in increased NK cell-mediated functional (lytic) activity by 12 -24 hr after administration (Senik et al., 1979). For

longer term studies (3 months post irradiation + bone marrow transplant), mice were injected with poly I:C daily from 1-20 days, and bi-daily from day 21-36, and twice weekly from day 37-90 (time of killing- 3 mo), and for analyses extending through 6 months, mice were injected as above for the first 36 days, and bi-weekly with poly I:C, beginning at day 37-119. Prolonged Poly I:C administration was carried out because it was expected that sustained NK cell enhancement would have, by 4 months (day 119), targeted any quiescent, non-proliferating leukemic cells not succumbing to irradiation and/or earlier recognition/location by host NK cells. No injections were administered from day 120 until the time of killing for final analysis - more than 6 months after irradiation and bone marrow transplant - at day 190. In all cases of poly I:C treatment, control mice were correspondingly injected with vehicle only.

Poly I:C and TNF-a Administration.

For work in Chapter 2, Poly I:C (Sigma Chemical Co.) was administered intraperitoneally in 0.1 ml PBS at a dose of 125 µg/mouse (Bo. Wt.:25 gm) once daily (early p.m.) for four days, beginning at day 5 of tumor growth. After irradiation, and concomitant with Poly I:C injections, some mice also received mouse recombinant (mr)TNF-a (Boehringer-Mannheim Biochemicals, Montreal, QC, Canada) at a dose of 50×10^3U (spec. Act.:6.0×10^7U/mg; MW: 18×10^3) in 0.1 ml vehicle (HEPES)/mouse. Based on previous studies (Bianchi et al., 1988; Bertini et al., 1989; McDonagh et al., 1992; Young & Wright, 1992), we have determined this dose and injection frequency to be the minimal effective, non-toxic dose in mice. Control mice were treated identically, but injected with the

HEPES vehicle only. TNF-a has a half life of 6-7, and 12 minutes in mice and rabbits (Beutler et al., 1985; Bemelmans et al., 1993), the largest proportions occurring in the liver and skin by 8 minutes after injection and rapidly degraded in the tissues by 30 minutes, after which it is cleared primarily by the kidneys (Beutler et al., 1985; Pessina et al., 1987; Bemelmans et al., 1993).

Melatonin (MLT) Administration.

Melatonin (Schiff Products, Inc., Salt Lake City, UT) was administered in the diet, ground homogeneously into pulverized Purina laboratory mouse chow, the standard diet. Mice of identical age, strain and sex were housed 2/cage and provided every evening (6:00 pm) with ground chow with/without (control) melatonin (0.0142 mg/mouse/day). Although the melatonin-containing food was available throughout the 24 hr daily cycle, it was consumed virtually exclusively during the night as evidenced at first light by the presence of empty food bins. Moreover, all mice remained inactive and engaged in the rest/sleep phase during the 12hr photoperiod. The normal 12hr/12hr light/dark cycle in the animal colony was not altered throughout the experimental period. The dose of melatonin/mouse employed here was selected based on existing dose/body weight studies in other strains of adult mouse, and moreover, it compares favorably with dose/body weight studies employing melatonin in adult humans. We established the optimum dose of melatonin, optimum time of day to begin exposure via the chow, and the daily rate of chow consumption per day per mouse (Currier et al., 2000; Yu et al., 2000). Daily ground chow consumption/ mouse/ day closely approximated 5.5 gm, with or without the apparently

undetectable presence of MLT in the chow. In pilot studies, inter-cage variation in food consumption among the experiment diet cages, or among the control-diet cages was ascertained per cage to be insignificant (by measurement) to undetectable at the end of each 24 hr feeding period. With respect to between-mice variations within each cage, it is assumed that the long exposure time (at least 7 days) of drug availability would have canceled out any minor fluctuations in consumption of melatonin by individual mice by the time of experiment conclusion.

Echinacea* purpurea *(E.p.) Administration.

A specific, commercially prepared, powder extract of *Echinacea purpurea* root (Phyto Adrien Gagnon, Sante Naturelle (A.G.) Ltee, La Prairie, QC, Canada) was homogenized in finely ground, standard chow such that each mouse, consumed 0.45 mg/day (dose/body weight adjusted from assorted anecdotal and experimental studies in humans and rodents) and that we have already proven to be effective (Sun et al., 1999). As with the administration of melatonin (above), chow was prepared and given fresh daily following the identical protocol (above). Control mice consumed the same, identically prepared, pulverized chow, without E. *purpurea*. There is, moreover, no maximum dose, at which this "nutriceutical", administered *in vivo* (taken extensively by humans for its prophylactic potential) appears to be toxic as defined by assorted criteria (Mengs et al., 1991, Lersch et al., 1992, Melchart et al., 1995). Moreover, the presence or absence of E. purpurea in the food bins had no influence on the quantity of chow consumed per day. Finally, any *between*-mouse variations in food consumption *within* either

the experimental or control groups would have been canceled by the relative long time period over which these studies occurred (at least 7 days).

MLT and E.p. Administration.

In some experiments (Chapters 5 and 7), mice consumed both melatonin and E. *purpurea* simultaneously homogenized into the finely ground, standard chow, administered as above, simultaneously, with all other parameters unchanged. In pilot studies, inter-cage variation in food consumption among the experimental diet cages (melatonin only, E.p. only, melatonin + E.p.) or among the control-diet cages was ascertained per cage to be insignificant (by measurement) or undetectable at the end of each 24 hr feeding period, and moreover, the consumption between the experimental cage groups (melatonin only, E.p. only or melatonin + E.p.), and the control diet cage groups did not vary. With respect to between-mice variations in each experimental cage, it is assumed that the long exposure time (at least 7 days) of agent(s) availability, would have canceled out any minor fluctuations in consumption of the agent(s) by these identical mice by the time of experiment conclusion. For work in Chapter 7, mice were given fresh ground chow with/without (control) melatonin from day 0 after tumor onset. Some mice were killed on day 9 thereafter, while other mice, to be taken at 3 months post tumor onset, continued to receive melatonin daily from day 0 (time of tumor onset) - day 50, after which their diet until 3 months was identical to control (pulverized chow only).

Arabinogalactan Administration.

Arabinogalactan (Sigma Chemical Co., St. Louis, MO) was injected intraperitoneally once daily for 7 or 14 days (500µg/0.1ml 7.2 ph PBS per mouse). Control mice were given the PBS vehicle only, following the above injection regimen.

Thyroxin Administration.

For work in Chapter 9, thyroxin (Sigma Chemical Co., St. Louis, MO), was administered to mice bi-daily for 10 days as 5 intraperitoneal injections (0.2µg/0.1ml 7.2 pH PBS), following a previously established protocol (Provinciali et al., 1991). Control mice were given the PBS vehicle only, following the above injection regimen.

Mouse Irradiation.

For work in Chapter 1, irradiation of tumor-bearing mice was accomplished by means of a Cesium (^{137}Cs) irradiator, delivering 115rad/min, on the fifth day after the first poly I:C injection or its vehicle (control), to leukemic mice. Radiation was delivered in 2 episodes of 4 min each (450rad/episode separated by 4 hr). For work in Chapter 2, one day after the last of 4 daily injections of Poly I:C, all mice, including control, were irradiated using a cesium (^{137}Cs) irradiator delivering 115rad/min, in 2 episodes of 4 min each (450rad/episode separated by 4 hr). TNF-a was then given in 4 daily injections beginning 12 hr after bone marrow transplant. Poly I:C, resuming 12 hr after transplant, continued daily for 8 more days. Thus, for the first 4 days after transplant, irradiated hosts received both

agents. TNF-a control mice (HEPES vehicle-injected), also continued to receive Poly I:C injections as above for 8 post transplant days. One day later (day 9) all mice (TNF-a- and vehicle-injected) were killed by cervical dislocation.

Preparation of Donor Bone Marrow Cells for Transplant.

Normal, unmanipulated, syngeneic mice of the same age and sex as the tumor-bearing recipients served as bone marrow donors. Donor mice were killed by cervical dislocation and both femurs were aseptically removed, flushed repeatedly with RPMI (Roswell Park Memorial Institute) 1640 medium containing 10% millipore filtered fetal calf serum (FCS). Single cell suspensions were layered over 1 ml newborn calf serum (NCS), allowing large particles (non-cellular debris and connective tissue) to sediment into the NCS for 5 min. The supernatant containing the free hemopoietic cells was centrifuged (5 min, 500xg, 4°C), the resulting cell pellet was resuspended in RPMI/FCS and the nucleated cells were counted with an electronic cell counter (Coulter Electronics, Hialeah, FL, USA) after lysing red blood cells with Zapoglobin (Coulter Diagnostics, FL, USA). Twenty million fresh bone marrow cells were injected aseptically in 0.1 ml PBS into each irradiated, leukemic recipient mouse via the tail vein the day following irradiation.

Preparation of Bone Marrow and Spleen Cells for Analysis.

Twenty-four hours following their final treatments (animals killed at different times depending on experimental protocol) mice were killed by cervical dislocation and their spleens and femurs removed aseptically. Spleens were pulverized by

pressing them through stainless steel screens into ice-cold RPMI (Roswell Park Memorial Institute) medium containing 10% millipore-filtered FCS (fetal calf serum). Bone marrow was obtained by removing both femoral extremities and repeatedly flushing the marrow plug from the bones into ice-cold medium. The spleen cells and the bone marrow cell mass were then subjected to gentle repeated pipetting to obtain single cell suspensions of these hemopoietic and immune cells. The suspensions were then layered onto 1 ml FCS to stand for 5 minutes during which time large, non-cellular debris settled to the bottom of the FCS. The upper suspension was then removed and washed by centrifugation for 5 minutes at 4°C and 1100 rpm, to produce a cell pellet. This pellet was then re-suspended in fresh medium and the total numbers of nucleated cells in the spleen and both femurs from each mouse, were counted by means of an electronic particle counter (Coulter Electronics, Hialeah, FL, USA) after lysing red blood cells with Zapoglobin (Coulter Diagnostics, FL, USA). The spleen and bone marrow cell suspensions were then adjusted to $40x10^6$cells/ml for immunostaining.

Immunoperoxidase Labelling of Bone Marrow and Spleen Cells.

Mature NK cells, all of which bear the surface molecule, ASGM-1 (Kasai et al., 1980; Beck et al., 1982; Stout et al., 1987), were processed for microscopic visualization by an indirect immunoperoxidase method in standard use in our laboratory (Christopher et al., 1991; Miller, 1992; Miller et al., 1992; Dussault & Miller, 1993, 1994, 1995a, b; Currier & Miller, 1998; Mahoney et al., 1998; Whyte & Miller, 1998; Sun et al., 1999; Currier et al., 2000). Although activated T blasts

may also bear the ASGM-1 surface molecule, such cells are readily morphologically distinguishable with our techniques, from the mature NK cells of interest. Moreover, our animals are young mice maintained under laminar flow conditions, 2 further conditions for extreme paucity of any such T blasts, especially in the bone marrow where they are virtually never found in healthy, young mammals and rarely found even in older, healthy mammals. The immunoperoxidase method employs the monoclonal primary antibody, rabbit anti-ASGM-1 (Wako Pure Chemicals, Dallas, TX, USA), conjugated to a biotinylated secondary antibody (goat anti-rabbit IgG) (Sigma BioSciences, MO, USA). Briefly, 100µl of cell samples (bone marrow or spleen from each mouse) were incubated with 100µl of primary antibody (concentration 1:40) or normal rabbit serum (control) for 30 min on ice. The cells were then washed twice by centrifugation (each wash: 5 min, 1,000 rpm, 4°C), the supernatant was discarded, and the resulting pellet containing the cells was resuspended in 100µl of RPMI/10%FCS. These cells were then incubated with the biotinylated secondary antibody (concentration 1:100) for 30 min on ice, washed again and resuspended in cytospotting medium (0.009% NaCl, 0.001% EDTA and 0.05% BSA in distilled water). The cells were then cytocentrifuged onto albumin-coated slides. The slides were rapidly air-dried to avoid cell crenation, fixed in ice-cold absolute methanol for 30 min and gradually rehydrated in a 1:1 methanol:PBS(7.2 pH) solution for 10 min followed by 100% PBS, pH 7.2 for 5 min. The slides were then bathed in 3% H_2O_2 (Fisher Scientific, Ontario, Canada) for 10 min at room temperature to block endogenous peroxidase activity, washed, and incubated with the avidin-biotin complex (Vector

Laboratories, Burlingame, Calif., USA) containing biotinylated-peroxidase enzymes for 45 min in humidity chambers. The slides were then washed once, as above, and finally bathed in a diaminobenzidine (chromogenic agent) solution (150 mg of DAB, 300ml PBS (pH 7.6), 100ul, 30% H_2O_2) for 13 min, washed twice in PBS (pH 7.2), air-dried, and stained with MacNeal's tetrachrome hematologic stain.

Identification of NK Cells and Non-NK Hematopoietic Cells.

Immunoperoxidase labeling of the ASGM-1 surface molecule permitted identification of NK cells as being lymphoid cells of small and medium size as well as of NK cells (small and medium lymphoid), by well-established criteria (Djeu et al., 1979; Herberman et al., 1979; Senik et al., 1979; Christopher et al., 1991; Dussault & Miller, 1993, 1994; Miller, 1994; Currier & Miller, 1998) in mice. The proportions of several other morphologically identifiable hemopoietic and immune cell populations (lymphoid cells, nucleated erythroid cells, monocytoid cells and myeloid (granulocytic) cells, precursor and mature forms) were also obtained by means of light microscopy, the lineage identification techniques having been well established several years ago in our laboratory (Miller and Osmond, 1974, 1975, 1976; Miller et al., 1978; Miller, 1982, 1992; Christopher et al., 1991), and continue in regular use in our laboratory as defined and accurate means of recording cell lineage/sub-lineage fluctuations under experimental and control conditions (Christopher et al., 1991; Miller et al., 1992, Dussault and Miller, 1993, 1994, 1995a, b, 1996; Currier and Miller, 1998; Mahoney et al., 1998; Whyte and Miller, 1998; Sun et al., 1999; Currier et al., 2000).

For some experiments all non-NK lymphoid cells in both organs were recorded in sized sub-populations (determined by means of an ocular micrometer) as follows: small <10μm) mature (non-NK) lymphocytes; large (>10μm, immature (precursor) lymphoid cells; mature granulocytes, immature (precursor) granuloid (myeloid) cells; monocytes (precursors of tissue macrophages; and nucleated erythroid cells (precursors of peripheral blood erythrocytes). Labeled lymphocytes were counted as a proportion of 2000 lymphocytes/organ/mouse. From the known total organ cellularity, the proportions of NK cells were converted to absolute numbers of NK cells/organ/mouse in each control and herb-fed animal. Next, from the proportions of each of several distinct subgroups, (lymphoid, myeloid, nucleated erythroid, monocytoid cells), recorded by scanning (reading) 2000 cells/cytospot (2-5)/ tissue/mouse/experiment (or control), the absolute numbers of each of these cell populations could be determined from the total known cellularity of each tissue (spleen, femurs) obtained previously by means of an electronic cell counter (Coulter Electronics, Hialeah, FL).

Although the current method of cell analysis is labor-intensive, no equipment available to date can classify accurately at least 6 different sub-populations of hemopoietic cells, several of which are often present in very low proportions.

Assay of Hemopoiesis in vitro.

For work in Chapter 3, from each of 10 additional DBA/2 mice, bone marrow cells were flushed from both femoral shafts, washed and prepared as single cell

suspensions by standard methods (above). From all mice $7.5x10^5$ sterile bone marrow cells were incubated per culture, in 2.0ml of medium (0.3% agar, 20%FBS, 5×10^{-5} M mercaptoethanol, 0.2ml lung conditioned medium in RPMI 1640), in 35ml Falcon petrie dishes for 7 days in 7.5% CO_2 at 37° in 100% humidity. At 7 days, all cultures were removed, and by means of an inverted microscope (Nikon Diaphot) colonies were read and enumerated from constant surface areas of each dish from both MLT-treated and control cultures. Lung conditioned medium (LCM), the source of granulocyte/macrophage – colony stimulating factor (GM-CSF), was prepared as described previously (Maestroni et al., 1994a). Briefly, 6 lungs, extracted under sterile conditions from DBA/2 mice and finely minced into 35 mm petrie dishes, were arranged in monolayers on the bottoms of the dishes, and incubated for 3 days in RPMI 1640, to which was added 20% fetal bovine serum (FBS) and 5×10^{-5}M mercaptoethanol (Dooley et al., 1988) for a total volume of 2.0 ml/dish. At 3 days, the supernatant was extracted, filtered and added to bone marrow containing cultures (above).

NK Cell-Mediated Cytotoxicity Assay of Functional Activity.

The propidium iodide (PI) uptake method was used to assay cell death by NK cell-mediated cytolysis. The results of this method compare precisely with those obtained by means of the ^{51}Cr-release method (Kroesen et al., 1992; Chang et al., 1993). Spleen cell suspensions were prepared from thyroxin- or E. purpurea-administered mice or control (untreated) mice, in ice-cold RPMI 1640 medium and prepared and enumerated using our standard methods (above). Clean, single cell suspensions of each spleen were adjusted to a final concentration of

5.0 x 10^5 cells/ml. These cells comprised the effector (E) cells in the assay. Target (T) cells were YAC-1 lymphoma cells, commonly used in our laboratory as a sensitive indicator of NK cell functional activity (capacity for lysis). Target cells were incubated overnight (37°C, 5% CO_2) with the green fluorescence membrane stain $DiOC_{18}(3)$ (Molecular Probes Inc., Oregon, USA) which labels only live cells with green stain fluorescing at the level of the cell membrane. One ml stain was mixed with 5.0 x 10^5 target cells in medium (above) at a 1:500 dilution. Labeled target cells were then centrifuged for 5 min at 1100 rpm, 4°C, rinsed free of residual $DiOC_{18}(3)$ stain in PBS, and adjusted to 2 x 10^4 cells/ml. The labeled target cells were resuspended in complete culture medium (above), and mixed with effector cells in an equal volume to give E:T ratios of 1:1, 5:1, 10:1, 13:1, and 25:1. Thus, the total final volume per well (24 well flat-bottom, polystyrene Corning #25820 microtitre plates) was 1.0 ml. Three replicate wells were assessed for each E:T ratio, for each organ from every animal in each experimental or control assay. The cell mixtures were then incubated (37°C, 5% CO_2) for 4 hr - the time needed to specifically assess NK cell-mediated lysis vs. lysis by other types of cells, i.e., Tc and macrophages. In addition, 0.5 ml of labeled target cells (above), were mixed with 0.5 ml of complete culture (without effectors) to serve as a control measuring spontaneous cell death. At 4 hr, the cells were concentrated by centrifugation at 250 x g for 10 min, and counterstained for 5 min at room temperature with the red, membrane-impermeable nucleic acid stain, propidium iodide (Molecular Probes Inc., Oregon, USA) (1:500 dilution in PBS; 50µl per well). Subsequently, 15µl of cell mixtures from each well at each E:T ratio for each treatment and control animal

is floated onto a glass microscope slide and overlaid with a 22 mm^2 glass coverslip. The final square with the trapped cell mixture is sealed with wax and viewed by means of immunofluorescence microscopy (Carl Zeiss, Inc.), employing the appropriate filter set for excitation and emission for the compounds used. Five hundred green-labeled target cells were counted per slide (each of which was the product of a single well), recording simultaneously those cells having green membrane stain and red nuclear stain ("G" and "R" cells in the formula below). Dead *effector* cells possess red nuclear stain but do not exhibit the green membrane stain. Live *target* cells will exhibit the green membrane stain only ("G" cells in the formula below). Thus, green membrane-labeled target cells observed containing the red dye (nuclear stain) as well, would have been membrane-punctured by NK cells in the lytic event, and are thus counted as dead target cells. The percentage of lysis (cytotoxicity) was then calculated as:

$$\%\text{Cytotoxicity} = \frac{\text{(Double-labeled) \{\# Green and Red cells\}}}{\text{\# Green cells (with effectors)}}$$

$$- \frac{\text{(Double-labeled) \{\# Green and Red cells\}}}{\text{\# Green Cells(without effectors) (measuring spontaneous death)}} \times 100$$

Standardization and Quantitation of NK Cell Functional Activity.

The percent cytotoxicity data across the ratios was not expressed in terms of lytic units (number of effector cells needed to kill a given number of target cells), used by others (Herberman et al., 1977; Santoni et al., 1979), to quantitate NK cell function activity. The "lytic units" method is inaccurate because it is based on the assumptions that NK activity across the range of E:T ratios (i) is completely *linear* for experimental and control data, and that (ii) the "lines" comparing experimental and control groups are *parallel*. Because this idealism is rarely the case, methods were previously developed in our lab (Miller, 1982; Nassiry & Miller, 1987; Miller et al., 1988; Christopher et al., 1991), and applied in the present study, an alternative method, subsequently used by others (Chatterjee-Hasrouni et al., 1984; Lala et al., 1985), for quantitating the level of NK cell mediated lytic activity. For each individual assay, regression lines were obtained from the data from the E:T ratios ranging from 1:1 to 10:1 (the ascending part of the curve), for both the treatment groups (thyroxin, E. *purpurea*) and their corresponding control groups. For each treated group, the values (% cytotoxicity) provided by the midpoint E:T ratio (5:1) from the regression line, were divided by the corresponding values provided by the regression line resulting from the data of the appropriate controls. The results for each experimental were expressed as the "relative NK activity", i.e. RNKA, as follows:

$$\text{RNKA} = \frac{\text{"Treatment" \% cytotoxicity (5:1)}}{\text{Control \% cytotoxicity (5:1)}}$$

RNKA is to this point independent of total cellularity of the spleen. RNKA, when corrected for changes in total numbers of nucleated cells in the organ of "treated" animals relative to corresponding control, provides a value of "total NK activity", i.e. TNKA, as follows:

$$\text{TNKA} = \text{RNKA} \times \frac{\text{total nucleated cells (treated)}}{\text{total nucleated cells (control)}}$$

This method of standardizing the results provides a method of eliminating the day-to-day experimental variations inherent in all NK assays. TNKA is expressed in arbitrary numbers.

Statistical Analysis.

Several mice of each group (experimental, control) were kept alive for the purpose of determining the effect of treatment(s) on life span. Kaplan-Meier Survival Analysis was used to assess the influence of treated vs. untreated control mice on life span. The influence of treatment(s) on NK cells, as well as on the various cell subpopulations in both the spleen and bone marrow, was determined by Student's t test (two-tailed). The differences between means of experimental and corresponding control groups were so compared and probability values of $p \leq 0.05$ or less were considered significant.

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INTRODUCTION TO CHAPTER 1

In an effort to boost NK cell numbers I first planned to utilize a well studied interferon (IFN) inducer, polyinosinic-polycytidylic acid (poly I:C). IFN is a powerful NK cell enhancer. Poly I:C is known to activate murine NK cells *in vivo*, however I wanted to examine the effect of its administration on young adult, irradiated, *leukemic* mice given a bone marrow transplant (BMT). This is a protocol commonly used for amelioration of human leukemias. The perceived added advantage of co-administering an NK cell enhancer would be that a much larger army of NK cells (bone marrow transplant-derived) would be on standby to prevent tumor regrowth from "residual disease" (no tumor eradication method, e.g., irradiation, ever eliminates 100% any tumor).

Poly I:C was given both pre-irradiation and post-irradiation. NK cells boosted prior to irradiation would survive irradiation. Additional abundant new supplies would also come from the BMT since their precursors would be present and subject to IFN-stimulation. This much larger, additional supply would then destroy any residual tumor surviving the irradiation procedure induced by *in vivo*, post-irradiation presence of poly I:C.

CHAPTER 1: Influence of an interferon inducer on bone marrow transplant reconstitution in irradiated, leukemic mice: Elevated natural killer cell numbers and improved life span.

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Abstract

Interferon-gamma and interferon inducers such as polyinosinic-polycytidylic acid (poly I:C) promote natural killer cell-mediated killing of a wide range of tumor cells both *in vitro* and *in vivo*. The aim of the present work was to administer poly I:C to irradiated, leukemic mice, both before and after syngeneic bone marrow transplant with the intent of boosting host natural immunity in the critical peritransplant period. Briefly, DBA/2 mice were injected with Friend-virus-induced erythroleukemia cells. After 5 days of tumor growth, some mice received daily injections of poly I:C for the next 4 days while control, leukemic mice received the saline vehicle only. All mice were then irradiated (450R x 2 at 4 hr intervals), and transplanted 1 day following irradiation, with bone marrow from age and sex matched, normal DBA/2 donor mice. After transplant, daily injections of poly I:C (or vehicle) continued for 8 more days. On day 9 post-transplant, treated and control mice were killed, and the total cellularity, total numbers of lymphoid cells, the total numbers of NK cells (identified by the presence of an immunolabelled, specific cell surface marker), were obtained from both the spleen and the bone marrow. Other, identically treated mice, subjected however, to several additional rounds of poly I:C treatment, were sampled at 3 months and 6 months after irradiation and bone marrow transplant. The results indicated that (a) poly I:C administered in the peritransplant period (before and after transplant), significantly increases the absolute numbers of NK cells in both the bone marrow and spleen of the transplanted host at all time intervals studied, (b) no erythroleukemic tumor cells were found, even as late as 6 months after transplant in the poly I:C treated hosts in spite of the fact that poly

I:C treatment in this group had been terminated more than two months prior to tissue sampling, and (c) survival was significantly improved by pre- and post-transplant treatment with poly I:C.

Introduction

Autologous bone marrow transplantation (ABMT) is increasingly being used as the treatment of choice in tumor-bearing patients, after chemical- or irradiation-mediated reduction/elimination of the host's tumor cells (Maestroni et al., 1992; Fefer et al., 1993; Trickett et al., 1993; Abdul-Hai et al., 1995; Dann et al., 1995). However, for variable periods of time following ABMT, the host immune system remains deficient (Witherspoon et al., 1984; Anderson et al., 1987). The slow kinetics of immunological reconstitution after ABMT remains problematic (Abdul-Hai, 1995). The rate of relapse after ABMT alone (unsupported) remains high and the slow regeneration of the elements of the immune system directly and indirectly involved in destroying residual disease in the early post-transplant period in the host, as well as the observed asynchronous restoration in numbers and function of these immune cells, may contribute to such relapses (Bilgrami et al., 1994; Abdul-Hai et al, 1995). Nevertheless, natural killer (NK) cells are among the first cells to recover their immunological function after bone marrow transplant (BMT) (Niederwieser et al., 1987; Keever et al., 1989), regenerating rapidly in the early post-transplantation period (Verma & Mazumder, 1993; Miller, 1994). Moreover, mature, lytic NK cells retain tumor cell killing capacity even after hemopoietically lethal doses of irradiation (950R) to the host, unlike other immune cells such as T, and B lymphocytes (Hochman et al., 1978; Gorelik & Herberman, 1982). Destruction of residual tumor cells by increasing the recipient's immune defenses in the peritransplant period, however, could potentially lead to a prolonged remission or even cure (Bilgrami et al., 1994).

Although success following tumor eradication depends upon the rate and completeness of hemopoietic and immune cell reconstitution following irradiation and ABMT (Maestroni et al., 1992), agents such as cyclosporin, interferon and interleukin-2 can be administered for the purpose of reducing relapse following transplant to aid in the destruction of minimal residual disease (Dann et al., 1995). Ex vivo bone marrow purging with pharmacological and/or immunological agents to remove neoplastic cells from the autograft prior to transplant is fundamental, although no technique of purging yet exists which can remove every tumor cell, nor does irradiation ensure complete eradication of every host-based tumor cell. Thus, any technique designed to boost tumor-lytic machinery both prior to and after irradiation + ABMT, would have obvious value.

Polyinosinic-polycytidylic acid (poly I:C), an analog of viral double stranded RNA, has been shown to increase NK cell killing of tumor cells *in vitro* and *in vivo* (Gidlund et al., 1978; Djeu et al., 1979; Senik et al., 1979), via its potent role as an interferon (IFN) inducer. IFN, itself, administered *in vivo*, inhibits tumor growth (Gresser & Tovey, 1978), including Friend virus-induced erythroleukemia (Rossi et al., 1975). *In vitro*, NK cells become potently lytic to tumor cells within minutes of exposure (Gidlund et al., 1978; Djeu et al., 1979; Herberman & Holden, 1979). The effect of poly I:C, however, via IFN induction, takes several hours since this agent stimulates macrophages (and other accessory cells) to produce IFN. Daily administration of poly I:C results in sustained, high levels of circulating IFN as well as enhanced NK cell-mediated lytic activity (Gidlund et al., 1978; Gresser & Tovey, 1978; Djeu et al., 1979; Herberman & Holden, 1979; Senik et al., 1979).

The aim of the present work was to employ bone marrow transplant techniques along with a novel drug administration protocol in an effort to ameliorate moderate-stage leukemia in mice. We hypothesized, thus, that poly I:C, given to leukemic host mice prior to irradiation+syngeneic BMT would stimulate production/function of mature NK cells themselves not susceptible to the impending irradiation. Continued treatment with poly I:C after irradiation + BMT would subsequently stimulate NK cells (via driving the macrophages/monocytes and any other responding accessory cells incoming with the BMT, to produce IFN). Host-based mature NK cells, enhanced prior to irradiation by poly I:C injections, would still be present and functional after transplant for a short time, given their rapid renewal rates in normal mice (Miller, 1982).

Our study has shown in poly I:C treated mice (a) a significant elevation in NK cell numbers in both the spleen and the bone marrow of leukemic, irradiated host mice given syngeneic bone marrow transplants, (b) no morphological evidence of any erythroleukemic tumor cells, microscopically assessed, at the time of host killing (9 days, 3 months and 6 months after irradiation and BMT), and, (c) a significantly prolonged life span relative to vehicle-treated, irradiated, transplanted hosts.

Results

No differences were found in the total cellularity or the total numbers of lymphoid cells in either the spleen or the bone marrow of poly I:C-treated mice and the vehicle-injected (control) mice 9 days after irradiation and bone marrow transplant (Table 1). On the other hand, the absolute numbers of NK (ASGM-1$^+$) cells in the spleens of poly I:C-injected and control mice were very different at this early post-transplant time interval, with NK cells in the drug-treated mouse spleens outnumbering those in the control spleens almost 3-fold ($p<0.001$) (Fig. 1). The absolute numbers of bone marrow localized NK (ASGM-1$^+$) cells in animals given poly I:C at this time interval was also significantly higher ($p<0.005$) than those of vehicle-injected mice (Fig.2). The fact that poly I:C produced no change in the total numbers of lymphoid cells in either organ (Table 1) indicates a selective enhancement of NK cells at the cost of other lymphoid cells in this early post-transplant period. In fact, combining the data of Figs. 1 and 2, and Table 1, it was found that NK cells in poly I:C -treated mice account for an average of 28% of all the lymphoid cells in the spleen vs. 10% in the spleens of vehicle-injected mice. In parallel, the numbers of NK cells in poly I:C-treated mouse bone marrow averaged 9.3% of all lymphoid cells compared to 4.9% of all bone marrow lymphoid cells in control mice.

Table 2 indicates that the spleens of poly I:C-treated mice killed at 3 and 6 months after irradiation and BMT, contained, at both intervals, approximately 3 times the number of cells in normal mouse spleen. Three months after BMT, the nucleated erythroid cells of the spleen accounted for approximately 30% of the total cells in the organ (Table 2). Six months after BMT, erythroid cell numbers

fell significantly to only 14.5% of the total cellularity of the spleen. No vehicle-injected mice survived until 3 months; consequently the comparison was made (Table 2) with normal, age-matched, unmanipulated mice. Interestingly, 3 and 6 months after BMT, the numbers of lymphoid cells were not significantly different in the spleen compared to each other, although in both cases, they were more than double the numbers in the normal mouse spleen (Table 2). By contrast, in the bone marrow, by 3 and 6 months of poly I:C treatment, the total cellularity was not significantly different relative to that of normal mice (Table 2). However, relative to normal mouse bone marrow, at 3 and 6 months of poly I:C treatment, the numbers of erythroid cells were approximately one third and one fifth, respectively, of normal numbers (Table 2). The total lymphoid cell numbers in the bone marrow at 3 months after poly I:C treatment were significantly elevated relative to normal, but by 6 months they had returned to approximately normal numbers (Table 2).

NK cells in the poly I:C-treated mouse spleens, having significantly increased numbers relatively early after BMT (Fig. 1), remained at virtually unchanged, consistently high levels in that organ even 6 months after BMT. In the bone marrow, on the other hand, although rising sharply in absolute numbers in the early (9 days) post-transplant period, NK cells declined progressively thereafter to levels significantly lower than the 9 day post-transplant + poly I:C values, by 6 months after BMT and poly I:C treatment (Fig.2).

Finally, survival was significantly improved ($p < 0.001$) in poly I:C-treated, bone marrow recipients vs. those receiving no post-transplant drug support (Fig. 3). No vehicle-injected bone marrow recipients remained alive at 3 months. All

poly I:C-treated mice surviving until 6 months were normal in phenotype and behaviour and were, at that time, killed for the purpose of assessing various hematological parameters (Table 2, Figs. 1,2).

Table 1.

Effect of Poly I:C on the total cellularity, numbers of lymphoid cells in the spleen and bone marrow of irradiated, bone marrow transplanted, leukemic[f] young adult male DBA/2 mice.

Organ[a]	Treatment	Total Nucleated cells ($\times 10^6$)	Total Lymphoid[b] cells ($\times 10^6$)
Spleen	poly I:C[c]	158.33±15.07[e]	38.11±3.58
	vehicle[d]	133.32±13.49	37.96±3.71
Bone Marrow (per femur)	poly I:C	13.37±0.96	3.80±0.36
	vehicle	16.76±1.00	4.45±0.31

[a]spleens and femurs were removed from all mice 9 days after transplant
[b]determined from differential counts of 2000-3000 total cells in each organ on stained cytospots preparations, and converted via the known total numbers of nucleated cells/organ to absolute numbers of lymphoid cells
[c]poly I:C (5μg/gm body wt. In 0.1ml PBS) given daily i.p. for 4 days prior to, and 8 days after syngeneic bone marrow cell transplant
[d]PBS (0.1ml), given daily i.p. for 4 days prior to, and 8 days after bone marrow transplant
[e]mean ± s.e. : 8-14 individual mice. In all cases, the same mice that provided the spleens also provided the femurs
[f]all experimental and control mice injected with 3×10^6 erythroleukemic cells 5 days prior to first poly I:C and vehicle injections

Table 2.

Total cellularity and numbers of nucleated erythroid and lymphoid cells in leukemic mice[f] irradiated and given syngeneic bone marrow transplants 3 months or 6 months earlier.

Organ[a]	Treatment	Total Nucleated Cells ($x10^6$)	Nucleated Erythroid[b] Cells ($x10^6$)	Total Lymphoid[b] Cells ($x10^6$)
Spleen				
(n=6)	poly I:C[c] (3 months)	320.8±29.9[e]	102.6±5.4	186.5±25.4
(n=5)	poly I:C (6 months)	342.9±52.5	49.9±7.6	221.9±31.9
(n=6)	normal[d]	121.3±11.6	13.1±1.6	85.6±8.3
Bone Marrow (per femur)	poly I:C (3 months)	10.6±1.1	0.5±0.4	4.8±0.6
	poly I:C (6 months)	10.5±1.4	0.3±0.06	2.5±0.4
	normal	12.9±1.4	1.5±0.3	3.2±0.7

[a]spleens and femurs were removed from poly I:C-treated mice at 3 or 6 months after irradiation and bone marrow transplant

[b]determined from differential counts of 2000-3000 total cells in each organ, on stained cytospots preparations, and converted via the known total numbers of nucleated cells/organ to absolute numbers of nucleated erythroid, or lymphoid cells

[c]given daily i.p. (5μg/gm body wt. In 0.1ml PBS) for 4 days prior to transplant, and, daily up to day 20 post transplant, followed by bi-daily injections from day 21-36, and then bi-weekly injections from day 37-90 for mice killed at 3 months. For mice killed at 6 months post transplant, poly I:C was administered as above up to day 120 after which no further injections were given until the time of kill: day 190.

[d]normal, unmanipulated same-strain and sex, 3-4 months old. No vehicle-injected control mice remained alive at 3 (or 6) months post irradiation and bone marrow transplant

[e]mean ± s.e., *$p<0.05$ (relative to normal values).

[f]all experimental and control mice injected with $3x10^6$ erythroleukemic cells 5 days prior to first poly I:C and vehicle injections

Fig. 1.

Absolute numbers of NK (ASGM-1$^+$) cells in the spleens of irradiated leukemic mice given poly I:C (or the PBS vehicle) for 4 days before, and for various periods after, irradiation and syngeneic BMT. Mean±s.e.: *$p<0.001$ at all intervals. ( ) = number of mice.

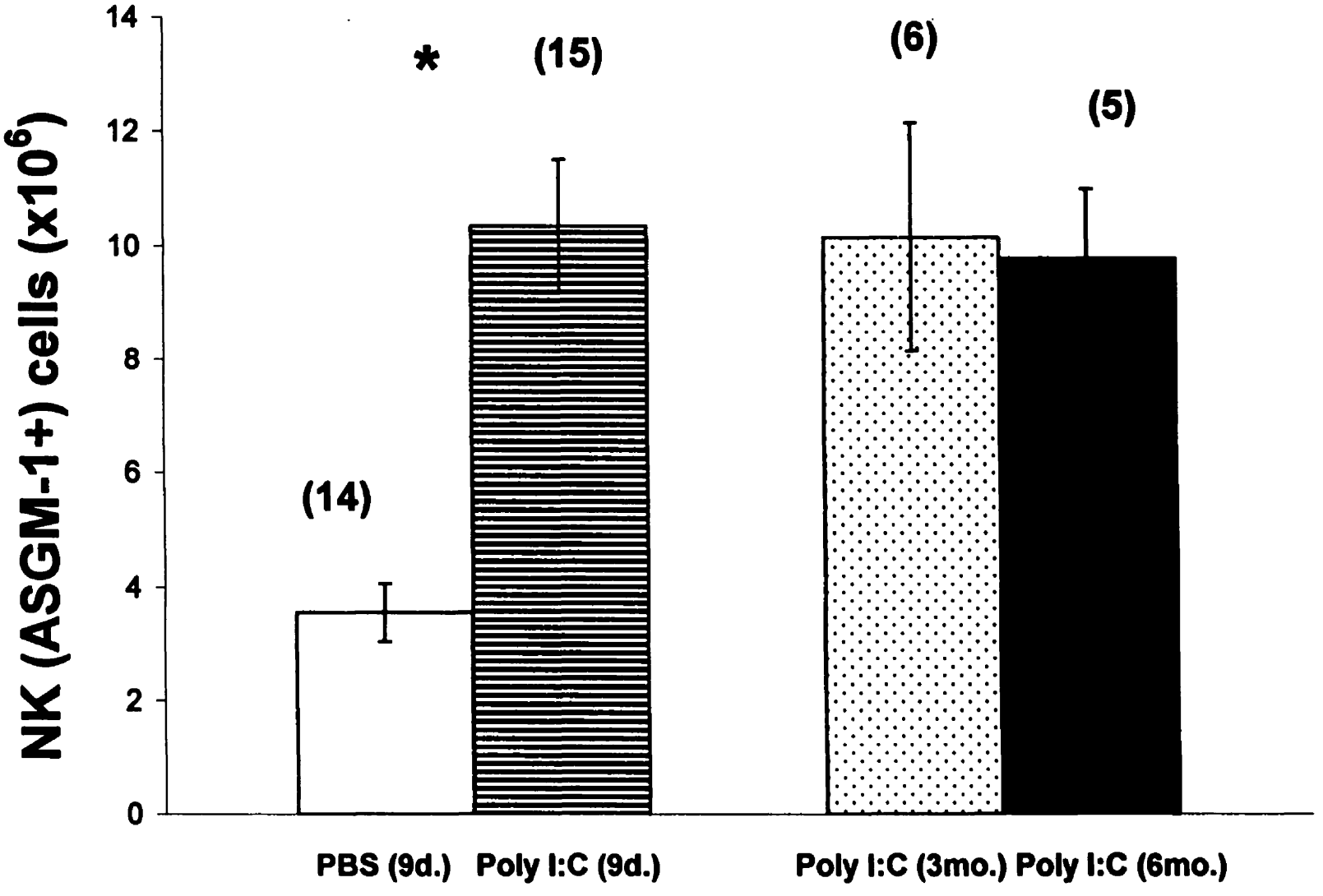

NK (ASGM-1+) cells (x10^6)
14
12
10
8
6
4
2
0
*
(15)
(14)
(6)
(5)
PBS (9d.)
Poly I:C (9d.)
Poly I:C (3mo.)
Poly I:C (6mo.)

Fig. 2.

Absolute numbers of NK (ASGM-1$^+$) cells in the bone marrow (1 femur) of irradiated, leukemic mice given poly I:C (or the PBS vehicle) for 4 days before, and for various periods after, irradiation and syngeneic bone marrow transplant. Mean±s.e.: *$p<0.005$ (9 days); **$p<0.0001$ (poly I:C - 9 days vs. poly I:C - 6 months). ( ) = number of mice.

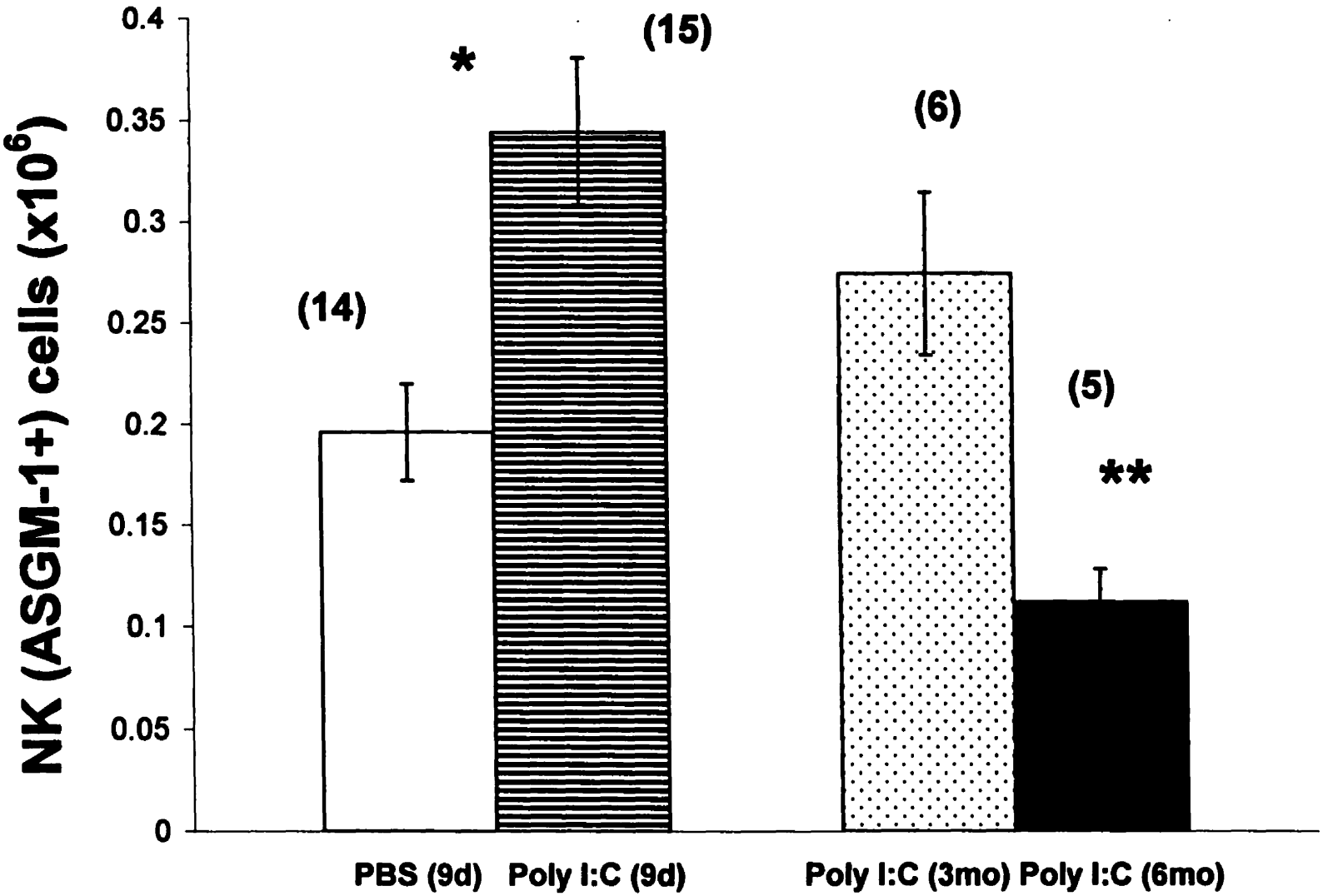
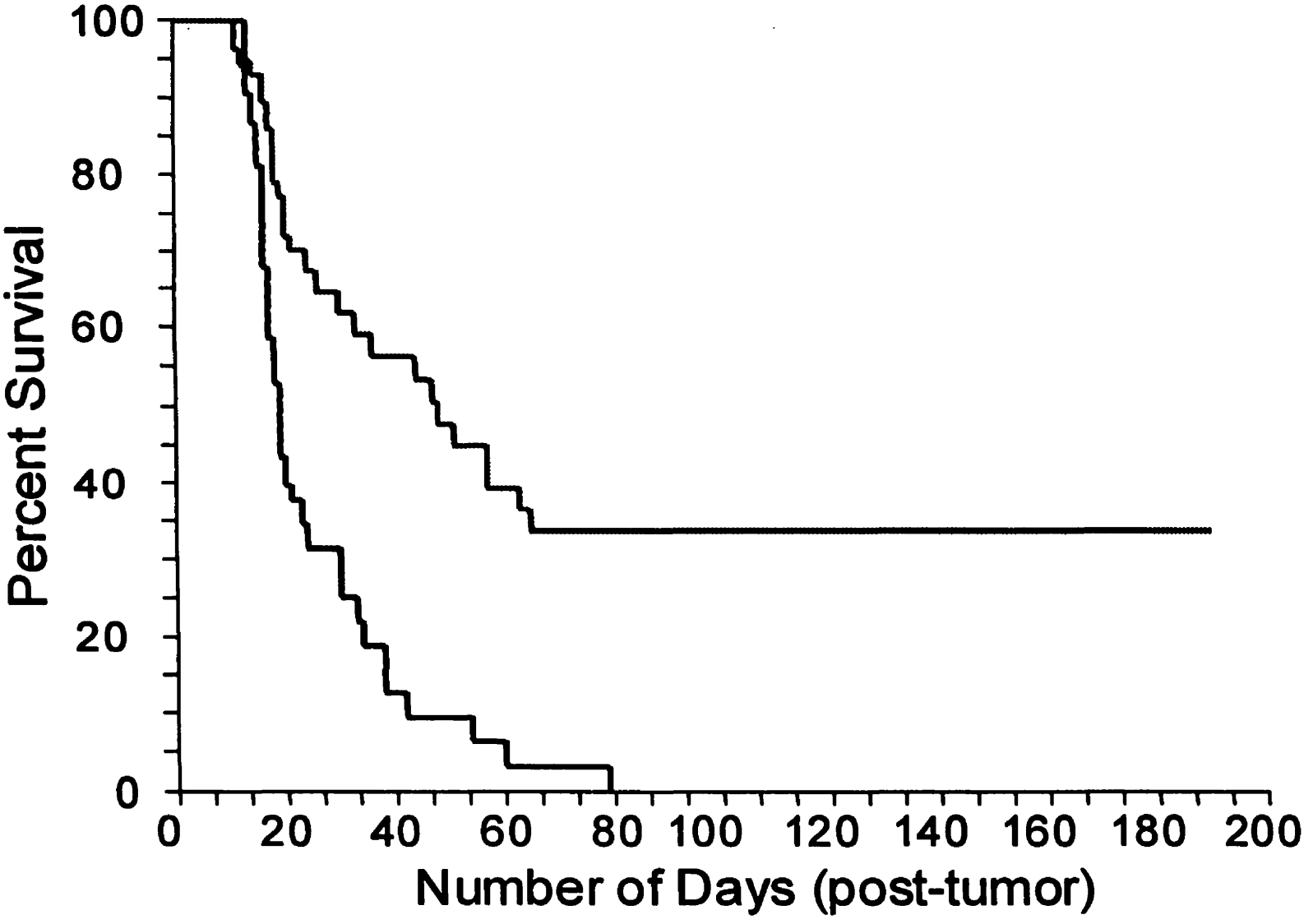

NK (ASGM-1+) cells (x10^6)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
(14)
*
(15)
(6)
(5)
**
PBS (9d)
Poly I:C (9d)
Poly I:C (3mo)
Poly I:C (6mo)

Fig. 3.

Effect of poly I:C treatment on life span of leukemic, irradiated mice given the agent (broken line), or its PBS vehicle (continuous line), for 4 days prior to, and for assorted time intervals after, syngeneic bone marrow transplant up to 6 months. *$p<0.001$ by Kaplan-Meier survival analysis statistics.


Percent Survival
100
80
60
40
20
0
0
20
40
60
80
100
120
140
160
180
200
Number of Days (post-tumor)

Discussion

The fact that the total cellularity of both the spleen and the bone marrow of vehicle (sham)-injected, bone-marrow-transplanted recipients is comparable to that found by us previously in normal (unmanipulated) mice of the same age and strain (Christopher et al., 1991; Dussault & Miller, 1994), suggests that by 9 days, at least, after BMT, hemopoietic and immune cell reconstitution is well underway. In the spleens of both vehicle-injected and poly I:C-treated mice, this organ cellularity consists almost exclusively of erythroid and granuloid precursor cells (unpublished observations) - distinctly contrasting with the composition of normal (unmanipulated) mouse spleens. By 8 days after BMT, lymphoid cells in the spleen in both vehicle and poly I:C-treated mice represent only 1/4 of the total cellularity, whereas in normal animals, these cells account for the majority of the total cell content of the DBA/2 spleen (Christopher et al., 1991; Dussault & Miller, 1994).

Given the paucity of lymphoid cells in the spleen, together with a predominance of erythroid and granuloid lineage precursor cells observed in the post-transplant, irradiated spleen, both in control and poly I:C-treated mice, it appears that the organ has resurrected its perinatal capacity as a major hemopoietic organ. The ability of the spleen to generate these lineages de novo, but not the lymphoid lineages, after irradiation and BMT, has been well established (Mekori et al., 1965; Curry & Trentin, 1967; Curry et al., 1967).

Unlike the spleen, the total numbers of lymphoid cells in the bone marrow of vehicle-injected transplanted mice were comparable with normal (unmanipulated) mice of the same age, sex and strain, representing

approximately 25% of the total numbers of cells in the mouse femur (Miller & Osmond, 1974; Christopher et al., 1991; Miller, 1992; Dussault & Miller, 1994). The bone marrow is the well-established generating center of the B lymphocyte lineage and B cells, thus, represents the vast majority of all lymphoid cells in that organ. NK cells (also of lymphoid morphology), however, representing 1-3% of bone marrow cells in the normal mouse are also produced de novo in that organ, and are, thus, represented in the generating lymphoid cells. Finally, pluripotent, hemopoietic stem cells, also believed to be of lymphoid morphology (Cudkowicz et al., 1964; van Bekkum et al., 1971), may contribute minimally to the total lymphoid cell numbers in the bone marrow, given that these stem cells account for substantially less that 0.5% of the total bone marrow cellularity.

Although the total lymphoid cell numbers are the same at 9 days after BMT in both vehicle-treated (control) mice, and poly I:C treated mice spleens, it is evident that the various lymphoid cell lineages are disproportionately represented. With respect to NK cells, the rapid increase in their numbers in control (vehicle-injected) mice supports our previous observations (Miller, 1994) demonstrating that 9-11 days after injecting normal, irradiated mice with FACS IV-separated, virtually pure pluripotential stem cells, NK cells could be found when/where few other cells of lymphoid morphology could be. Moreover, NK cells in such regenerating hosts, achieved normal levels rapidly thereafter, while other immune cells of lymphoid morphology lagged well behind (Miller, 1994).

In the present study, poly I:C, while not altering the total lymphoid cell numbers of the spleen or the bone marrow by 9 days after transplant, did, on the other hand, result in significantly elevated numbers of NK cells both in host

spleen and bone marrow, indicating its selective stimulation, *in vivo*, of NK cell regeneration. The high levels of NK cells in the bone marrow at 3 months after BMT and poly I:C treatment, necessarily reflects a consistent up-regulation in new NK cell generation during this period. It is well established that NK cells *normally* decline, to low or absent levels, beginning at about 3 months (Herberman, 1977; Itoh et al., 1982; Albright & Albright, 1983).

An apparently peculiar event has occurred in the mice 6 months after BMT with respect to their NK cells. NK cell numbers in the bone marrow were significantly fewer at this post-transplant interval than at either 9 days or 3 months after BMT, yet the splenic NK cell numbers of these poly I:C treated mice at the 6 month post-transplant interval remained at virtually the same consistently high levels observed at 9 days and 3 months of poly I:C treatment. It is believed that this reduction in bone marrow NK cell numbers is explicable on the basis of the natural age-related decline in bone marrow production of this lineage, as well as the progressive age-related resistance to immunostimulants, already well demonstrated by ourselves and others (Thoman & Weigle, 1982; Dussault & Miller, 1994; Dussault & Miller, 1995). Since, under normal conditions, the spleen receives its entire input of new NK cells from the bone marrow, it must be postulated that under the highly atypical conditions of the present protocol, the spleen may not have fully relinquished production of NK cells to the bone marrow early after the BM transplant. Moreover, it has been demonstrated that under atypical conditions, the spleen is, itself, capable of de novo production of NK cells (Biron et al., 1983; Miller & Shatz, 1991).

By 3 months in poly I:C-treated mice (by which time no vehicle-injected mice remained alive), the spleen is heavily engaged in erythropoiesis, although by 6 months the spleen is relinquishing this role as normal. By 3 and 6 months post-transplant, moreover, not only had the other lymphocytes, i.e., T and B lineage, reseeded the spleen, but their presence in numbers which were 2.5-3.0 times normal, reflects the immunostimulating properties of poly I:C.

Poly I:C administration prior to irradiation and BMT ensures an abundance of mature NK cells in the host at the time of irradiation and transplant. These mature NK cells, however, would soon die out in the host (mature NK cells are radioresistant) in the immediate post-transplant period, given their short life span (Miller, 1982). The NK cell lineage is one without long-lived, recirculating, memory components (Haller & Wigzell, 1977; Seaman et al., 1978; Miller, 1982). Thus, by 8 days after BMT, all NK cells in the host would have been derived from the donor transplant.

After irradiation and transplantation, the targets of poly I:C are brought in with the transplant and also include, nevertheless, the host-based, radioresistant accessory cells, fixed cells including fibroblasts and endothelial cells, and bone marrow stromal cells, the latter being effective producers of a variety of growth factors (Charbord et al., 1996; Fauteux & Osmond, 1996; Tsuji et al., 1996). The advantage of administering a general growth factor inducer such as poly I:C, versus a single cytokine, is thus apparent. We have already shown that one such cytokine, IL-2 (generated in the presence of poly I:C), is a powerful stimulator of new NK cell production, as well as NK cell lytic function, in leukemic mice (Christopher et al., 1991; Dussault & Miller, 1993).

Whether or not the significant elevation in NK cells accounts exclusively, for the significant survival at this post-treatment stage, is not known. However, the leukemia, which is the subject of the present study, is known to be NK-sensitive and T cell insensitive (Hagner, 1984; Lust et al., 1984; Nabi et al., 1986). The tumor is non-responsive to the immunostimulants generated *in vivo* in the presence of poly I:C and, in fact, this leukemia appears to proliferate only in response to internal autocrine mechanisms (Lacombe et al., 1987; Stage-Maroquin et al., 1996), involving only epo and epoR. The consistently elevated levels of NK cells in poly I:C- treated mice, may well have been adequate to eliminate from the beginning, any remaining residual disease.

Although survival has been significantly improved by the protocol implemented in this study, it is still far from 100%. While longer-term survival advantage has at least in part resulted from the abundant NK cell armament (augmented pre-and post-transplant) in these treated hosts, the greatest numbers of deaths occurs in the immediate post-transplant period, consistent with death from hemorrhage in the vital organs/brain commonly seen after clinical transplants. In mice surviving for 3 and 6 months, microscopic analysis of their spleens indicated no morphological evidence of erythroleukemic blasts characteristic of tumor re-growth. Absence of blast re-growth is further evidenced by our observations of sub-normal numbers of any nucleated erythroid cells, including erythroblasts, in the bone marrow, signaling, in that organ no detectable erythroleukemia re-growth.

In summary, a novel approach has been employed in which a general growth factor-stimulating agent has been administered *in vivo*, both before and

after irradiation and BMT in leukemic hosts. The results have demonstrated that with the present protocol, important criteria for disease amelioration in the recipients have been fulfilled: (i) elimination of tumor re-growth, (ii) reconstitution of the hemopoietic and immune cell lineages, and, (iii) significantly increased survival.

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INTRODUCTION TO CHAPTER 2

In Chapter 1 the results showed that administration of poly I:C, combined with syngeneic bone marrow transplant, significantly increased NK cell numbers and life span over control animals not receiving poly I:C. However, survival rate was not 100% in the treated group. Of those animals that did die, the majority expired shortly (within one to five days) after irradiation and bone marrow transplant. This is a well-documented clinical phenomena deriving from hemmorhage, especially in the brain. In an attempt to further increase NK cell numbers I examined the effect of administration of tumor necrosis factor alpha (TNF-a) to the protocol utilized in Chapter 1. TNF-a is a known NK cell enhancer and is in fact produced by NK cells themselves. TNF-a also, in controlled dose/time administration regimens, is a powerful driver of hemopoietic reconstitution. It was the belief that administration of TNF-a following irradiation and BMT, would further drive the hemopoietic reconstitution even greater than that mediated by poly I:C administration. This would help eliminate post-irradiation complications in irradiated, leukemic, bone-marrow-transplanted mice, with the anticipated advantage of further increasing NK cell numbers and prolonging life span.

CHAPTER 2: TNF-a further augments natural killer cells when co-administered with an interferon inducer to irradiated, leukemic, bone-marrow-transplanted mice.

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Abstract

We and others have demonstrated that the analogue of viral double-stranded RNA, i.e., poly I:C, a macrophage activator which results in interferon (IFN) production, is a powerful NK cell stimulant. We have previously shown that poly I:C, administered in the post-transplant period, significantly boosts NK cell numbers in leukemic, irradiated mice given a syngeneic bone marrow transplant (SBMT). The cytokine, tumor necrosis factor-alpha (TNF-a), also stimulates NK cells directly through receptor-ligand mechanisms. We have combined in the present study, the NK-enhancing properties of IFN (poly I:C-induced), and TNF-a by administering poly I:C to leukemic mice 4 days before and 8 days after irradiation and SBMT, concomitant with TNF-a during the first 4 days immediately following transplant. All mice were sampled at day 9 following irradiation, transplantation and treatment. NK cells were identified by immunoperoxidase labeling methods and other lymphoid cells, nucleated erythroid cells, monocytes and granuloid (myeloid) cells were identified in tetrachrome stained cytospots by microscopy. The absolute numbers of cells in each category were obtained in both the spleen and bone marrow. The results revealed that TNF-a, added to the poly I:C administration protocol, significantly boosted NK cell numbers 2.4-fold over that achieved by poly I:C alone. There was, on the other hand, no change, in either the bone marrow or spleen, in the numbers of non-NK lymphoid cells, nucleated erythroid cells or granulocytes when TNF-a was added to the poly I:C injection protocol. In contrast, monocytes fell significantly in number in both organs, when TNF-a was added to the poly I:C injection regimen (above). Since the role of NK cells in the transplanted recipient

is two-fold: (i) the destruction of residual tumor cells, and (ii) the production of hemopoiesis-driving cytokines, it appears, that at least under the conditions of the present protocol, two stimulants are indeed better than one with respect to NK cell enhancement.

Introduction

Syngeneic (autologous) bone marrow transplantation is gaining wide acceptance clinically as the preferred method (vs. allogeneic) of reconstituting the hemopoietic and immune systems of tumor-bearing humans/animals after ablation of their tumor bulk by irradiation or chemical means (Maestroni et al., 1992; Fefer et al., 1993; Trickett et al., 1993; Abdul-Hai et al., 1995; Dann et al., 1995). However, even after syngeneic reconstitution, the immune system remains deficient (Witherspoon et al., 1984; Anderson et al., 1987), resulting from the slow kinetics of restoration of the primary cell lineages in the immune system (Abdul-Hai et al., 1995). The rate of relapse of unsupported, syngeneic, transplanted recipients is high, and is due, in large measure, to this slow re-establishment of cells which are directly and/or indirectly involved in residual tumor cell combat in the early post-transplant period. The probability of relapse is further aggravated by the asynchronous restoration of the numbers and functional potential of these cells (Bilgami et al., 1994; Abdul-Hai et al., 1995). Nevertheless, natural killer (NK) cells (directly involved in tumor cytolysis) are among the earliest to reconstitute from a bone marrow transplant (Niederwieser et al., 1987; Keever et al., 1989), and regenerate rapidly in the early days after the engraftment (Verma & Mazumder, 1993; Miller, 1994). Another advantage that NK lineage cells have (unlike cells of the T and B lineages), derives from the ability of mature, tumor cytolytic NK cells, to survive lethal irradiation, retaining their functional (tumor lytic) capacity after irradiation (Hockman et al., 1978; Gorelik & Herberman, 1982). Clearly, destruction of residual tumor cells by increasing the recipient's immune defenses in the critical pre- and post-transplant

period would provide a distinct survival advantage to the host (Bilgami et al., 1994).

The rate and completeness of hemopoietic and immune cell reconstitution following irradiation and bone marrow engrafting determines success following tumor eradication (Maestroni et al., 1992). However, agents such as cytokines, i.e., interferon and interleukin-2, immune boosters which target NK cells, can be administered for the purpose of impeding tumor relapse (Dann et al., 1995).

Consequently, any agent, or combination thereof, which would drive at least those cells responsible for front-line defense against residual tumor cells while bone marrow reconstitution is underway, would obviously offer survival advantage. We have recently demonstrated (Currier & Miller, 1998) that indeed, the interferon inducer, poly I:C, (polyinosinic-polycytidylic acid), an analogue of viral double-stranded RNA with well known NK function-augmenting ability (Djeu et al., 1979; Senik et al., 1979; Gresser & Tovey, 1978), successfully boosts NK cells in the days immediately following irradiation and bone marrow transplantation. We showed furthermore (Currier & Miller, 1998), that this protocol resulted in significantly improved life span vs. untreated, syngeneic-bone marrow-transplanted hosts.

Another agent highly touted for its therapeutic potential in assorted cancer treatment protocols is tumor necrosis factor-alpha (TNF-a), a member of a rapidly growing superfamily of cytokines, which are the products of activated monocytes and macrophages (Bemelmans et al., 1996; Branch & Guilbert, 1996). This multi-functional cytokine mediates its activity by binding to cell membranes in a receptor ligand fashion (Ding & Porteu, 1992; Grell et al., 1993).

As pleiotropic regulators of hemopoiesis, such receptor-ligand interactions induce a variety of responses ranging from functional activation of the target cell, to proliferation, differentiation, and apoptosis (Slordal et al., 1989; Furmanski & Johnson, 1990; Sidhu & Bollon, 1993; Mayani et al., 1995; Selleri et al., 1995; Walczak et al., 1999). TNF-a alone, however, has a transient anti-tumor effect, with re-growth of the tumor soon resulting (Lejeune, 1995). However, it has been well established (Aggarwal et al., 1985; Fiers et al., 1986; Johnson et al., 1988; Sohumra, 1998) that profound tumor amelioration can occur in tumor-bearing humans and mice, if TNF-a is combined with interferon-gamma (IFN-g). Additionally, TNF-a directly stimulates/activates NK cells (Degliantoni et al., 1985; Yang et al., 1990; Chan et al., 1991; D'Andrea et al., 1992) to produce interferon, thereby further augmenting the tumor defense armament. Indeed, NK cells themselves can produce TNF-a (Murphy et al., 1992; Jewett & Bonavida, 1993; Bemelmans et al., 1996).

Moreover, another advantage of NK cell boosting, derives from the fact that stimulated NK cells themselves secrete a number of cytokines which are hemopoiesis stimulants, namely GM-CSF, G-CSF, M-CSF, and the cytokines IL-1, IL-2, IL-4 (Scala et al., 1984; Trinchieri et al., 1984; Pistoia et al., 1985; Numerof et al., 1988; Dinarello, 1989; Murphy et al., 1992; Zlotnik et al., 1992; Arase et al., 1993; Chen & Paul, 1997), all vital to the speedy reconstitution of engrafted bone marrow in irradiated hosts. Given the well-known toxicity of TNF-a when administered chronically or in large doses (Beutler & Cerami, 1987; Bertini et al., 1989; Fiers, 1991; Molloy et al., 1993; Hieber & Heim, 1994), it was of utmost importance for the present protocol, to select the optimal, non-toxic

dose/duration regimen for TNF-a administration *in vivo*. That selected for the present work was based on the results of several studies employing TNF-a in rodents, especially mice (Bianchi et al., 1988; Johnson et al., 1988; Bertini et al., 1989; Slordal et al., 1989; McDonagh et al., 1992; Young & Wright, 1992). Given that the cytokine, interferon, is beset with the same problems of toxicity *in vivo*, when administered as bolus inocula, and, moreover, synergizes with TNF-a (Murphy et al., 1988; Demetri et al., 1989), we elected to administer an interferon inducer, namely poly I:C which acts via macrophage stimulation to produce interferon at sustained, elevated, yet physiologically tolerated levels (Gidlund et al., 1978; Gresser & Tovey, 1978; Djeu et al., 1979; Senik et al., 1979).

We have combined the positive features of IFN and TNF-a, testing their collective potential on our bone marrow transplant protocol. The aim of the present study was to employ our standard bone marrow transplant techniques in irradiated, leukemic mice, administering a combination of NK cell stimulants, in an effort to further boost NK cells beyond the already super-normal level which we had previously achieved employing the interferon-inducer, poly I:C alone (Currier & Miller, 1998). We hypothesized, that TNF-a, given in conjunction with poly I:C, to leukemic mice *prior* to irradiation would stimulate production/function of mature NK cells (radiation-resistant), and that treatment with both agents *after* irradiation and bone marrow engraftment would stimulate new NK cell production from the transplant, along with general hemopoiesis there from, by molecular mechanisms unique to each agent. Thus, poly I:C would directly target (pre- and post-irradiation) cells of the monocyte/macrophage lineage, resulting in interferon production. Host-based mature, interferon-sensitive NK cells, enhanced prior to

irradiation by poly I:C and TNF-a, would still be present and functional for at least 24 hr in the irradiated host, given their radioresistance, yet short life span (Miller, 1982; Biron et al., 1983). TNF-a, which acts directly on NK cells, via receptor-ligand interactions (Ito et al., 1999), results in their secreting cytokines to drive hemopoiesis in the incoming bone marrow transplant.

Results

The absolute numbers of NK (ASGM-1$^+$) cells in the spleens of irradiated, leukemic mice injected with both TNF-a and poly I:C, were significantly higher ($p<0.00027$) at 9 days post-transplantation than were those of mice administered poly I:C (control) only (Fig. 1). In the bone marrow, however, the absolute numbers of these cells were similar in mice injected with both TNF-a + poly I:C and in mice given poly I:C only (Fig. 1). Monocytes were the only other lineage in the spleen to be significantly affected by the addition of TNF-a to the poly I:C regimen (Fig. 2). In striking contrast, however, with NK cells, the absolute numbers of monocytes in both the spleen and bone marrow of mice treated with both agents was significantly reduced relative to mice given poly I:C only ($p<0.00043$:bone marrow, virtually absent: spleen). No other cell lineage, in either the spleen or the bone marrow was positively or negatively influenced by the additional presence of TNF-a in the poly I:C injection regimen (Fig. 3-5).

Fig. 1.

Absolute numbers of NK (ASGM-1$^+$) cells in the spleen and bone marrow of irradiated, leukemic mice 9 days after syngeneic bone marrow transplant (SBMT). Control mice (open columns) received poly I:C 4 days prior to and 8 days after SBMT. Experimental mice (solid columns) received TNF-a for 4 days immediately following SBMT along with the poly I:C regimen (above). Mean±s.e.; ( ) = number of mice; *$p<0.00027$

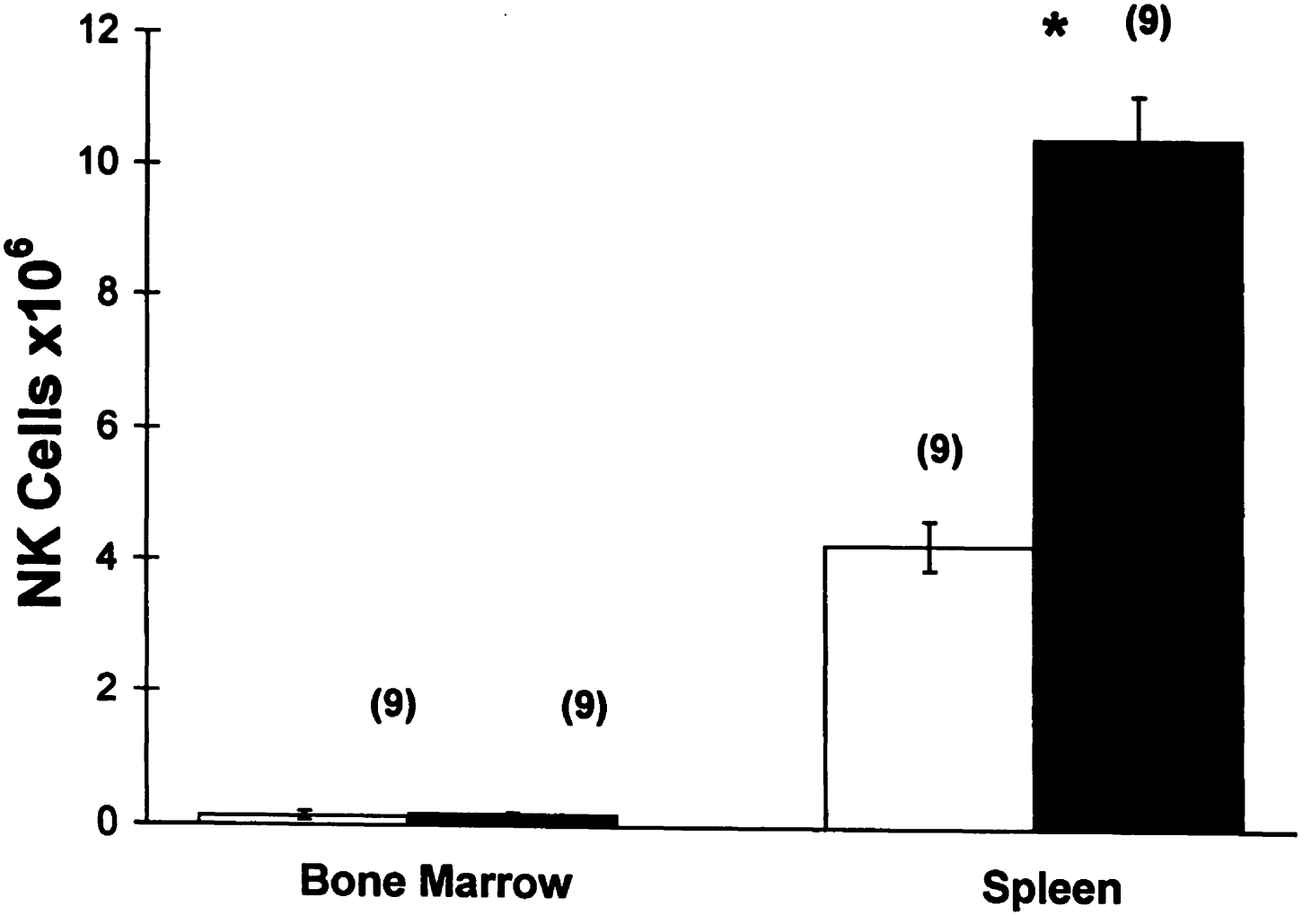

12
10
8
6
4
2
0
NK Cells x10^6
(9)
(9)
(9)
*
(9)
Bone Marrow
Spleen

Fig. 2.

Absolute numbers of monocytes in the spleen and bone marrow of irradiated, leukemic mice 9 days after syngeneic bone marrow transplant (SBMT). Control mice (open columns) received poly I:C 4 days prior to and 8 days after SBMT. Experimental mice (solid columns) received TNF-a for 4 days immediately following SBMT along with the poly I:C regimen (above). Mean±s.e.: 9 mice; ( ): number of mice; *$p<0.00043$ (bone marrow), *absent (spleen)

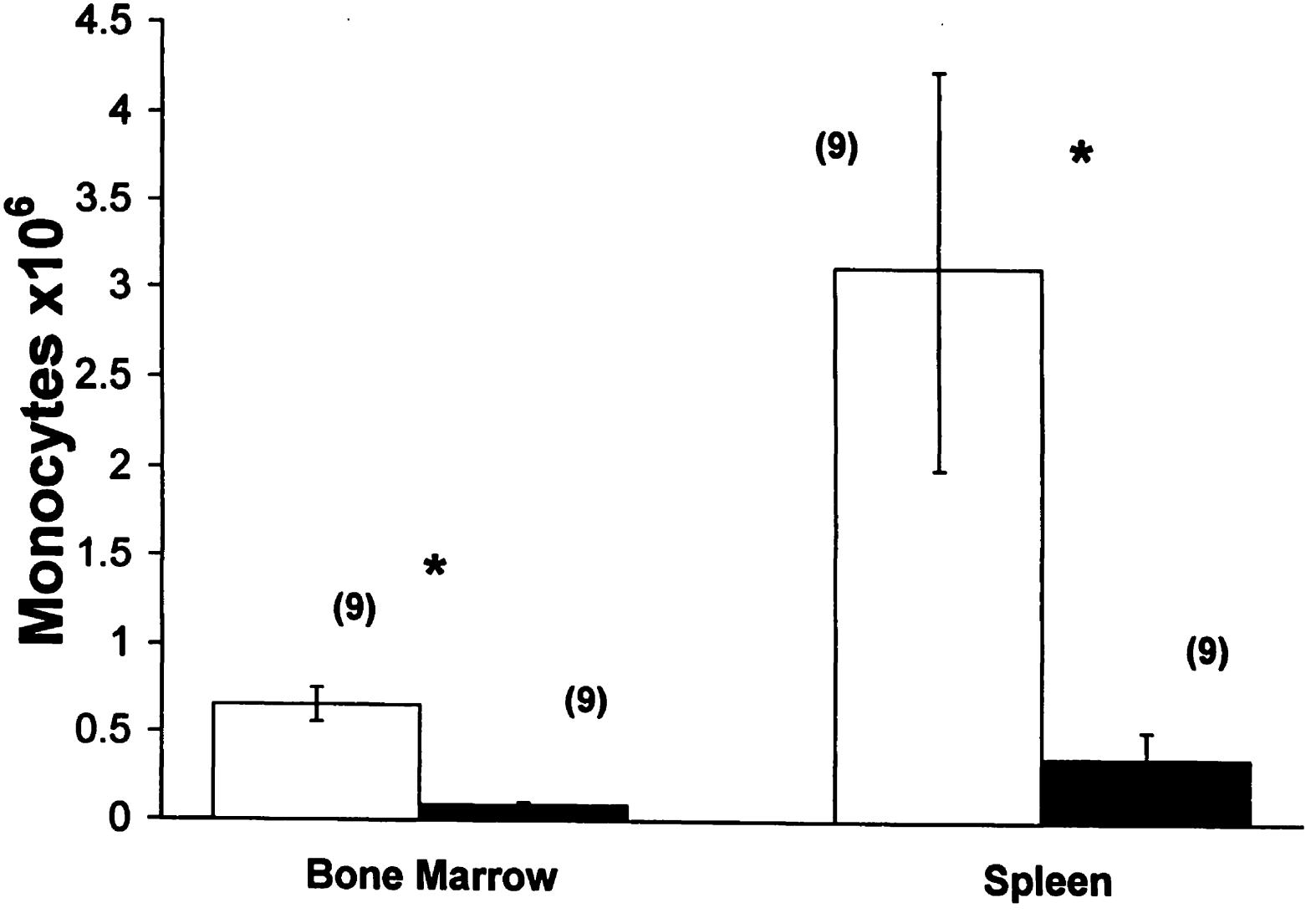

Monocytes x10^6
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
(9)
*
(9)
(9)
*
(9)
Bone Marrow
Spleen

Fig. 3.

Absolute numbers of non-NK lymphoid cells, comprising a mixture of T and B cells (mature and proliferating forms) in the spleen, and comprising predominantly B cells (mature and proliferating forms) in the bone marrow of irradiated, leukemic mice 9 days after syngeneic bone marrow transplant (SBMT). Control mice (open columns) received poly I:C 4 days prior to and 8 days after SBMT. Experimental mice (solid columns) received TNF-a for 4 days immediately following SBMT along with the poly I:C regimen (above). Mean±s.e.: 9 mice; ( ): number of mice.

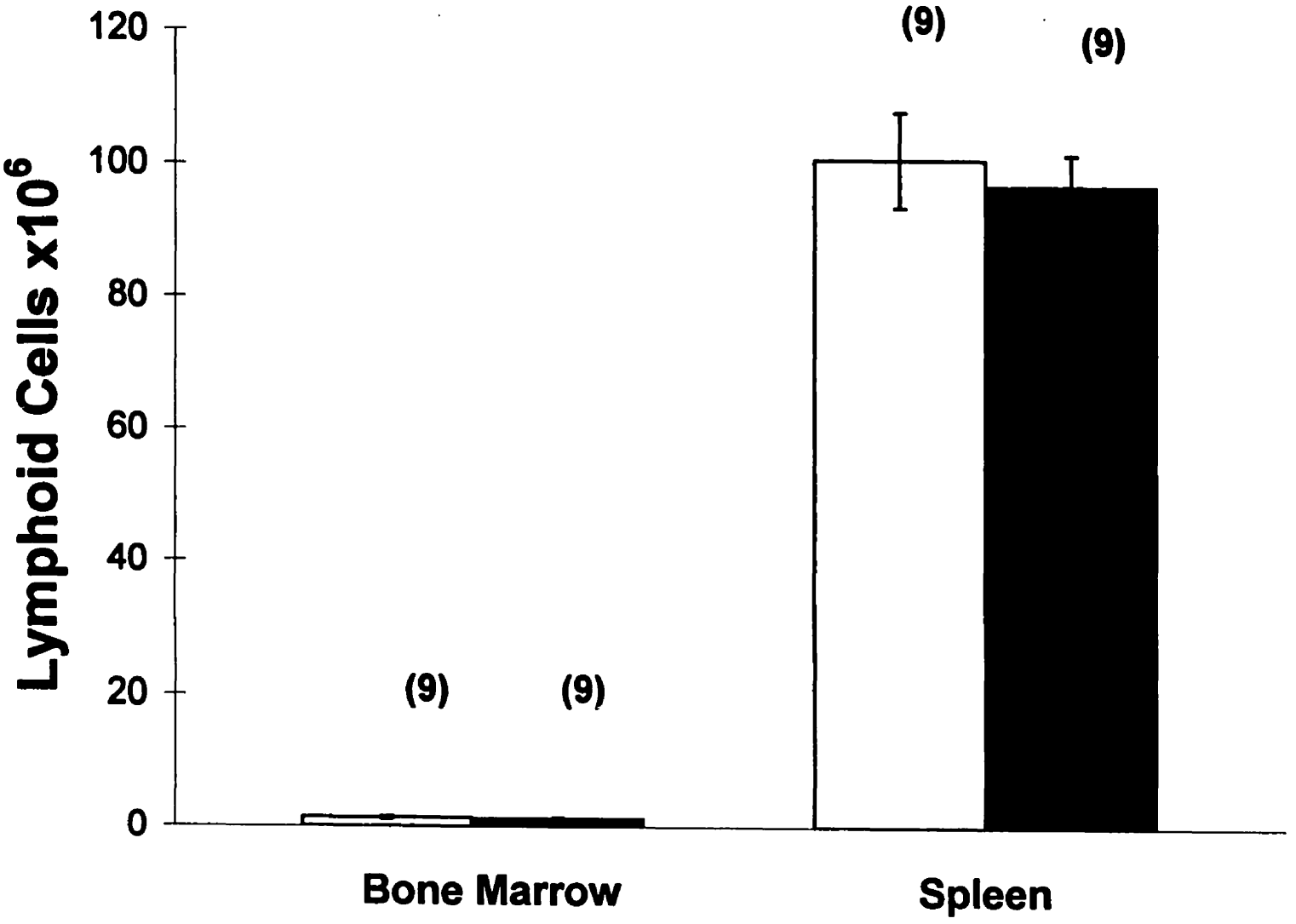

120
100
80
60
40
20
0
Lymphoid Cells x10^6
(9)
(9)
(9)
(9)
Bone Marrow
Spleen

Fig. 4.

Absolute numbers of myeloid (granulocytic) cells, including proliferating and mature forms, in the spleen and bone marrow of irradiated, leukemic mice 9 days after syngeneic bone marrow transplant (SBMT). Control mice (open columns) received poly I:C 4 days prior to and 8 days after SBMT. Experimental mice (solid columns) received TNF-a for 4 days immediately following SBMT along with the poly I:C regimen (above). Mean±s.e.: 9 mice; ( ): number of mice.

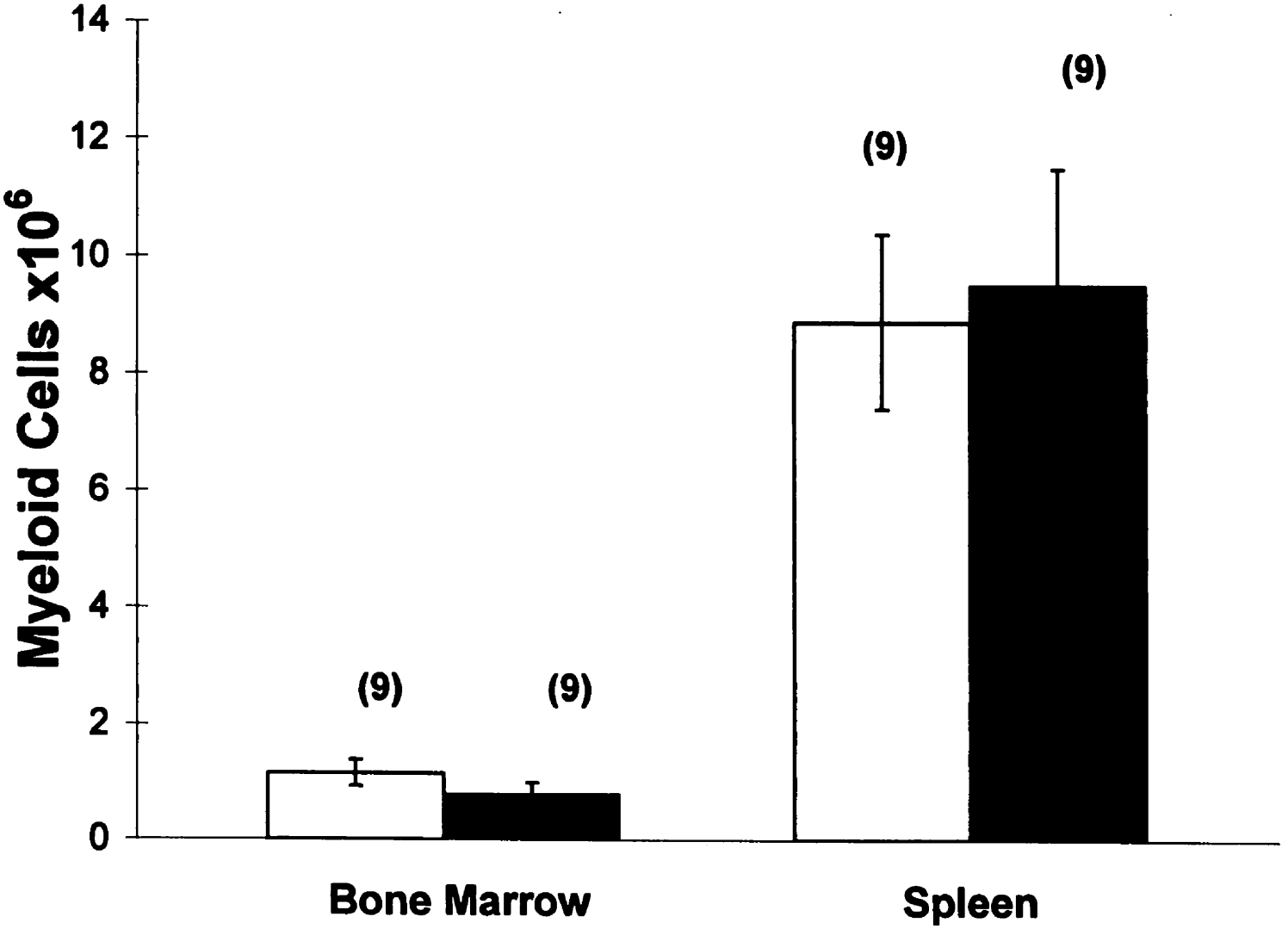
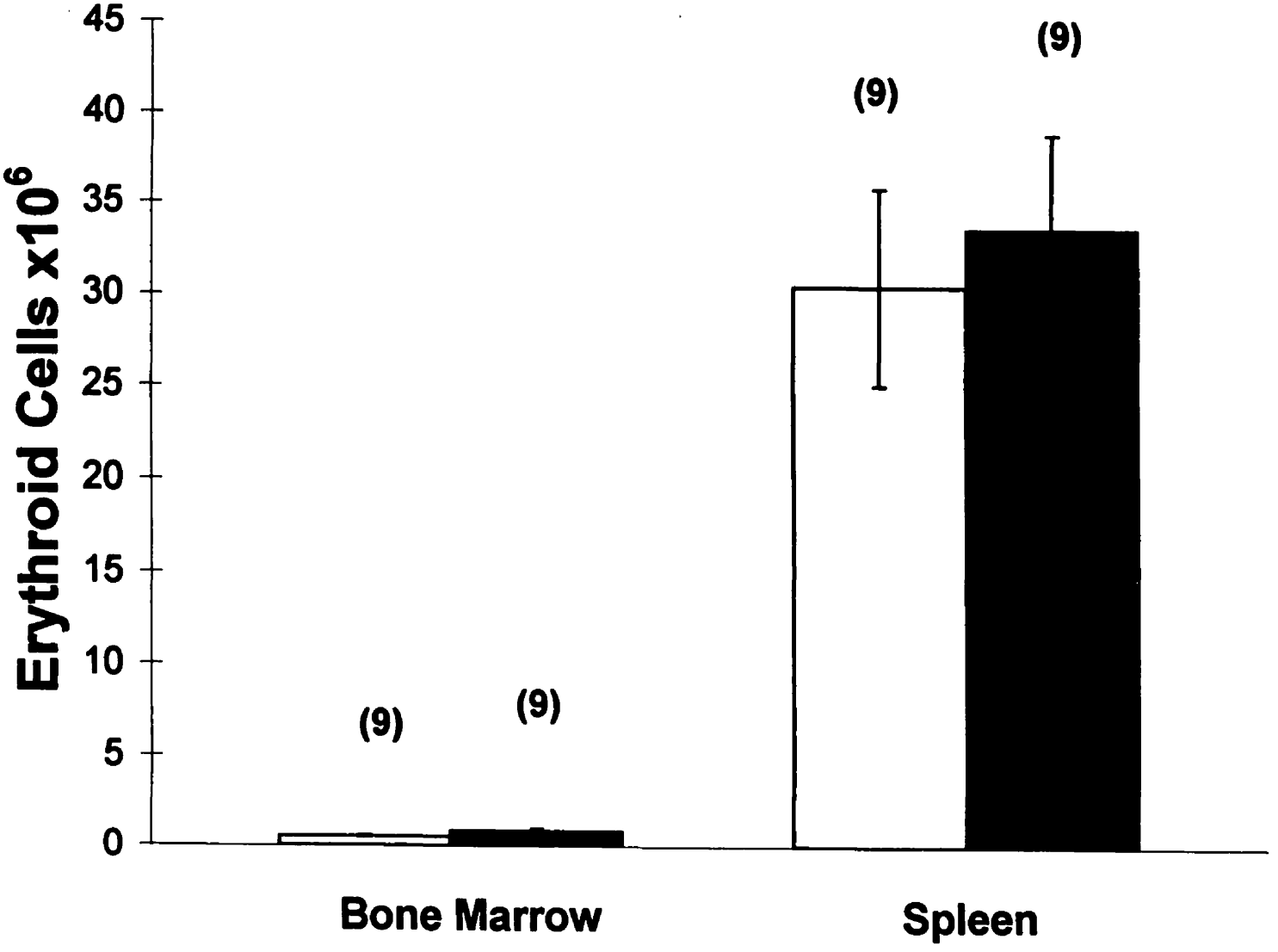

Myeloid Cells x10^6
14
12
10
8
6
4
2
0
(9)
(9)
(9)
(9)
Bone Marrow
Spleen

Fig. 5.

Absolute numbers of nucleated erythroid cells (precursors to blood-borne erythrocytes) in the spleen and bone marrow of irradiated, leukemic mice 9 days after syngeneic bone marrow transplant (SBMT). Control mice (open columns) received poly I:C 4 days prior to and 8 days after SBMT. Experimental mice (solid columns) received TNF-a for 4 days immediately following SBMT along with the poly I:C injection regimen. Mean±s.e.: 9 mice; ( ) number of mice.


Erythroid Cells x10^6
45
40
35
30
25
20
15
10
5
0
(9)
(9)
(9)
(9)
Bone Marrow
Spleen

Discussion

In the present study, we have sought to investigate the possibility that in animals bearing tumors, combinations of treatments may act additively or even synergistically, to augment NK cells, the first line of defense in tumor combat. We have previously found that absolute NK cell numbers were increased 2.8-fold (vs. untreated controls) in the spleens of poly I:C-treated, irradiated, leukemic mice, employing the identical peri-transplant injection protocol used in the present study (Currier & Miller, 1998). In the present study, the addition of TNF-a to this protocol, resulted in a 2.4-fold increase in NK cell numbers (vs. poly I:C-injected controls), indicating that the combined treatment has had a definite advantage with respect to NK cells. Given that both IFN-g (the cytokine produced by poly I:C administration), and TNF-a are powerful NK cell stimulants, acting via different receptors, it is not surprising that the increment in NK cell numbers mediated by both agents appears additive or synergistic. In support of the possibility of such "double stimulation" are our previous observations (Christopher et al., 1991), which indicated that NK cells in similar leukemic mice could be augmented 1.5 - 4.0 fold by the combined administration of 2 other powerful NK cell stimulants, i.e., interleukin-2 and indomethacin vs. either alone. The fact that NK cells were elevated in the spleen may reflect *in situ* production of these cells in that organ given (i) that TNF-a drives NK cell proliferation and development (Jewett & Bonavida, 1993; Ito et al., 1999), and (ii) that the spleen, under abnormal conditions, i.e., those occurring under the conditions of the present protocol, can convert to a generation site for new NK cells (Pollack & Rosse, 1987; Miller & Shatz, 1991).

Monocytes were the only other cell lineage to be profoundly influenced by the combined treatment. The virtual absence of monocytes in the bone marrow may have resulted from (a) lack of new cell production, or (b) early and rapid apoptosis of monocytes after their production in this organ of their central generation. Their decreased numbers in the spleen (a major organ of monocyte trafficking) would reflect this lack of monocyte efflux from the bone marrow. While TNF-a and IFN are stimulants of monocyte function, this work indicates that monocyte precursors (bone marrow), and hence, monocyte production, appears to be significantly interrupted in the presence of both agents together, but not in the presence of IFN-g (poly I:C induced) alone. The observation that monocytes alone were rendered virtually absent in both the bone marrow and spleen, while all other hemopoietic and immune lineages were not further influenced by the addition of TNF-a to the treatment protocol, may attest to a lack or inadequacy of one specific cytokine which drives monocyte production, i.e., GM-CSF. This particular cytokine has been shown, at least in humans, not to be generated *in vivo* by TNF-a administration while others are, including granulocyte and peripheral macrophage stimulating cytokines (Logan et al., 1996).

TNF-a is considered to be an inhibitor of hemopoiesis (Furmanski & Johnson, 1990; Sidhu & Bollon, 1993; Mayani et al., 1995). By contrast, however, we have found in this study, in the presence of the combined treatment, no decrease in either the bone marrow or spleen, in the numbers of myeloid (granulocytic), nucleated erythroid, or lymphoid (almost exclusively B lymphocytes: bone marrow; and T and B lymphocytes: spleen) cell numbers. That the levels of these 3 major hemopoietic cell lineages have not been

inhibited or reduced, especially in their bone marrow generation site, may be in large measure, or even exclusively, due to the NK cell. Upon TNF-a stimulation, NK cells produce a host of hemopoiesis-stimulating cytokines (Scala et al., 1984; Trinchieri et al., 1984; Pistoia et al., 1985; Numerof et al., 1988; Dinarello, 1989; Murphy et al., 1992; Zlotnik et al., 1992; Arase et al., 1993; Chen & Paul, 1997), all of which would drive post-bone-marrow-transplant hemopoiesis including new NK cell production. In support of this hypothesis are previous observations in which spleen cells, fibroblasts and others in culture stimulated with TNF-a, produce a supernatant rich in hemopoiesis stimulating cytokines (Peetre et al., 1986; Zucali et al., 1988; Le Bousse-Kerdiles et al., 1992).

Thus, there appears to have been compensatory reactions in progress in the simultaneous presence of IFN (poly I:C-driven) and TNF-a. While these fundamental hemopoietic cell lineages may have been inhibited by TNF-a administration directly (Munker et al, 1987; Furmanski & Johnson, 1990; Sidhu & Bollon, 1993; Mayani et al., 1995), the production of several proliferation-stimulating cytokines by TNF-a-stimulated NK cells appears to have tipped the balance in favor of hemopoiesis. Unlike poly I:C which can be administered long term (weeks or months), with distinct survival advantage (Currier & Miller, 1998), the toxicity of TNF-a prohibits this. However, the advantage which TNF-a does offer when administered even in the short term, along with poly I:C, is evident. That is, it provides for a greater increase in NK cell numbers than that achieved by poly I:C alone. Since the role of NK cells in the transplanted host is twofold: (a) destroy residual tumor cells and, (b) produce hemopoiesis stimulants, it becomes clear that 2 such stimulants are better than 1.

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INTRODUCTION TO CHAPTER 3

The work presented in Chapters 1 and 2 dealt with the administration of the IFN inducer poly I:C, and the cytokine TNF-a, all in an effort to boost NK cell numbers and increase life span in leukemic mice. The neurohormone melatonin (MLT) is known to synchronize eukaryotic organisms with the photoperiod. As well, its *in vitro* effects on T helper cells of the immune system have recently been examined. Yet, very little is known of its effects *in vivo* on those cells mediating a 'natural resistance', NK cells. NK cells rely on T helper cells for activation and are exquisitely sensitive to the cytokine profile they produce. It was thought that adminstration of MLT to normal, young adult mice would enhance NK cells indirectly through T helper cell involvement while avoiding the direct toxicity of exogenously administered cytokines or cytokine inducers.

CHAPTER 3: Exogenous melatonin: Quantitative enhancement in vivo of cells mediating non-specific immunity.

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Abstract

Melatonin (MLT), a biogenic indoleamine and neuromodulator produced by the pineal gland, is known to activate T helper cells by means of direct binding to melatonin receptors on both Th1 and Th2 cells. The present *in vivo* study aimed to investigate the effect of exogenously administered MLT on the hemopoietic and immune cell populations of the bone marrow and spleen in healthy, young adult male mice at two distinct MLT exposure intervals. The neurohormone, administered daily through the diet (7-14 days), was homogenized into finely ground chow. Control mice received ground chow without MLT. The results revealed cell lineage-specific, quantitative, MLT exposure-time-dependent changes in both the bone marrow and spleen. NK cells and monocytes (both components of the non-specific immune system functioning as the first line of defense against neoplasia and virus infected cells) were significantly increased in the bone marrow by both 7 and 14 days of dietary melatonin. The quantitative increment in these two cell populations, in the organ of their production, i.e., the bone marrow, indicates that new cell proliferation/production may have been stimulated by MLT. In the spleen, as in the bone marrow, NK cell levels remained significantly elevated at both 7 and 14 days after melatonin exposure. However, the number of monocytes in the spleen did not maintain, at day 14 of MLT exposure, the high levels observed after 7 days of MLT, in spite of their sustained, high numbers at 14 days in the bone marrow. This suggests that the progeny of the apparently increased monocyte production in the bone marrow (elevated absolute numbers therein) had localized in anatomical sites (other than the spleen) also common to these cells. Thus, the selective, positive influences

of *in vivo* administered, exogenous melatonin on cells mediating non-specific immunity suggests a plausible mechanism for numerous claims that it is responsible for tumor amelioration in patients.

Introduction

The neuroendocrine-immune system axis is one that has gained considerable attention in recent years. Among the neuromodulators of the various components of immunity is the pineal hormone, melatonin, (N-acetyl-5-methoxytryptamine), a biogenic indoleamine long known to act as a chronomodulator in biological systems, acting to functionally synchronize eukaryotes with the photoperiod. Endogenous MLT production follows a strict circadian rhythm, being maximally produced during the dark periods of the 24 hr cycle, being broken down soon after production, having species-dependent (hamster, rat, dog, monkey, human), and even age-dependent half lives in the blood ranging from 7.5 - 59 minutes (Mallo et al., 1990; Pang et al., 1990; Cavallo & Ritschel, 1996; Yellon, 1996; Yeleswaram et al., 1997; Brown et al., 1997). The more recently recognized interactions of melatonin with the immune system primarily have dealt with its effects on cells mediating specific immunity, primarily T helper lymphocytes (Cutando & Silvestre, 1995; Gonzalez-Haba et al., 1995; Garcia-Maurino et al., 1997; Lissoni et al., 1998; Maestroni, 1998a,b,c; Rafii-el-Idrissi et al., 1998), and functional T lymphocyte enhancement in the periphery (Pioli et al., 1993; Champney et al., 1997; Garcia-Maurino et al., 1997; Demas & Nelson, 1998). Evidence, however, is even more scant with respect to the interplay of melatonin and another prominent cell lineage mediating non-specific immunity, i.e., natural killer (NK) cells, which act in the first line of defense against tumorigeneic and virus-infected cells.

In the presence of MLT, in old mice, the thymus and T cell mediated immune functions in the periphery recovered to young adult levels (Mocchegiani

et al., 1996). MLT has also been shown to have anti-apoptotic properties among normal (non-tumor) cells (Maestroni et al., 1994a,b; Maestroni, 1998a), while another advantage attributed to this neurohormone is its ability to counter the toxicity of conventional anti-cancer therapeutic agents (Maestroni et al., 1994a,b; Cutando & Silvestre, 1995; Lissoni et al., 1995; Lissoni et al., 1997 a,b,c). Moreover, MLT itself has no known side effects (Lissoni et al., 1996; Wichmann et al., 1996; Lissoni et al., 1998), and has no stimulatory influence on tumor cell growth (Maestroni et al., 1994a,b).

MLT, by itself, appears to act on sub-cellular events pertaining to cell function, having no *direct* role in stimulating proliferation of any hemopoietic or immune cell lineage studied to date (Provinciali et al., 1997; Rogers et al., 1997). Moreover, MLT has no direct affinity for B cells, the latter not possessing MLT receptors, while other evidence indicates that MLT does not enhance humoral immunity (Gonzalez-Haba et al., 1995, Champney et al., 1997; Demas & Nelson, 1998). MLT has a role in general hemopoiesis stimulation as well (Maestroni & Conti, 1996; Maestroni, 1998 a, b, c). Receptors for MLT have been found on cells of the monocyte/macrophage lineage and the binding of MLT to these receptors results in increased production of GM-CSF, enhanced antigen presentation (spleen macrophages), and increased macrophage function (Pioli et al., 1993; Lissoni et al., 1994; Maestroni et al., 1994a,b; Garcia-Maurino et al., 1997; Williams et al., 1998).

With respect to any influence of MLT on NK cells, there is some evidence in mammals, which indicates that MLT *in vivo* produces enhanced peripheral (mature) NK cell-lytic function (Poon et al., 1994). Although, it is completely

unknown whether this results from direct mechanisms, i.e., receptor dependent interactions between MLT and NK cells, or, indirect mechanisms, i.e., via T helper cell-enhanced IL-2 production, the latter being a well known, exquisite stimulant of NK cells (Christopher et al., 1991; Naume & Espevik, 1991; Biron et al., 1992).

MLT production is progressively reduced with advancing age (Pierpaoli, 1991; Pierpaoli et al., 1991; Cavallo & Ritschel, 1996; Lesnikov & Pierpaoli, 1996; Inserra et al., 1998), just as are the cells of the immune system. Thus, the potential clinical importance is apparent for maintaining prophylactically, youthful levels both of MLT and of cells mediating immunity, especially those which act in the first line of defense in tumor immunosurveillance, i.e., NK cells and monocytes.

To date, there has been no *in vivo* quantitative, information on the various hemopoietic and immune cell lineages in the spleen and bone marrow of animals given exogenous melatonin over a range of exposure intervals. The present work, however, has now demonstrated cell lineage-specific effects mediated by both short and longer periods of exposure to exogenous melatonin. We have shown that the specific cell lineages which have been quantitatively stimulated either directly or indirectly by melatonin *in vivo*, are those which mediate immunosurveillance against tumorogenesis and virus infection, i.e., NK cells and monocytes.

Results

Table 1 indicates that in general, the cell populations of both the bone marrow and the spleen were unaffected by 7 days of dietary MLT. There was, in both organs, an elevated total cellularity (significant only in the bone marrow) resulting from 7 days of dietary MLT (Table 1). In the presence of MLT for 7 days, only the progenitor cells of the myeloid (granulocytic) cells were significantly elevated (133% of control), and moreover, only in the bone marrow.

The *in vivo* increment in the numbers of myeloid cells was confirmed to be the result of *de novo* production of such cells in the bone marrow in the presence of MLT, rather than an influx of myeloid cells from other organs, i.e., spleen, gut, etc. by *in vitro* experiments. That is, the absolute number of myeloid colonies in cultures of MLT-treated, 7-day bone marrow cultures (111.0 ±6.8) was significantly higher ($p < 0.000046$) than identically prepared cultures without MLT (4.6 ±0.9). Moreover, when 50 randomly selected colonies (10/mouse bone marrow culture from each of the 5 MLT-treated mice), were smeared, stained and microscopically analyzed, not a single colony was found to contain cells of either erythroid or lymphoid morphology. All colonies contained cells of mature and precursor myeloid (granulocytic) cells and monocytoid cells. The same was true of control colonies, in spite of their total numbers over comparable surface areas being significantly fewer.

Figs. 1 and 2 indicate that those cells which were consistently and positively enhanced by the presence *in vivo* of MLT, in both organs, were those responsible for mediating non-specific immunity, i.e., NK cells and cells of the monocyte/macrophage lineage. Figs. 3 and 4 indicate that the additional 7 days

of dietary MLT resulted in the maintenance of significantly elevated NK cell numbers in both organs. However, by contrast, after 14 days of exogenous MLT, monocyte/macrophage cells, in the spleen only, fell precipitously to 34.6% of control levels (Figs. 3,4).

Table 1.

The effect of melatonin administration to young, adult male mice on the population sizes of the hemopoietic and immune cell lineages in the spleen and bone marrow.

Treatment	Total Nucleated Cells ($x10^6$)	Total Lymphoid Cells ($x10^6$)	Mature Granulocytes ($x10^6$)	Precursor Myeloid Cells ($x10^6$)	Nucleated Erythroid Cells ($x10^6$)
Spleen					
MLT[a]	182.5±9.99[c]	139.5±7.40	7.61±1.86	1.30±0.39	35.12±2.15
Vehicle[b]	166.0±11.0	119.0±7.62	4.95±0.87	1.25±0.17	40.33±3.39
Bone Marrow (per femur)					
MLT	23.5±1.26*	8.0±0.75	8.44±0.45*	1.87± 0.12*	4.92± 0.6
Vehicle	19.4± 0.70	6.64±0.44	7.16±0.37	1.40±0.07	4.13±0.19

[a]given daily (18:00 hr) in the ground chow diet for 7 days
[b]ground chow administered as above, without melatonin
[c]Mean s.e.:6-7 mice
[d]determined from differential counts of 2,000 total cells in each organ from each mouse, on stained cytospot preparations and converted, via the known total numbers of nucleated cells/organ (enumerated by means of an electronic cell counter), to absolute numbers of cells in each morphologically identifiable series.
*$p<0.05$

Fig. 1.

Absolute number of NK cells and monocyte/macrophage lineage cells in the spleen of melatonin-fed, 7 days (closed bars), and control mice (open bars). Mean±s.e.: 6 mice. *$p<0.01$ (NK cells) - 0.001 (monocyte/macrophage cells).

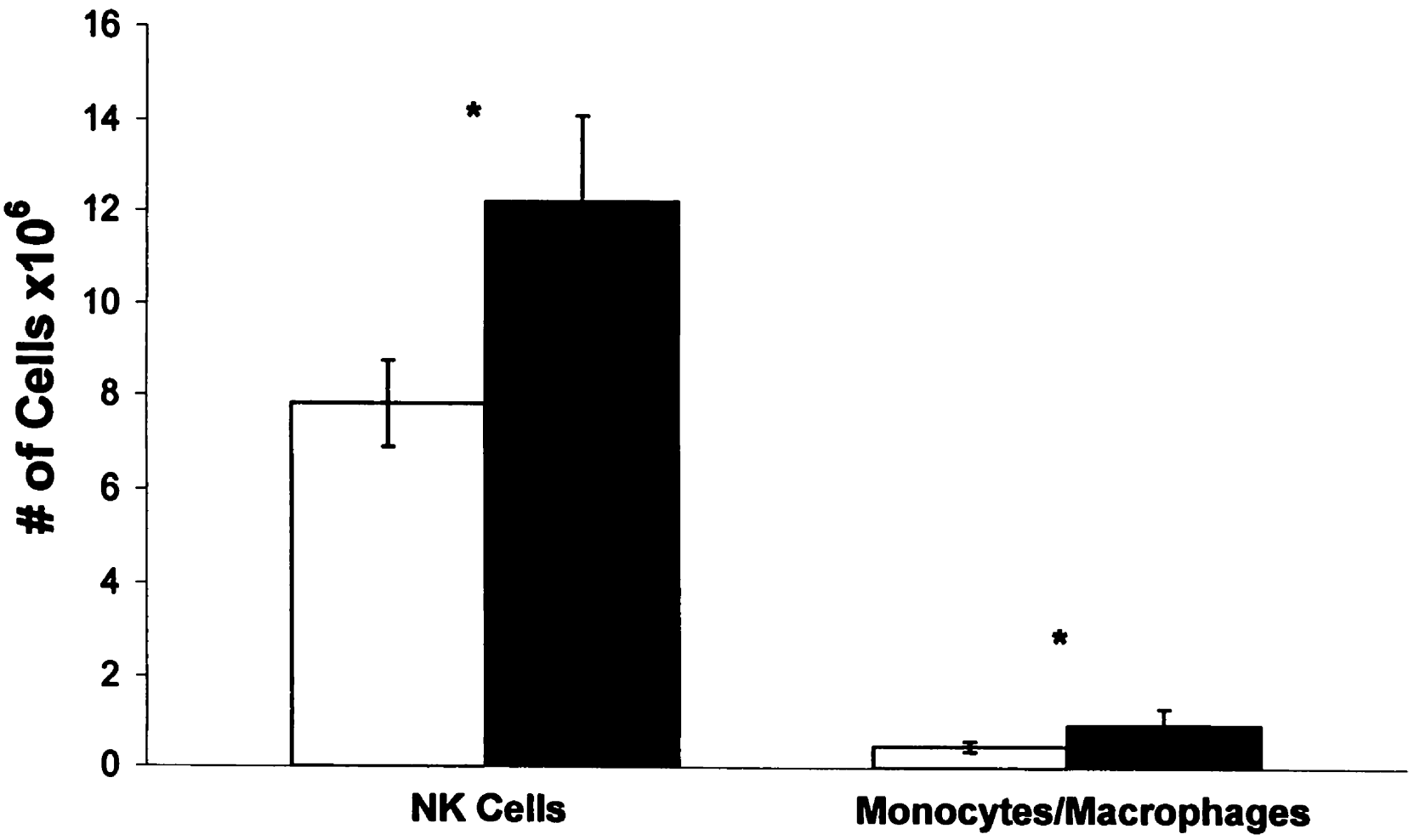

16
14
12
10
8
6
4
2
0
# of Cells x10^6
*
*
NK Cells
Monocytes/Macrophages

Fig. 2.

Absolute number of NK cells and monocyte/macrophage lineage cells in the bone marrow of melatonin-fed, 7 days (closed bars), and control mice (open bars). Mean±s.e.: 6 mice. *$p<0.005$ (NK cells) - 0.01 (monocyte/macrophage cells).

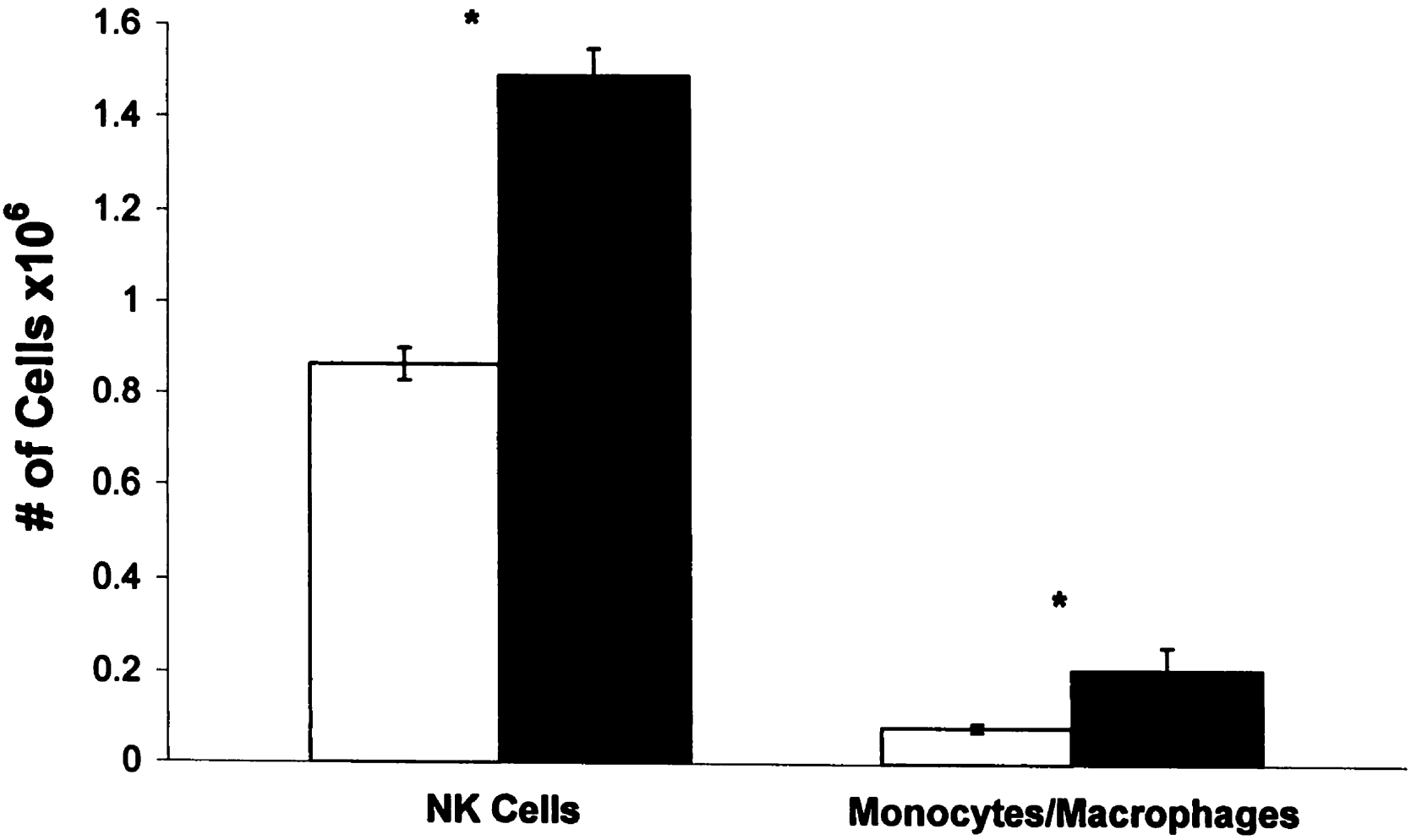

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
# of Cells x10^6
*
*
NK Cells
Monocytes/Macrophages

Fig. 3.

Absolute number of NK cells and monocyte/macrophage lineage cells in the spleen of melatonin-fed, 14 days (closed bars), and control mice (open bars). Mean±s.e.: 6-7 mice. *$p<0.02$ (NK cells) - 0.007 (monocyte/macrophage cells).

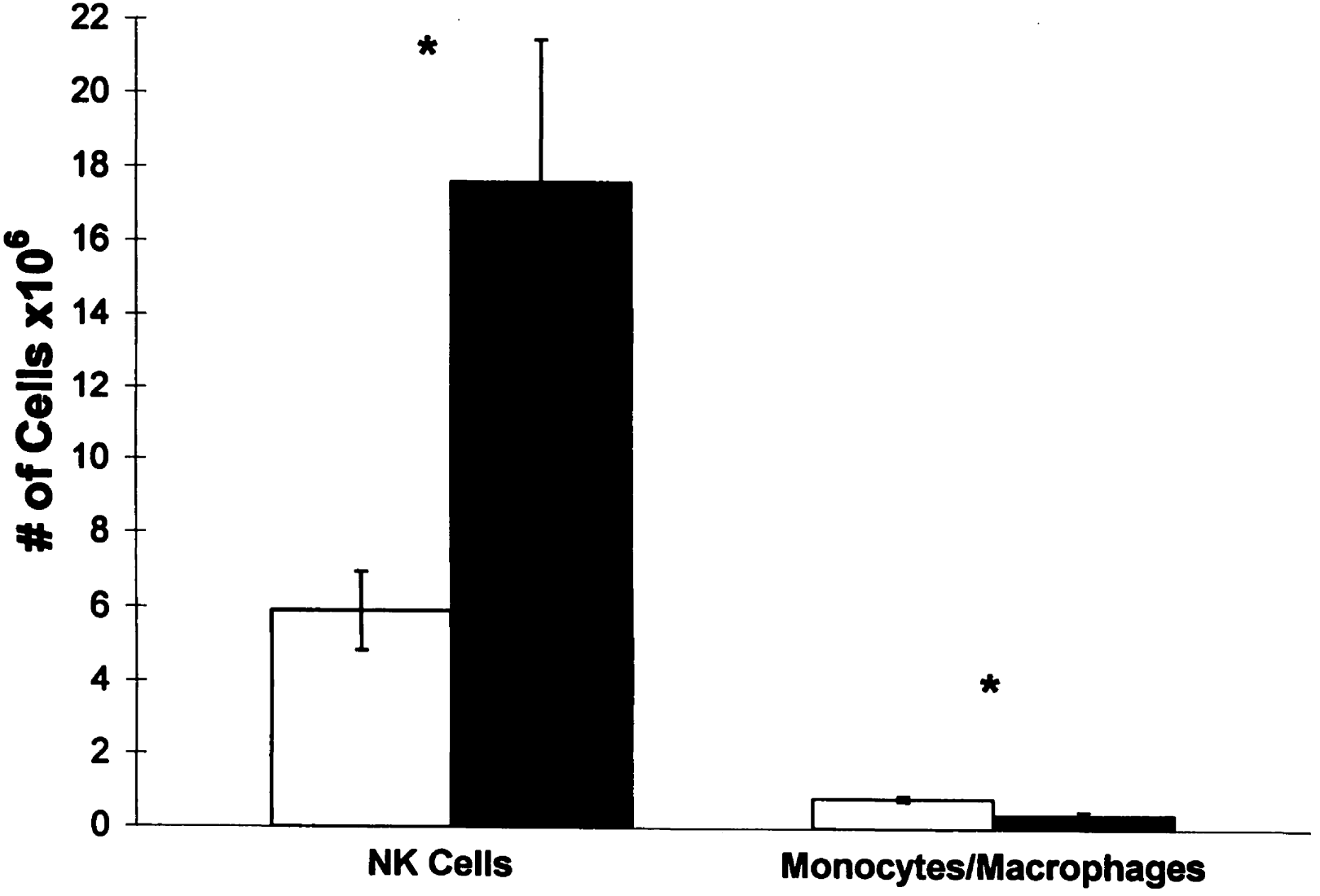
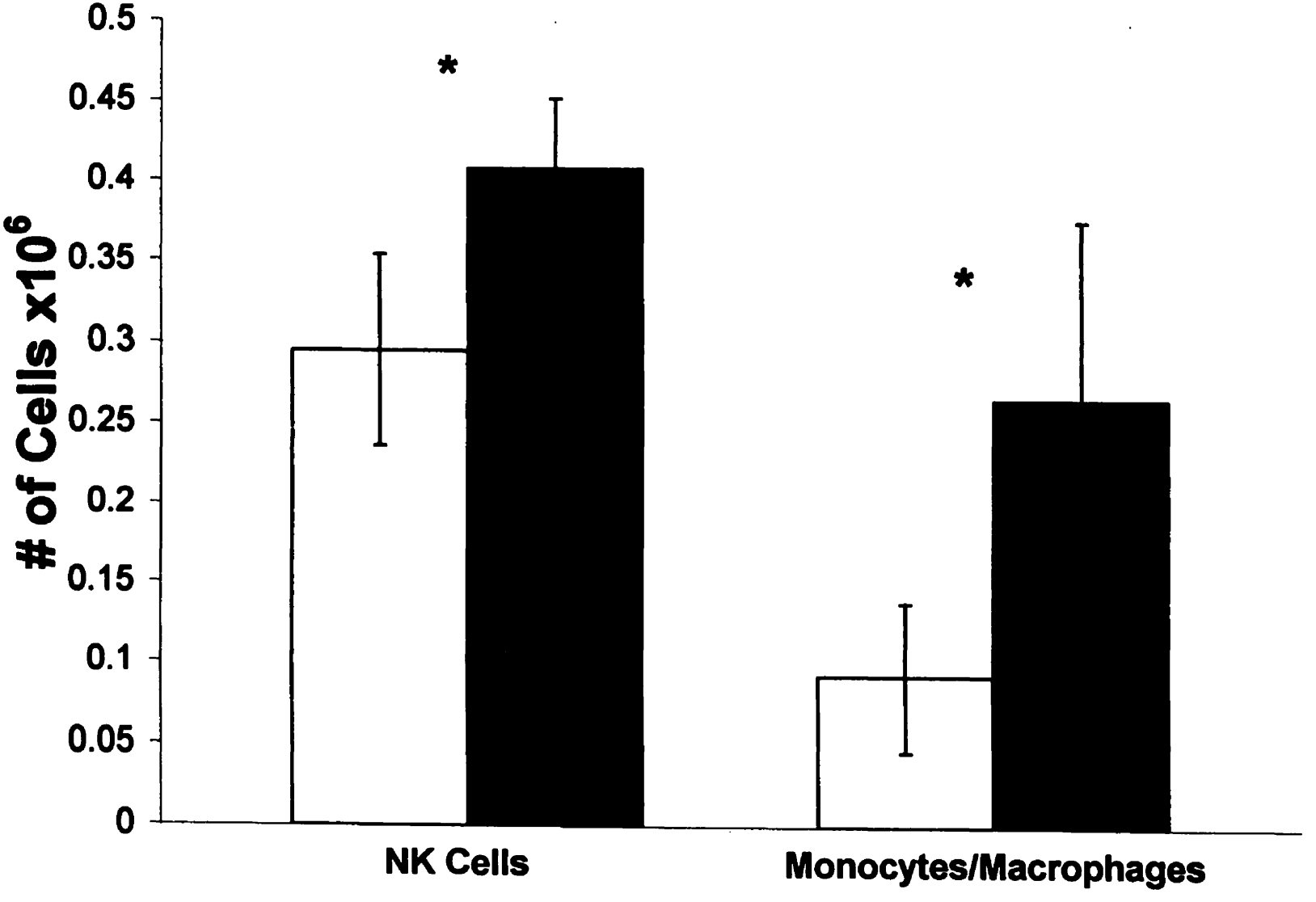

22
20
18
16
14
12
10
8
6
4
2
0
# of Cells x10^6
*
*
NK Cells
Monocytes/Macrophages

Fig. 4.

Absolute number of NK cells and monocyte/macrophage lineage cells in the bone marrow of melatonin-fed, 14 days (closed bars), and control mice (open bars). Mean±s.e.: 6-7 mice. *p=0.05 (NK cells) - 0.003 (monocyte/macrophage cells).


0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
# of Cells x10^6
*
*
NK Cells
Monocytes/Macrophages

Discussion

This study has provided a quantitative, *in vivo* analysis of the influence of exogenous melatonin on hemopoietic and immune cells in the organs of their generation (bone marrow), and function (spleen). Short and/or longer term exposure to this indoleamine, has revealed lineage-specific and MLT-exposure-duration changes, most notably in cells mediating non-specific immunity, i.e., NK cells and monocytes. That NK cells were so influenced by exposure to melatonin, may reflect, at least in part, the fact that NK cells are exquisitely sensitive to cytokines produced by melatonin-stimulated T helper cells. Specifically, interleukin-2, -6, -12 and interferon-gamma, all products of melatonin-activated T helper cells, can augment NK cell function and/or stimulate new NK cell production, (Senik et al., 1979; Hinuma et al., 1986; Christopher et al., 1991; Dussault & Miller, 1993; Cho et al., 1996; Wu et al., 1996). To date, it is unknown if NK cells themselves possess receptors for MLT as do T helper cells. Monocyte production, on the other hand (resulting in our observations of increased absolute numbers of cells of this lineage), may be driven either directly (Boranic et al., 1997), since cells of this lineage do possess receptors for MLT, or indirectly, in response to the triggered cascade of monocyte-sensitive stimulants such as IL-3, -4, -6 and GM-CSF (Maestroni et al., 1994a,b), set in place by MLT activation of T helper cells. Stromal cells possess receptors for kappa opioid cytokine peptides (Maestroni, 1998a, b), proteins structurally similar to interleukin-4 (Maestoni & Conti, 1996; Maestroni, 1998c), and the products of direct ligand (MLT)-receptor (MLTR) mediated stimulation of T helper cells. Opioid neuropeptides from mouse bone marrow cultured stromal cells appear to

regulate the proliferation of hemopoietic cells including monocyte-lineage cells (Boranic et al., 1997; Maestroni, 1998a).

The only other cells to be significantly increased in absolute number were the precursors (and consequently, their mature progeny), of the granulocyte lineage, and, moreover, only those of the bone marrow were so influenced. This suggests the influence of opioid-stimulated bone marrow stromal cells was also occurring *in vivo* to drive proliferation in this lineage, as with monocytes. Since there is a common stem cell for granulocytes and monocytes, their similar responses to GM-CSF (granulocyte-monocyte colony stimulating factor) is not an unusual observation. Moreover, the results of the bone marrow culture assays clearly support the *in vivo* observations, and indicate that the substantial increase in myeloid precursors occurring *in vivo* after 7 days of MLT, do indeed reflect *in situ* generation of these cells rather than some MLT-altered, inter-organ trafficking of myeloid precursors.

There is no evidence *in vitro* or *in vivo* that melatonin stimulates any cells of the erythroid lineage, either directly or indirectly. Nucleated erythroid cells do not themselves possess receptors for melatonin. Our present data confirm, *in vivo*, that even after sustained, daily exposure to MLT for periods of to 2 weeks, erythropoiesis remained unaffected. This is evidenced by the absolute numbers of nucleated erythroid cell precursors remaining steadfastly at control levels throughout MLT exposure.

The influence of MLT on non-NK lymphocytic cells in the bone marrow, the site of B lymphocyte genesis (Hermans et al., 1989; Jacobsen et al., 1990; Osmond, 1990), and the spleen (almost exclusively composed of mature,

functional B and T lymphocytes), has been similarly inconsequential. Although our data provides the first *in vivo* observations supporting existing *in vitro* evidence indicating that MLT does not affect B lymphocyte proliferation or humoral (B cell mediated) immunity (Gonzalez-Haba et al., 1995; Champney et al., 1997; Rogers et al., 1997; Atre & Blumenthal, 1998), there is some evidence that humoral immunity can at least indirectly be influenced by melatonin (Maestroni et al., 1986).

In summary, the present work has revealed a selective, positive influence of melatonin on cells mediating non-specific immunity, i.e., NK cells and monocyte/macrophage lineage cells, both populations well established as the first line of defense against neoplastically transformed and virus infected cells. The results suggest, thus, a plausible mechanism for the growing body of evidence implicating melatonin in tumor amelioration in patients (Lissoni et al.,1992; 1994; 1997a; b; c; Fraschini et al., 1998).

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INTRODUCTION TO CHAPTER 4

In Chapter 3 I found that administration of MLT to healthy, young adult mice, resulted in significantly increased numbers of NK cells and monocytes in both the bone marrow and the spleen, after one or two weeks of feeding, versus untreated mice. MLT is nonetheless a hormone, and inherently its administration as a therapy is beset with significant drawbacks and side effects, i.e., it is ineffective during the daylight hours.

For this reason, I continued my attempt to boost natural resistance with examination of a plant product with plentiful circumstantial evidence that it ameliorates virus-mediated conditions such as the common cold and influenza, namely Echinacea *purpurea*. However, virtually no evidence existed examining the *in vivo* effects of E. *purpurea* on those cells mediating natural resistance, NK cells. Further, E. *purpurea* has been shown to be entirely non-toxic when given at a wide range of doses. For these reasons, I aimed to examine the effects of E. *purpurea* administered via the chow to healthy, young adult mice, for one or two weeks, with the intent of establishing the effect of this herb over short and longer term administration on all cells of the hemopoietic and immune cell lineages.

CHAPTER 4: The American Coneflower: A prophylactic role involving non-specific immunity.

Linda Z- Y Sun, Nathan L. Currier and Sandra C. Miller, Ph.D.

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Abstract

In humans, considerable circumstantial evidence exists which indicates that soluble root extracts of the American coneflower, genus *Echinacea,* may act to ameliorate virus-mediated afflictions such as the common cold, influenza and even AIDS and virus-based tumors. The present study was designed to quantify, in normal mice, *Echinacea-mediated,* quantitative, dynamic changes with time on both mature and precursor cells, of all the hemopoietic and immune lineages in the spleen and bone marrow. Briefly, a specific, commercially prepared potent extract of *Echinacea* root was provided daily in the diet for either 1 week or 2 weeks with the aim of establishing a possible mechanism of action for this phytochemical. The results revealed that natural killer (NK) cells and monocytes, both mediators of non-specific immunity and well-demonstrated killers of virus-containing cells, were numerically, significantly increased in both the bone marrow (generation center of both cell types) and the spleen (mature cell organ of immune function), as early as 1 week after beginning the dietary herb. In striking contrast to our observations with NK cells and monocytes, the sizes of all other hemopoietic and immune cell populations in these 2 organs remained steadfastly at control levels, even after 2 weeks of daily dietary *Echinacea.* The work has shown, thus, the exquisitely specific nature of *Echinacea*-derived phytochemicals, in acting as powerful stimulants only of those cells responsible for non-specific immunity – known to act as the first line of defense against virus-infected/transformed cells. Secondly, the observations that these cells were elevated in their bone marrow birthplace, indicates that at least one mechanism of action of this herb, is to stimulate *in situ* new cell

production. Finally, the significant elevation of these 2 fundamental immune cell populations, in perfectly normal animals, strongly suggests a prophylactic role for this herb.

Introduction

Variously prepared extracts of the roots and other parts of *Echinacea* species *(E.angustifolia, E. purpurea),* have gained considerable interest recently for their reported health benefits including amelioration of an assortment of pathologies such as inflammations, bacterial/viral infections, tumors and AIDS (Stimpel et al., 1984; Tragni et al., 1985; Lersch et al., 1990; 1992; Roesler et al., 1991a, b; Steinmuller et al., 1993; Hill et al., 1996). While all parts of Echinacea species plants appear to have medicinal value, the roots are most concentrated in medicinal properties. These species were originally used by the native Americans for the treatment of insect and animal bites and assorted skin irritations, given that at least part of the healing values of Echinacea species derives from its potent anti-inflammatory qualities (Tragni et al., 1985; Hill et al., 1996). Although herbal medicine was practiced by the American physicians in the 19th and early 20th centuries, Echinacea was never approved by the American Medical Association since rigorous experimental evidence of its medicinal efficacy did not exist, and, in fact, the healing properties of this herb were virtually forgotten with the development of antibiotics (Combest and Nemecz, 1997). Subsequently, however, with the debut of the science of immunology concomitant with an assortment of techniques for measuring the functional responses of different immune cells, at least in culture, herbs such as Echinacea were re-discovered, and immune-stimulation was advanced as a possible mechanism for their medicinal value in so many disease-defense processes. During the past 2 decades, much of the concentrated effort in studying the medicinally relevant herbs has been aimed at biochemically

dissecting out the many and varied chemical compounds which, individually, may act uniquely on specific immune cells. These relatively exhaustive studies have indicated that such compounds include: high molecular weight polysaccharides such as arabinogalactan, inulin, heteroxylan, essential oils such as germacrene, vanillin, humulene, borneol; the alkylamides such as echinacein, isobutylamides (penta- and hexa-decadienes); polyacetylene, tannins, Vitamin C and flavonoids.

It appears that at least one family of active ingredients in these plants is the alkamides, shown to inhibit 5-lipoxygenase and cyclooxygenase (Wagner et al., 1989; Muller-Jakic et al., 1994), key enzymes in the production of prostaglandin. Prostaglandins are inhibitory to natural killer (NK) cells. On the other hand, acid arabinogalactan, a 75,000 MW polysaccharide extractable from *Echinacea* species, was shown to be effective against *Leishmania, in vitro,* via direct stimulation of macrophages, cytotoxic for these microorganisms (Leuttig et al., 1989). While *Echinacea* species contain a host of other physiologically beneficial components (above), some of their most important immunoenhancing elements may be those that interfere with prostaglandin formation.

We have found recently, that *in vivo* administration of inhibitors of prostaglandin such as indomethacin, significantly stimulated NK cells in leukemic mice, concomitant with cure and/or significantly longer life span (Christopher et al., 1991; Dussault & Miller, 1993). The literature is completely deficient in information which records and/or quantifies the behaviour of hemopoietic and immune cells, *in vivo,* during short or long term *Echinacea* exposure. Nothing is known of the population dynamics *in vivo* of monocytes or NK cells (first lines of defense in viral infections or tumor growth) in animals or humans given either

whole plant or purified extracts of *Echinacea* species, under controlled conditions of exposure time and environment. To date, various extracts from these plants have been assessed for their ability to stimulate, directly or indirectly, the function of macrophages (animal and human) *in vitro/in vivo* (Stimpel et al., 1984; Leuttig et al., 1989; Wagner & Jurcic, 1991; Fry et al., 1998). Similar assays show little or no change in animals or humans (peripheral blood), in the functional activity of T or B lymphocytes in the presence of *Echinacea* (Stimpel et al., 1984; Leuttig et al., 1989; Roesler et al., 1991b; Elsasser-Beile et al., 1996). There is some *in vitro* evidence that NK cells from animals, and from the blood of normal and AIDS-inflicted humans, can be augmented functionally by extracts from *Echinacea* species (Lersch et al., 1990; See et al. 1997).

The present study was undertaken to investigate any absolute changes in cells of the immune system as well as other hemopoietic cells that may result from dietary intake of *Echinacea*. A specific, commercially prepared extract of the root (most potent part) of *Echinacea purpurea* was used, *in vivo,* under controlled conditions. Thus, inbred mice of known strain, age, sex and environment were used throughout. From mice fed the dried root daily (onset of feeding, dosage and exposure time standardized), the hemopoietic and immune cell populations of the bone marrow and spleen, were quantified with time. The results showed that *Echinacea*-mediated influences were exclusively, and positively embued upon those cells (natural killer cells and monocytes) responsible for targeting tumors and virus-infected cells.

Results

Consumption of this specific root extract of *Echinacea purpurea* by mice for either 1 or 2 weeks had a significant ($p<0.05$-0.01) enhancing influence on the absolute numbers of NK cells in the organ of their production, the bone marrow (Fig. 1). Moreover, the spleens of *Echinacea-fed* mice also revealed, by 2 weeks, significant ($p<0.01$) increments in the absolute numbers of NK cells, relative to normal-diet-fed (control) mice (Fig.2). Monocytes, also mediators of non-specific immunity were increased ($p<0.01$) as well, by 2 weeks of *Echinacea* exposure, in both the bone marrow and the spleen (Figs. 3 and 4). Of striking significance is our observation that all other hemopoietic and immune cell populations, in both the bone marrow and spleen, remained steadfastly at control levels irrespective of the dietary presence of *Echinacea* for either 1 or 2 weeks (Figs. 5-9). Since the data obtained for each population (Figs. 5-9) in each organ was virtually identical at 1 week and at 2 weeks of dietary *Echinacea,* comparable data from the 2 feeding periods were pooled.

Fig. 1.

Total number (mean±s.e.) of NK (ASGM-1+) cells (lymphocyte morphology) in the bone marrow of control diet- and *Echinacea-fed,* young adult DBA/2 male mice. ( ) = number of mice. One week E.p. fed mice (open bar), one week control diet (closed bar), two weeks E.p. fed mice (dotted bar), two weeks control diet (horizontal striped bar). *$p<0.01$

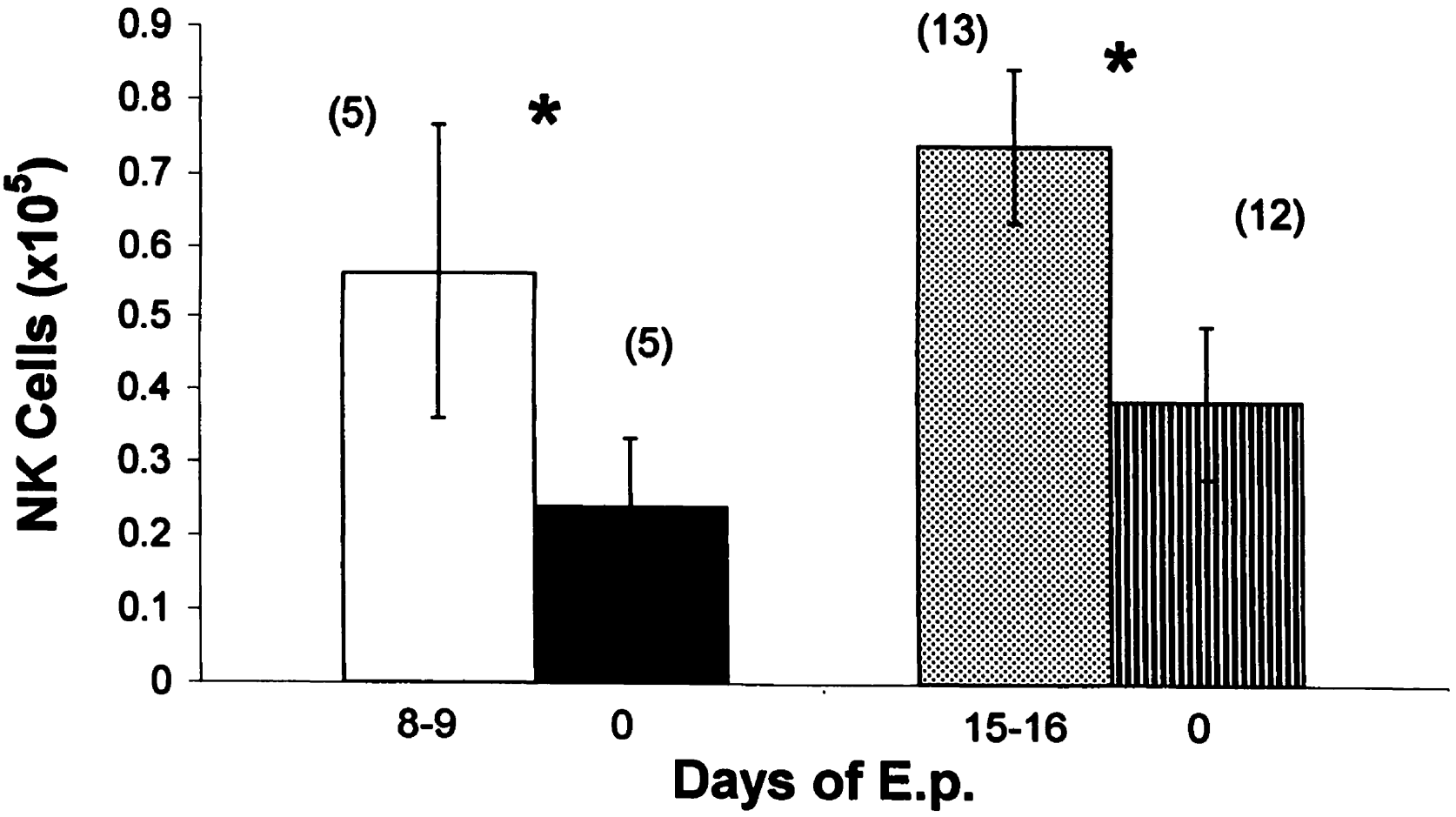

NK Cells (x10^5)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
(5)
*
(5)
(13)
*
(12)
8-9
0
15-16
0
Days of E.p.

Fig. 2.

Total number (mean±s.e.) of NK (ASGM-1 +) cells (lymphocyte morphology in the spleen of control diet- and *Echinacea* –fed, young adult DBA/2 male mice. ( ) = number of mice. One week E.p. fed mice (open bar), one week control diet (closed bar), two weeks E.p. fed mice (dotted bar), two weeks control diet (horizontal striped bar). *$p<0.01$

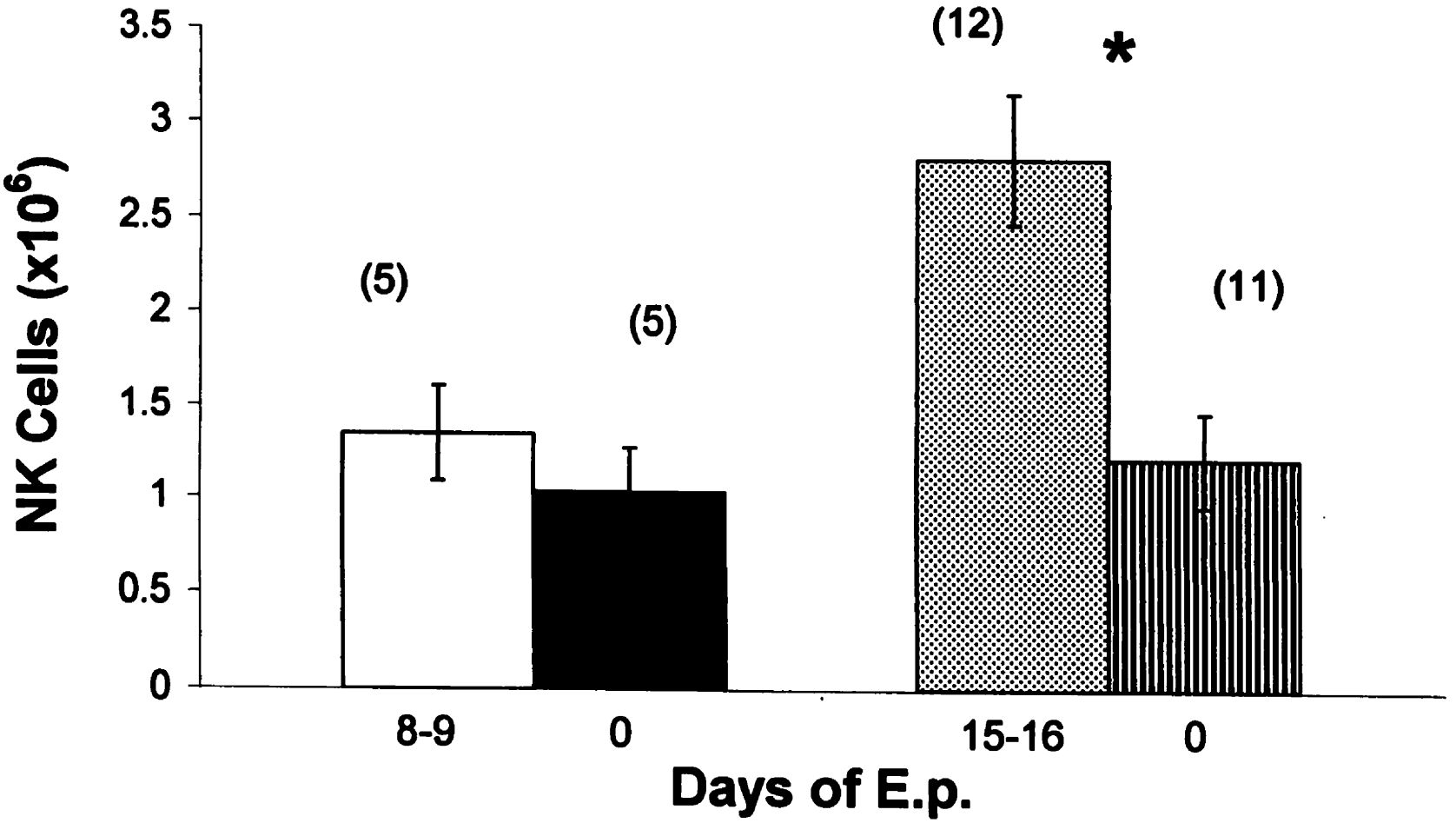

NK Cells (x10^6)
3.5
3
2.5
2
1.5
1
0.5
0
(5)
(5)
(12)
*
(11)
8-9
0
15-16
0
Days of E.p.

Fig. 3.

Total number (mean±s.e) of monocytes (precursors to tissue macrophages) in the bone marrow of control diet- and *Echinacea* –fed young adult DBA/2 male mice. ( ) = number of mice. One week E.p. fed mice (open bar), one week control diet (closed bar), two weeks E.p. fed mice (dotted bar), two weeks control diet (horizontal striped bar). *$p<0.01$

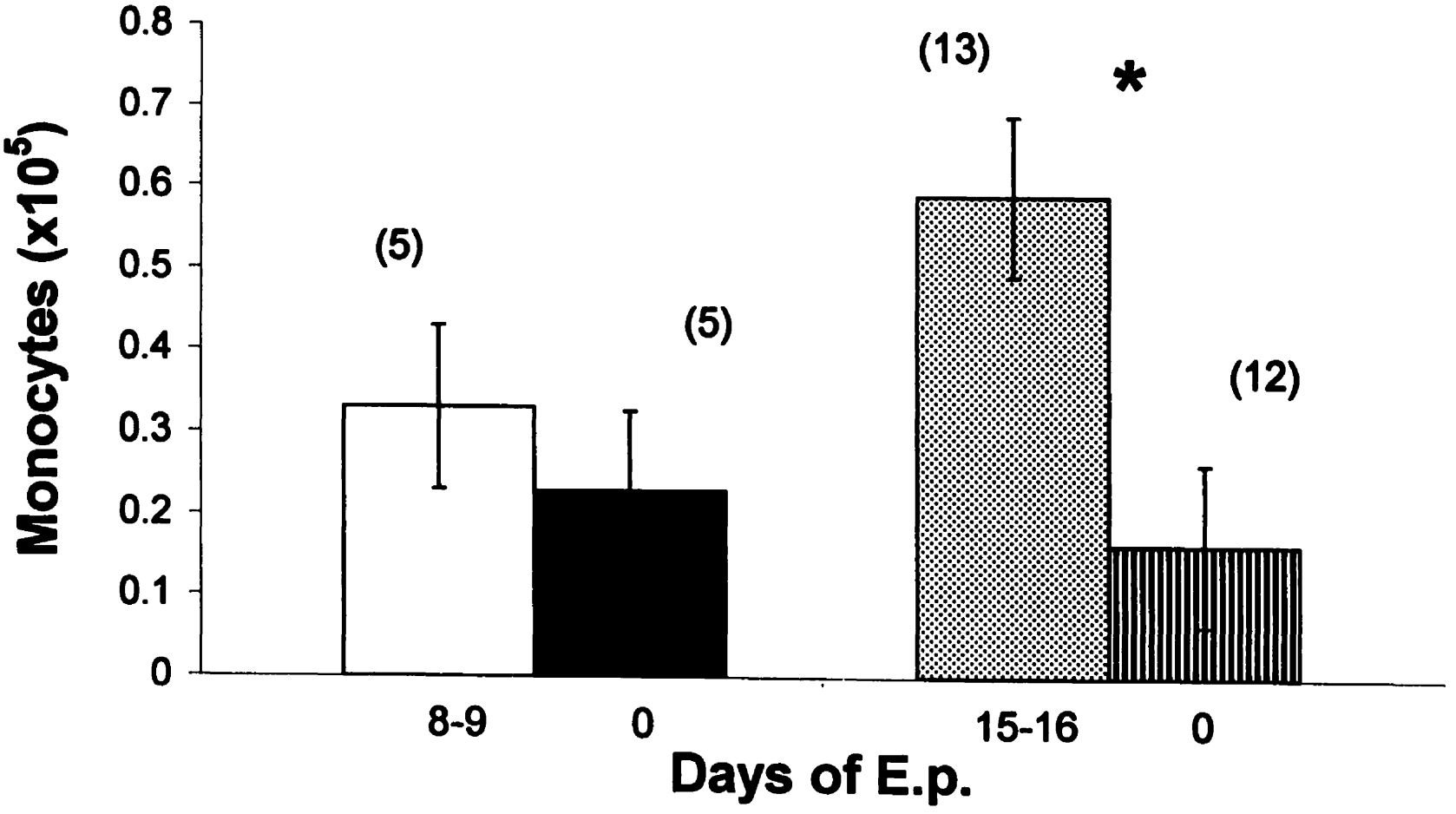

Monocytes (x10^5)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
(5)
(5)
(13)
*
(12)
8-9
0
15-16
0
Days of E.p.

Fig. 4.

Total number (mean±s.e.) of monocytes in the spleen of control diet- and *Echinacea* –fed, young adult DBA/2 male mice. ( ) = number of mice. One week E.p. fed mice (open bar), one week control diet (closed bar), two weeks E.p. fed mice (dotted bar), two weeks control diet (horizontal striped bar). *$p<0.01$

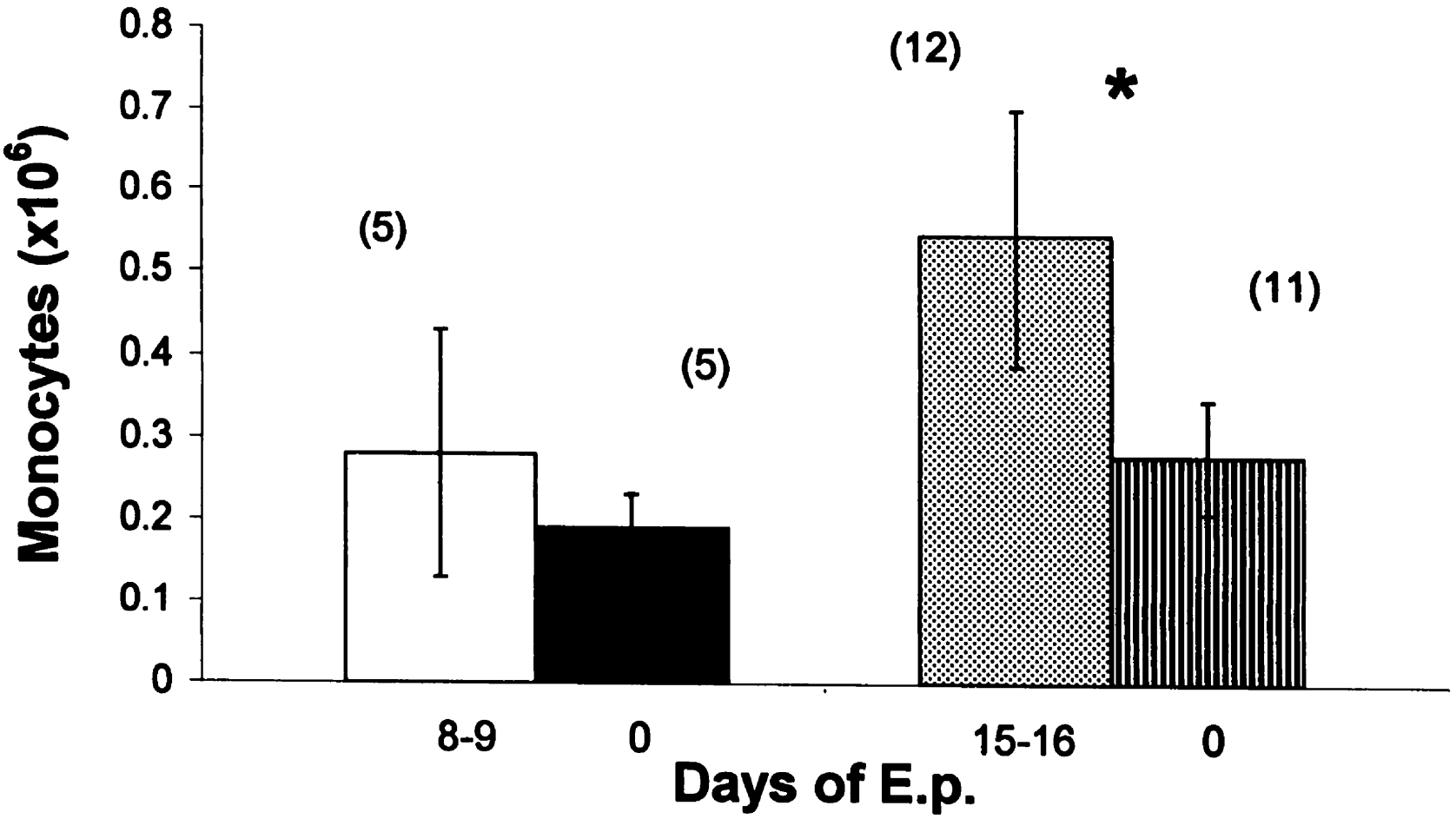

Monocytes (x10^6)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
(5)
(5)
(12)
*
(11)
8-9
0
15-16
0
Days of E.p.

Fig. 5.

Total number (mean±s.e.) of non-NK lymphocytes (large precursors and small progeny) in the bone marrow of control (ctrl) diet fed- and *Echinacea* –fed, young adult *DBA/2* male mice. ( ) = number of mice. Large precursors one and two week E.p. fed mice (open bar), large precursors one week control diet (closed bar), small precursors one and two week E.p. fed mice (dotted bar), small precursors two weeks control diet (horizontal striped bar).

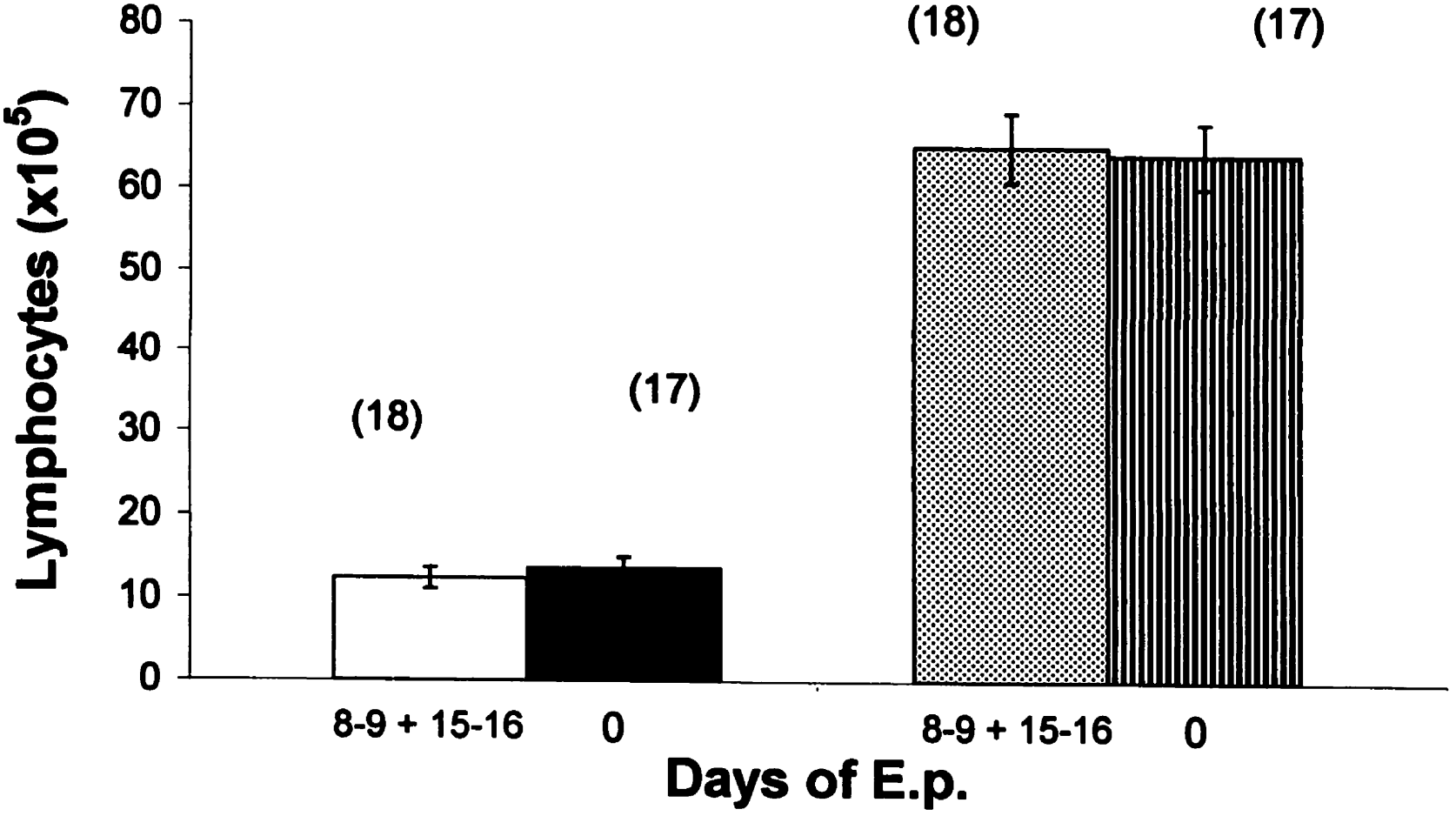

Lymphocytes (x10^5)
80
70
60
50
40
30
20
10
0
(18)
(17)
(18)
(17)
8-9 + 15-16
0
8-9 + 15-16
0
Days of E.p.

Fig. 6.

Total number (mean±s.e.) of non-NK lymphocytes (large precursors and small progeny) in the spleen of control (ctrl) diet fed- and *Echinacea* –fed, young adult *DBA/2* male mice. ( ) = number of mice. Large precursors one and two week E.p. fed mice (open bar), large precursors one week control diet (closed bar), small precursors one and two week E.p. fed mice (dotted bar), small precursors two weeks control diet (horizontal striped bar).

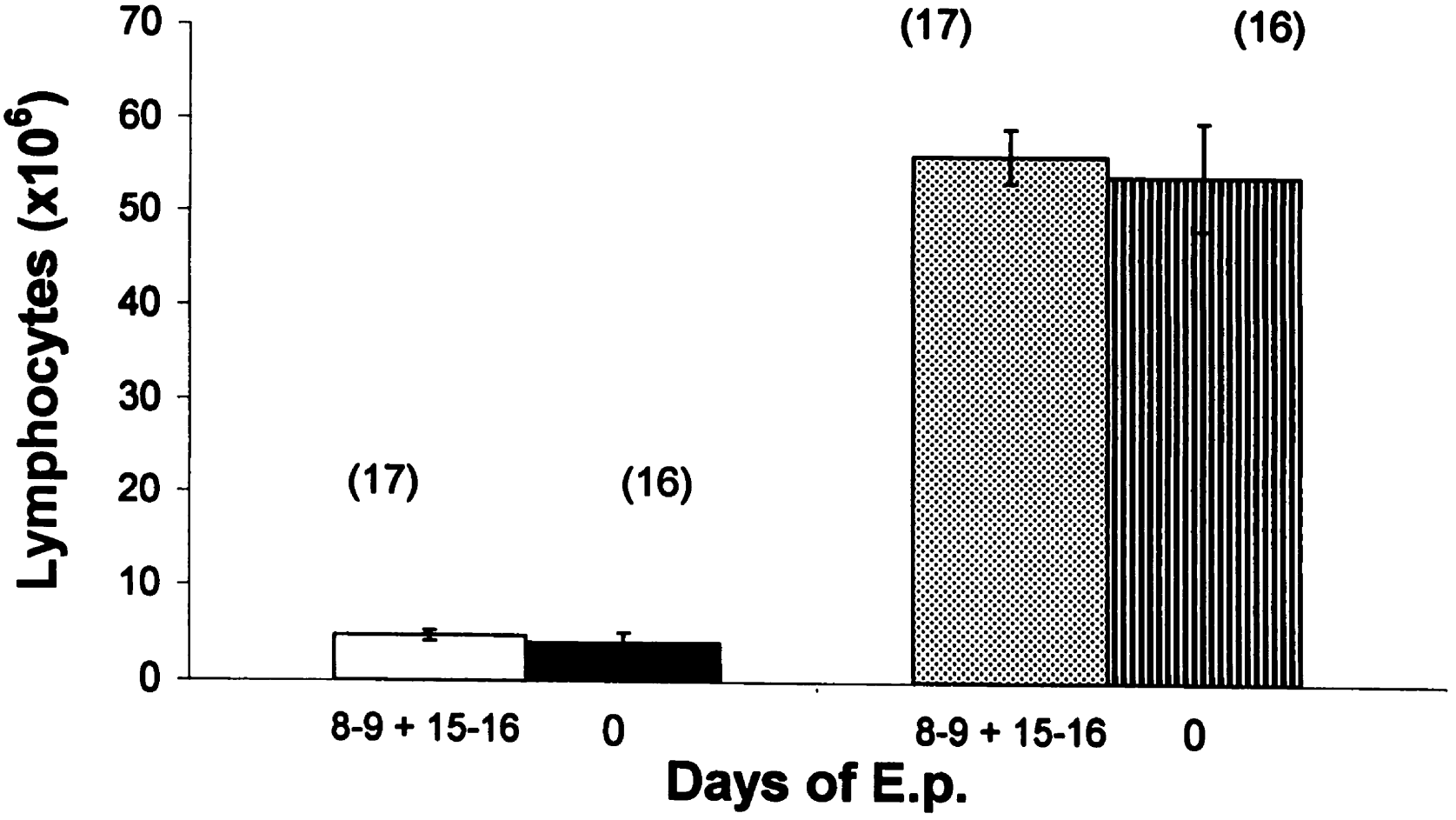

Lymphocytes (x10^6)
70
60
50
40
30
20
10
0
(17)
(16)
(17)
(16)
8-9 + 15-16
0
8-9 + 15-16
0
Days of E.p.

Fig. 7.

Total number (mean±s.e.) of precursor (pre.) and mature cells in the granuloid (myeloid) lineage in the bone marrow of control (ctrl) diet fed- and *Echinacea* – fed, young adult *DBA/2* male mice. ( ) = number of mice. Precursor cells one and two week E.p. fed mice (open bar), precursor cells one week control diet (closed bar), Mature cells one and two weeks E.p. fed mice (dotted bar), Mature cells two weeks control diet (horizontal striped bar).

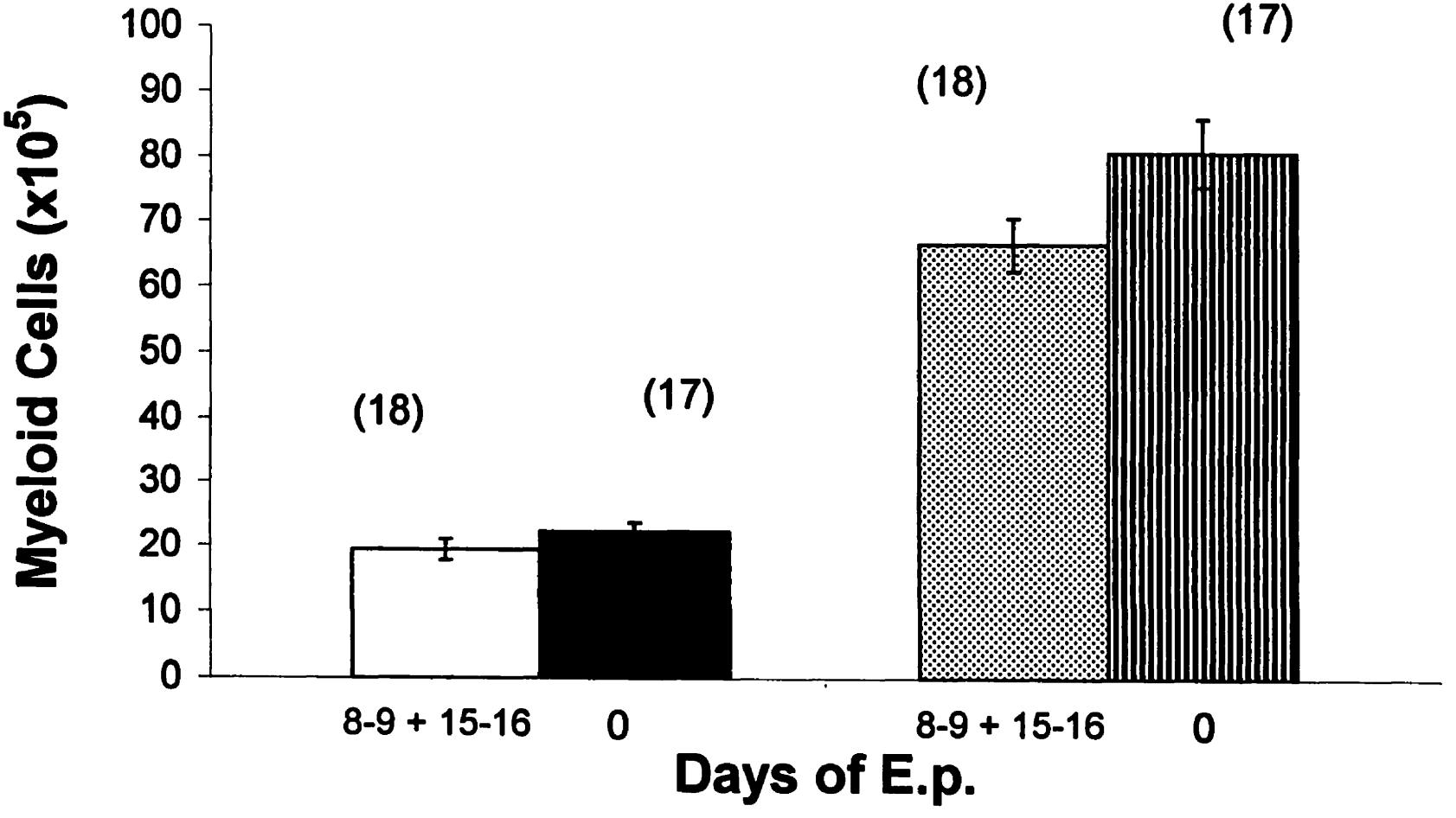

Myeloid Cells (x10^5)
100
90
80
70
60
50
40
30
20
10
0
(18)
(17)
(18)
(17)
8-9 + 15-16
0
8-9 + 15-16
0
Days of E.p.

Fig. 8.

Total number (mean±s.e.) of precursor (pre.) and mature cells in the granuloid (myeloid) lineage of the spleen of control (ctrl) diet fed- and *Echinacea*-fed, young adult *DBA/2* male mice. ( ) = number of mice. Precursor cells one and two week E.p. fed mice (open bar), precursor cells one week control diet (closed bar), Mature cells one and two weeks E.p. fed mice (dotted bar), Mature cells two weeks control diet (horizontal striped bar).

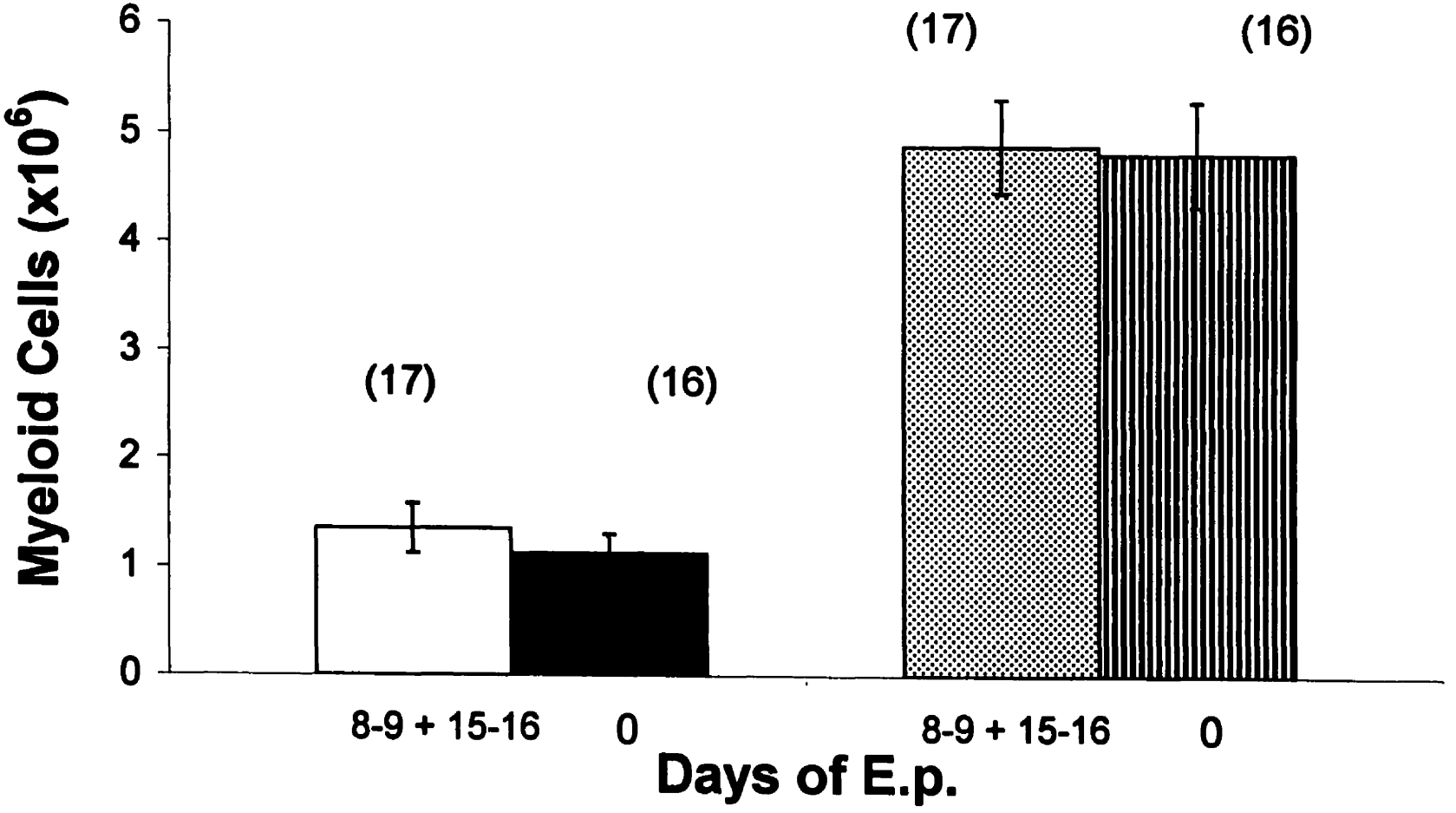
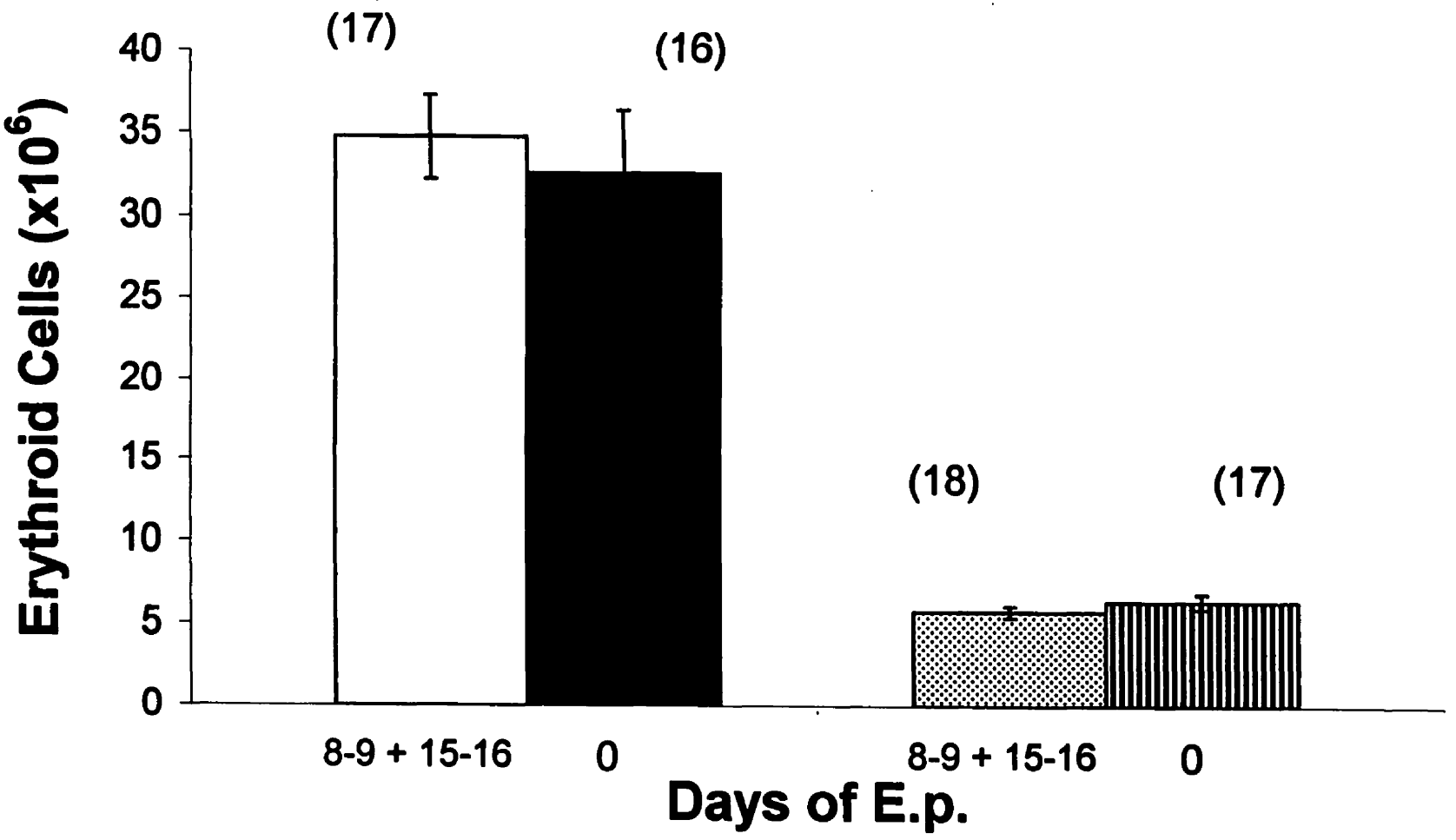

Myeloid Cells (x10^6)
6
5
4
3
2
1
0
(17)
(16)
(17)
(16)
8-9 + 15-16
0
8-9 + 15-16
0
Days of E.p.

Fig. 9.

Total number (mean±s.e.) nucleated erythroid cells (precursors of peripheral red blood cells) in the spleen and bone marrow of control (ctrl) diet fed- and *Echinacea* –fed, young adult *DBA/2* male mice. ( ) = number of mice. Spleen one and two week E.p. fed mice (open bar), spleen one week control diet (closed bar), bone marrow one and two weeks E.p. fed mice (dotted bar), bone marrow two weeks control diet (horizontal striped bar).


(17)
(16)
(18)
(17)
40
35
30
25
20
15
10
5
0
Erythroid Cells (x10^6)
8-9 + 15-16
0
8-9 + 15-16
0
Days of E.p.

Discussion

The results have revealed, firstly, that this particular commercial extract of *Echinacea* root, administered via the diet, was indeed active. This is significant to establish, prior to investigating the herb's mechanism of immunoenhancement, given the wide range of potencies, or lack thereof, in the various commercially available Echinacea extracts (Carr et al., 1998). Secondly, the effects of *Echinacea* appear to be unique, affecting only cells mediating non-specific immunity, i.e., NK cells and monocytes. The increase in NK cells in the bone marrow necessarily indicated enhanced production of these cells given that the bone marrow is the well established birth site of all new NK cells (Haller & Wigzell, 1977; Hackett et al., 1986; Pollack & Rosse, 1987), the spleen being the recipient of all such newly produced, bone marrow derived NK cells (Miller, 1982). Since the spleen is not the normal birth site of NK cells, and since there is a unidirectional trafficking of new NK cells from the bone marrow to the spleen via the blood (Miller, 1982), the elevated NK cell numbers of NK cells in the spleen necessarily reflects new NK cell influx vs. *in situ* (spleen) production. There is no long-lived, recirculating, i.e., "memory" component to the NK cell-mediated limb of the immune response (Seaman et al., 1978; Zoller et al., 1982), a well-established phenomenon for both T cell- and B cell-mediated limbs of the immune response and the reason for the success of vaccination. Since the elevated numbers of NK cells in the spleen cannot be explained by recirculation of long-lived, memory NK cells, then stepped-up production within, and efflux from, the bone marrow must be occurring under the influence of *Echinacea*. The observed results may reflect herb-mediated inhibition of *in vivo*-produced

prostaglandins (Wagner et al., 1989; Muller-Jakic, 1994), or increased production of macrophage-derived stimulants (Leuttig et al., 1989), or both, given that both phenomena occur in the presence of *Echinacea*. It is well established is that prostaglandin inhibition (Kendall & Targan, 1980; Lala et al., 1986), and macrophage-derived cytokines (Minato et al., 1980; Djeu et al., 1981), have a positive influence on NK cells. Under the influence of the dietary herb, the significant increase in cells of the monocyte/macrophage lineage, producers of the powerful NK stimulants, gamma-interferon, and the cytokines interleukin-12 and interleukin-15 (Brunda & Gately, 1994; Puzanov et al., 1996; Condiotti & Nagler, 1998), may well be responsible for the increase in bone marrow-based NK cells.

The bone marrow is also the source (production site) of new cells of the monocyte/macrophage lineage, which, like NK cells, leave that organ and travel via the blood to the secondary (peripheral) organs such as the spleen. A significant increase in monocyte numbers in the bone marrow was apparent only after 2 weeks of *Echinacea* consumption, and may reflect a slower production of new cells in that lineage vs. NK cells (absolute increase by 1 week). By 2 weeks, however, monocyte numbers in both the bone marrow and spleen, clearly reflect the production-stimulating influence of this herb. The increases in monocytes reflect their response to a host of autostimulants such as interferons, interleukins 1 and 12, and TNF-alpha, all products of monocytes themselves (Gallily et al., 1985; Warren & Ralph, 1986; Kovacs et al., 1988; Verma & Mazumder, 1995). Furthermore, NK cells also produce interferon (Ye et al., 1995), which may, in turn, further drive monocyte lineage in the bone marrow

(birth site) and/or the spleen.

Our observations of virtually unchanged levels of other (non-NK) lymphoid cells/lymphocytes in both the spleen and the bone marrow, at both *Echinacea* feeding intervals (1 and 2 weeks), support and extend, now with *in vivo* evidence, previous *in vitro* indications that *Echinacea* was ineffective in enhancing T or B lymphocyte functional reactions to cell-specific antigens (Roesler et al., 1991b).

In summary, the present study has provided a quantified, *in vivo* analysis of the immunoenhancing effects of a specific, commercially prepared *Echinacea purpurea* root extract administered in the diet. The analysis has incorporated controlled parameters of herb exposure time and dosage, host animal pedigree, age, health, sex, and living environment. The results have shown a singular positive influence of *Echinacea purpurea* root extract on only those cells which are well established as being the "first line of defense" against tumor and virus-infected cells *in vivo,* i.e., NK cells and monocytes. In stunning contrast to this increase in numbers of NK cells and monocytes, the population size of all other hemopoietic and immune cells, in both the bone marrow and spleen, remained steadfastly at control levels under the influence of the *Echinacea* extract. Given the significant elevation in the absolute numbers of new NK cells and monocytes in their bone marrow birth site, it appears that one mechanism of action of *Echinacea* is to stimulate the proliferating precursor cells of these 2 lineages. Finally, the fact that these results were found in normal, healthy young animals, suggests a potentially prophylactic role for this non-toxic phytopharmaceutical.

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INTRODUCTION TO CHAPTER 5

In Chapter 3 I found that administration of MLT for one and two weeks boosted NK cell numbers and monocytes, while in Chapter 4 I found similar results in E. *purpurea* administered mice. In Chapter 5 I aimed to administer MLT and E. *purpurea*, alone and together, to healthy, young adult mice in order to determine their effects on NK cells and other hemopoeitic cell populations. The hypothesis was that 2 agents together would be better NK cell/monocyte immunoenhancers than either agent alone.

CHAPTER 5: The effect of combined administration of melatonin with a second immunostimulant on cells mediating non-specific immunity.

Nathan L. Currier, Maryline Sicotte, and Sandra C. Miller

Contents, in part, have been submitted for publication to Journal of Leukocyte Biology

Abstract

The neuroendocrine-immune system axis is one that has gained considerable attention in recent years. Melatonin (MLT), a neurohormone is a powerful immunostimulant. A second type of immunostimulant is contained in root extracts of Echinacea species. To date, however, there exists no information on the reactions of immune cells when confronted with relatively long-term exposure to a combination of immunostimulants i.e., MLT and the root extract of E. *purpurea*. Microisolator-housed mice of identical age, strain, sex were administered both the neurohormone and the herbal root extract daily, ground simultaneously into the chow for 7 or 14 days. All experimental and control (untreated chow) mice were killed at one day after the last feeding and the population sizes of cells mediating non-specific immunity, i.e., natural killer (NK) cells and monocytes, in both the bone marrow and spleens were determined by means of tetrachrome (hematological) staining, combined with immunostaining methods. The results revealed that while MLT, administered alone for either 7 or 14 days, significantly stimulated NK cells and monocytes in the spleen and bone marrow, these populations were uniformly decimated when the second immunostimulant, E. *purpurea*, was co-administered along with MLT for the 7 or 14 days. The study has revealed that combinations of immunostimulants, at least when one of them is the neurohormone melatonin, taken prophylactically over sustained periods of time, can have profoundly negative effects on the major populations of immune cells responsible for anti-viral and anti-tumor lytic activities.

Introduction

The neurohormone, melatonin (MLT), coordinates circadian biological rhythms (Nelson & Demas, 1996; Mazzoccoli et al., 1997), and is produced by the pineal gland almost exclusively during the hours of darkness. The neuroendocrine-immune system axis is one that has gained considerable attention in recent years. Among its many actions, MLT plays an immunoregulatory role (Guerrero & Teiter, 1992; Liebmann et al., 1997). Most commonly studied aspects of MLT- immune system interactions involve T helper lymphocytes, where MLT interacts directly with membrane receptors as well as with nuclear receptors (Lissoni et al., 1994; Gonzalez-Haba et al., 1995; Garcia-Maurino et al., 1997; Rafii-el-Idrissi et al., 1998). One or both types of receptors may be activated, resulting in enhanced release of T helper cell-derived cytokines (Cutando & Silvestre, 1995; Lissoni et al., 1998; Maestroni, 1998a, b, c). Administration of MLT in humans, hamsters and mice results in T cell mediated functional enhancement in the periphery (Pioli et al., 1993; Nelson & Demas, 1996; Champney et al., 1997; Garcia-Maurino et al., 1997). Moreover, in the presence of MLT, in old mice, the thymus and T cell mediated immune functions in the periphery recover (Mocchegiani et al., 1996). Evidence, however, is much more scant and is mostly indirect with respect to the effect of melatonin on another prominent cell lineage - one responsible for mediating non-specific immunity, i.e., natural (NK) cells. To date, MLT itself has no known side effects (Lissoni et al., 1996, 1998; Wichmann et al., 1996) and does not stimulate tumor growth (Maestroni et al., 1994a, b).

A second immunostimulant, primarily targeting NK cells (Lersch et al., 1992; See et al., 1997; Sun et al., 1999), is a root extract of the plant E. *purpurea*. Extracts from this plant have become extremely popular recently for their reported (albeit anecdotal) health benefits including abatement of virus-mediated infections, assorted inflammations, tumors and AIDS (Stimpel et al., 1984; Tragni et al., 1985; Roesler et al., 1991; Lersch et al., 1992; Steinmuller et al., 1993; Hill et al., 1996). Nothing is known, however, of the effect on the mediators of non-specific immunity, or even of specific immunity (T, B lymphocyte-mediated), when 2 or more such powerful immunostimulants are co-administered *in vivo*. To date, there exists no quantitative information, either *in vitro* or *in vivo*, on the behavior of immune cells when confronted with relatively long-term exposure to a *combination* of immunostimulants, i.e., MLT and E. *purpurea*.

Melatonin and Echinacea species phytocompounds are currently available, in an unrestricted manner, on an "over-the-counter" basis. However, the effect of combining 2 or more powerful immunostimulants, such as those above, has never been formally tested on any aspect of the immune system.

The present study thus, was undertaken to provide information of the effect on cells mediating non-specific immunity, of co-administering 2 immunostimulants. We used two popular immunostimulants currently in widespread prophylactic use in the Western World, i.e., melatonin and E. *purpurea*. Our results have shown that two is not better than one, and, moreover, at least with respect to melatonin and E. *purpurea*, taken in combination, such agents may have the potential to pre-dispose the normal host

to negative immunological consequences such as seriously compromising precisely the immune mechanisms which are responsible for anti-viral/anti-tumor defenses.

Results

NK cells are typically found, in a strain-independent manner, with frequencies of 3-7% of all the cells in the spleen and 1-3% of the total cellularity of the bone marrow (Keissling et al., 1975; Tam et al., 1980; Koo et al., 1981; Christopher et al., 1991; Whyte & Miller, 1998), in accordance with what we have also found in this study. Fig. 1 indicates that NK cells in the spleen were significantly elevated in absolute numbers over control at both 7 and 14 days of melatonin exposure. However, when melatonin was co-administered along the immunostimulant E. *purpurea*, the NK cell population in that organ was decimated (Fig. 1). Precisely this trend in effect, of single vs. combined administration of the 2 agents was found in the bone marrow (Fig.2).

In normal mice of all strains we have studied, monocytes occur in very low numbers again in accordance with our observations in the present study. Typically, these cells account for approximately 2% of all cells in the normal mouse spleen (Miller & Kearney, 1997; Sun et al., 1999). Fig. 3 indicates that 7 days of administration of the neurohormone resulted in a profound increase in the size of the monocyte population in the spleen, which, however, was not sustained by 14 days exposure (Fig. 3). Nevertheless, when the second immunostimulant, an extract from E. *purpurea*, was co-administered along with melatonin, the effect on monocytes was uniformly negative, i.e., their population size was significantly reduced, relative to control (and to MLT-treated mice) at both 7 and 14 days (Fig. 3). In the bone marrow (Fig. 4), in contrast with the spleen, MLT administration alone, transiently but significantly reduced monocyte numbers. However, by 14 days, exposure to MLT alone, monocyte numbers in

the bone marrow had almost tripled (Fig. 4). Nevertheless, by both 7 and 14 days co-administration of the neurohormone with E. *purpurea*, monocyte numbers in the bone marrow had virtually disappeared (Fig. 4).

Finally, Figs. 5 and 6 reveal the temporal effect resulting from the combined administration of both these agents on NK cells and monocytes. The population sizes of both NK cells (Fig. 5) and monocytes (Fig. 6) were significantly reduced - only in the spleen - as a function of exposure time of the 2 agents together. NK cells and monocytes in co-treated bone marrow (birth site of NK cell and monocytes) on the other hand, (although significantly reduced relative to untreated controls, or to MLT-treated mice), appeared more resilient to the exposure-time effect (Fig. 5, 6).

Table 1.

The effect of 7 days of simultaneous administration of dietary MLT and E. *purpurea* on the hemopoietic cells lineages of the spleen and bone marrow

Organ	Nucleated Erythroid ($\times 10^6$)[a]	Mature Granulocytic ($\times 10^6$)	Precursor Granulocytic ($\times 10^6$)	Lymphocytes ($\times 10^6$)	Monocytes ($\times 10^6$)
Spleen	7.11±0.64[b]	2.10±0.31	10.97±0.96	92.38±5.74	0.17±0.06
Bone Marrow (/femur)	2.98±0.30	0.57±0.08	4.88±0.47	1.34±0.08	0.001±0.0

MLT and E. *purpurea* were given daily in the ground chow as 14.2µg and 0.45 mg, respectively.
[a]determined from differential cell counts of 1000 total nucleated cells/organ (enumerated by means of an electronic cell counter), to absolute numbers of cells of each morphologically identifiable lineage.
[b]Mean ± s.e.: 9 mice

Fig. 1.

Absolute numbers of NK cells in the spleen of mice exposed to MLT alone or MLT + E. *purpurea* for 7 or 14 days, normalized against the respective means of 7 untreated, control mice. ( ): number of mice. [a] $p<0.01$; [b] $p>0.05$; [c] $p<0.02$; [d] $p<0.05$. Range of standard errors: 7.1-15.5% of the means of the absolute numbers in (7) or (10) mice.

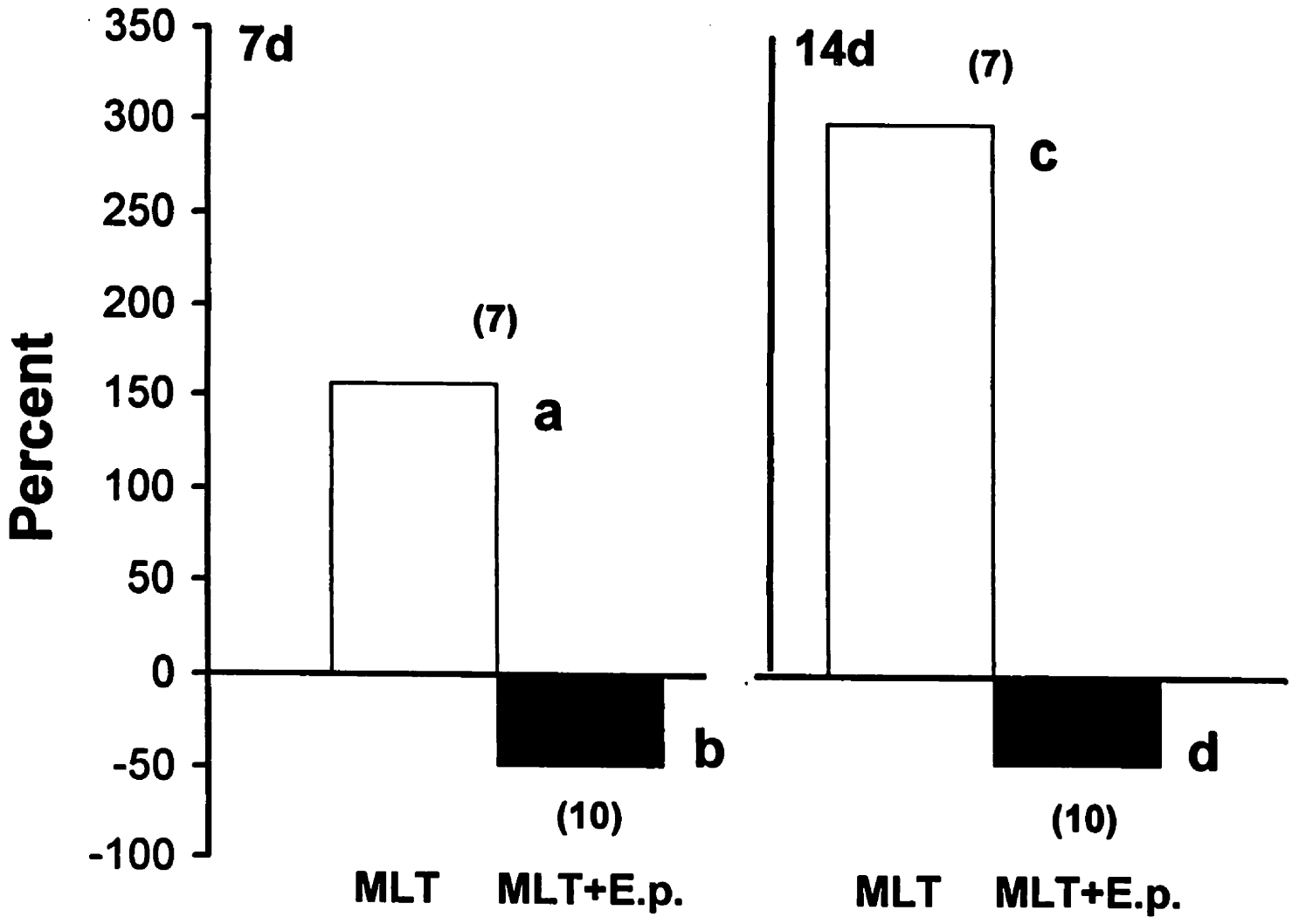

Percent
350
300
250
200
150
100
50
0
-50
-100
7d
(7)
a
b
(10)
MLT
MLT+E.p.
14d
(7)
c
d
(10)
MLT
MLT+E.p.

Fig. 2.

Absolute numbers of NK cells in the bone marrow of mice exposed to MLT alone or MLT + E. *purpurea* for 7 or 14 days, normalized against the respective means of 7 untreated, control mice. ( ): number of mice. [a] $p<0.005$; [b] $p<0.00003$; [c] $p=0.05$; [d] $p<0.04$. Range of standard errors: 4.0-19.9% of the means of the absolute numbers in (7) or (10) mice.

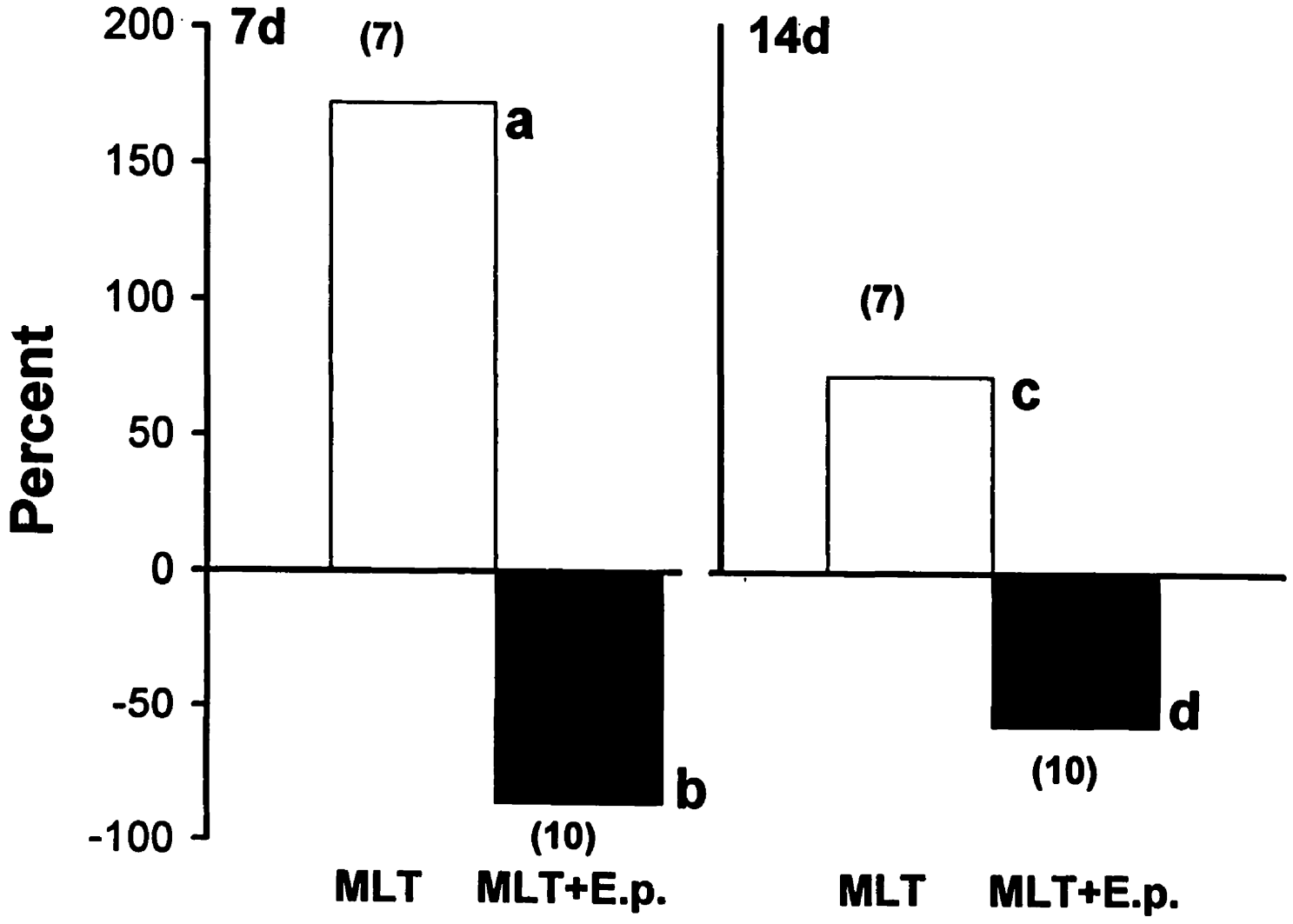

200
150
100
50
0
-50
-100
Percent
7d
(7)
a
b
(10)
MLT
MLT+E.p.
14d
(7)
c
d
(10)
MLT
MLT+E.p.

Fig. 3.

Absolute numbers of monocytes in the spleen of mice exposed to MLT alone or to MLT + E. *purpurea* for 7 or 14 days, normalized against the respective means of 7 untreated, control mice. ( ): number of mice. [a] $p<0.001$; [b] $p<0.032$; [c] $p<0.007$; [d] $p<0.006$. Range of standard errors: 9.0 - 28.9% of the means of the absolute numbers in (7) or (10) mice.

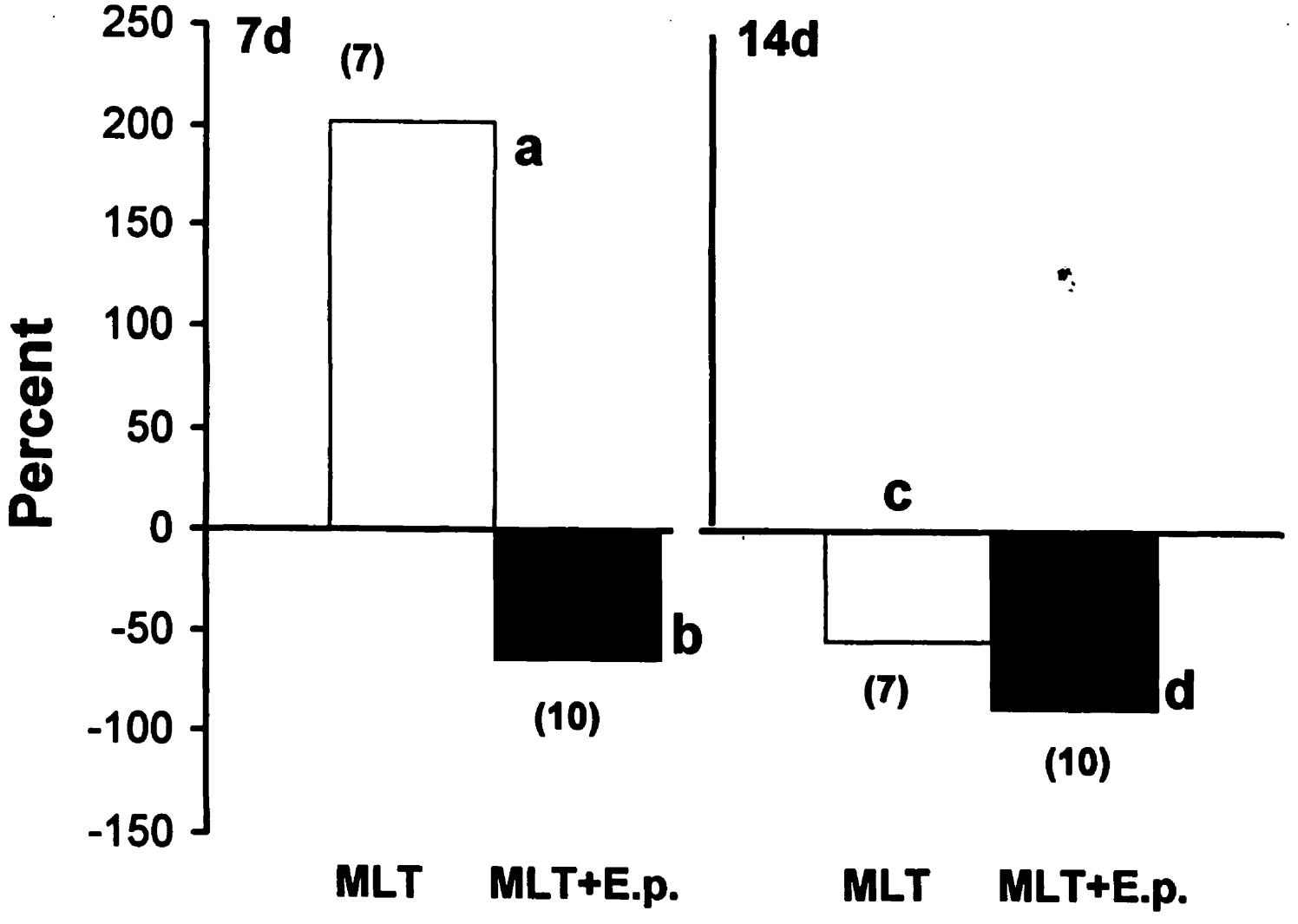

250
200
150
100
50
0
-50
-100
-150
Percent
7d
(7)
a
b
(10)
14d
c
(7)
d
(10)
MLT
MLT+E.p.
MLT
MLT+E.p.

Fig. 4.

Absolute numbers of monocytes in the bone marrow of mice exposed to MLT alone or to MLT + E. *purpurea* for 7 or 14 days, normalized against the respective means of 7 untreated, control mice. ( ): number of mice. [a] $p<0.01$; [b] $p<0.0017$; [c] $p<0.003$; [d] $p<0.002$. Range of standard errors: 12.5- 23.8% of the means of the absolute numbers in (7) or (10) mice.

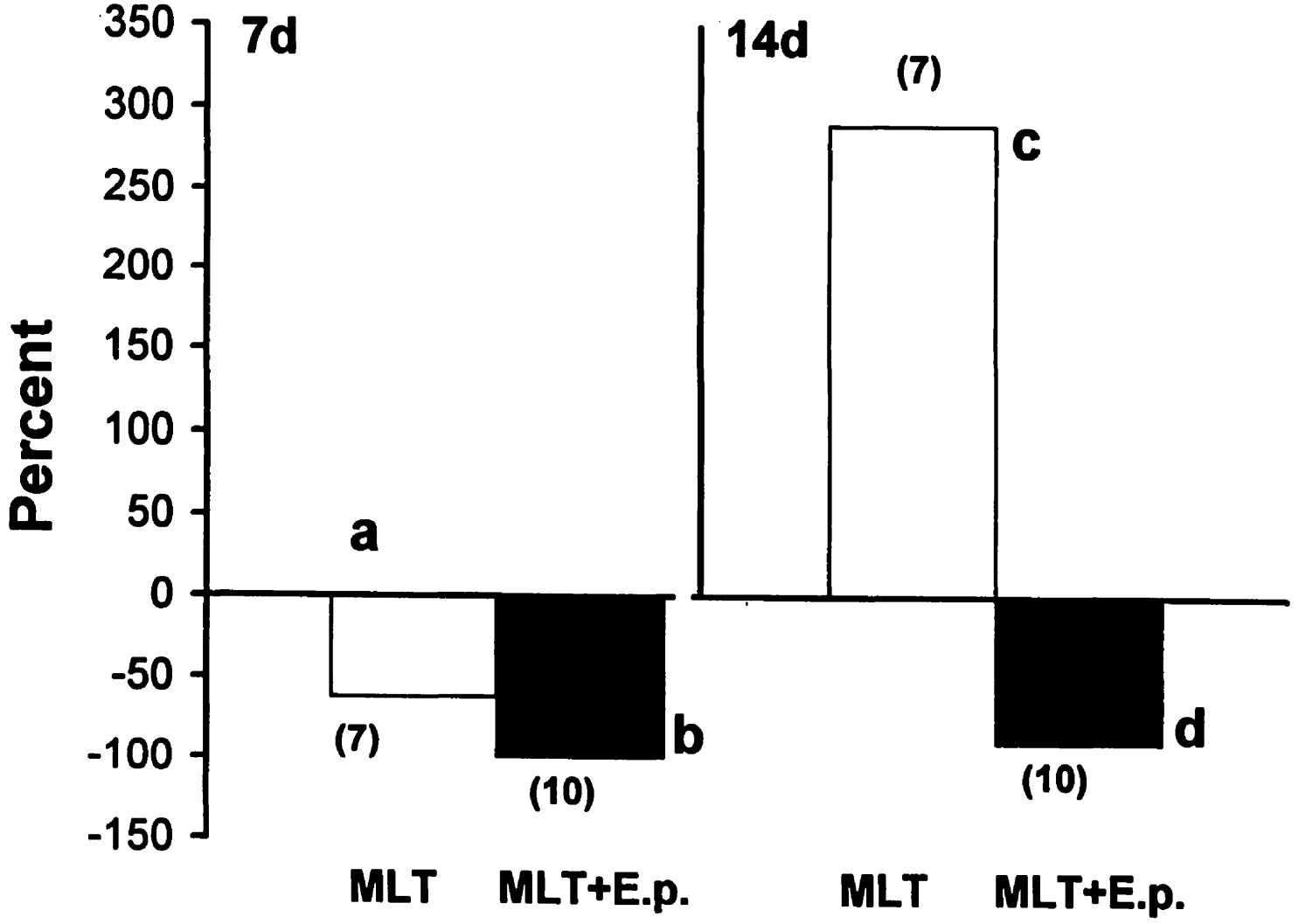

350
300
250
200
150
100
50
0
-50
-100
-150
Percent
7d
14d
(7)
c
a
(7)
b
(10)
(10)
d
MLT
MLT+E.p.
MLT
MLT+E.p.

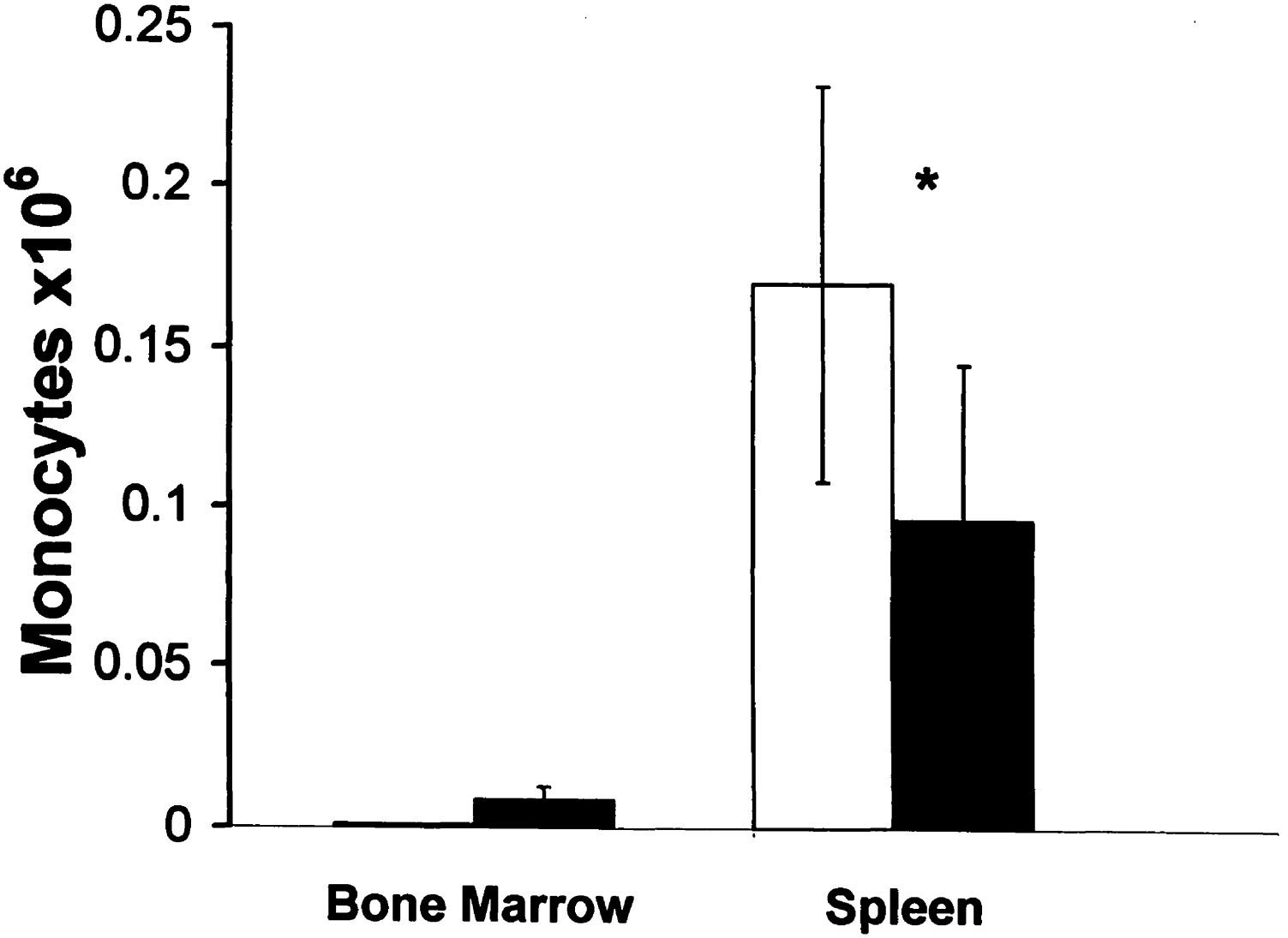
Fig. 5.

Comparison of the absolute numbers of NK cells in the spleen and bone marrow of mice exposed to MLT + E. *purpurea* for 7 days vs. 14 days. Mean ± s.e.: 10 mice. *$p<0.04$. 7 day values (open bars), 14 day values (closed bars).


NK Cells x10^6
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Bone Marrow
Spleen

Fig. 6.

Comparison of the absolute numbers of monocytes in the spleen and bone marrow of mice exposed to MLT + E. *purpurea* for 7 days vs. 14 days. Mean ± s.e.: 10 mice. *$p<0.05$. 7 day values (open bars), 14 day values (closed bars).


0.25
0.2
0.15
0.1
0.05
0
Monocytes x10^6
*
Bone Marrow
Spleen

Discussion

The present study represents the first *in vivo* analysis of the influence of the co-administration of immunostimulants, employing in the present study, MLT and E. *purpurea,* on cells mediating non-specific immunity in the spleen and bone marrow. We have found in this study, little or no influence of MLT, when administered alone, on any hemopoietic cells (unpublished observations), other than NK cells and monocytes, the mediators of non-specific immunity. From evidence *in vitro*, MLT appears to stimulate GM-CFU, acting through MLT receptors on bone marrow stromal cells, the latter producing, subsequently a host of cytokines (Maestroni et al., 1994a, b; Maestroni, 1998a, b, c). NK cells are exquisitely sensitive to cytokines produced by melatonin-stimulated T helper cells. Specifically, interleukin-2, -6, -12 and interferon-gamma, all products of melatonin-stimulated T helper cells, can augment NK cell function and/or stimulate new NK cell production (Senik et al., 1979; Hinuma et al., 1986; Christopher et al., 1991; Dussault & Miller, 1993; Cho et al., 1996; Wu et al., 1996). To date, it is unknown if NK cells possess receptors for melatonin.

With respect to monocytes, which do possess receptors for melatonin (Boranic et al., 1997), melatonin alone appeared to invoke somewhat different response in the bone marrow vs. the spleen - unlike NK cells, whose numbers in the presence of MLT, were uniformly augmented in an exposure- time-independent and organ-independent manner. An anti-apoptotic activity has already been ascribed to melatonin (Maestroni et al., 1994b; Sainz et al., 1995; Maestroni & Conti, 1996; Provinciali et al., 1996; Maestroni, 1998a), and it is possible that accumulation of monocytes in the bone marrow (their normal birth

site), after 14 days sustained MLT exposure, accompanied by a significant reduction in the numbers of monocytes in the spleen (a site of function), reflects this anti-apoptotic action of MLT. A halt in the continued development and/or dispersal of functional monocytes to the spleen would readily explain our observations. Moreover, the results of *in vitro* studies recently performed by us (Currier et al., 1999) using melatonin-treated bone marrow, indicate a very substantial increase and accumulation of precursors of the myeloid lineage, including thus, those of monocytes.

We have recently found that phytocompounds from the American coneflower, E. *purpurea*, had a significant and highly positive influence on both NK cells and monocytes, when administered for 7 or 14 days, while all other hemopoietic and immune cells lineages (including both precursor forms and their mature progeny) remained steadfastly at control levels (Sun et al., 1999). E. *purpurea*'s powerful immunoenhancing properties derive from a mixture of phytochemicals ranging from interferon inducers (Carr et al., 1998; Leuttig et al., 1989) to prostaglandin inhibitors (Wagner et al., 1989). All agents falling under the headings of interferon inducer or prostaglandin inhibitor are exquisite stimulants of NK cell numbers/function (Kendall & Targan, 1980; Minato et al., 1980; Lala et al., 1986).

With respect to the consistently negative effect on NK cells and monocytes observed when both MLT and E. purpurea are present together, the possibility of toxicity, or "overdose effect" may be entertained. Profoundly negative effects on NK cells, such as those found in this study, are similar to what we have found with 2 other co-administered, immunostimulatory agents

(indomethacin, interleukin-2), each of which, when acting alone, strongly enhances production/ function of these cells (Christopher et al., 1991; Dussault & Miller, 1993).

The results presented in this work illustrate that immunoenhancing agents, taken prophylactically in combination with the immunostimulating neurohormone, melatonin, can have severe and undesirable consequences for the major cell lineages acting in the first line of defense in anti-viral and anti-tumor immune responses. We are currently investigating specific molecular agents, isolated from phytocompounds in E. *purpurea*, with the intent of localizing which one(s) may be interacting with melatonin to so effectively decimate the populations of these 2 vital immune cell lineages - NK cells and monocytes.

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INTRODUCTION TO CHAPTER 6

The results from previous chapters show the effect of E. *purpurea* administered alone, or together with MLT, on NK cell populations in young adult mice. E. *purpurea*, being a plant product contains numerous possible immunostimulating chemicals. In fact, much research has gone into dissecting out these individual components and examining their immunostimulating properties. One such chemical, the polysaccharide arabinogalactan, a component of E. *purpurea* root extract, warranted further investigation, since there is some limited *in vitro* evidence for its immunostimulating properties, involving cells of the monocyte/macrophage lineage, which upon stimulation appear to produce several NK cell enhancing cytokines. Nothing is known however of its *in vivo* effects on NK cells or any cell lineage. Many NK cell-activating receptors are C-type lectin-like which bind carbohydrates for activation. For this reason, arabinogalactan may even *directly* stimulate NK cells. I investigated the *in vivo* role of arabinogalactan in healthy, young adult mice for one or two weeks, using standard administration protocols, to determine its effect on all immune and hemopoeitic cell populations.

CHAPTER 6: Effect of the polysaccharide arabinogalactan, a component of E. *purpurea*, on immune and hemopoietic cell lineages of the spleen and bone marrow.

Nathan L. Currier and Sandra C. Miller

Abstract

Work in our lab has recently demonstrated the natural killer (NK) cell enhancing properties of a commercially available extract of the herb Echinacea *purpurea*. A growing body of evidence exists identifying the possible immunostimulating compounds within this plant product. We wanted to determine, *in vivo,* the specific effects of one such compound, the complex carbohydrate arabinogalactan on NK cells and other immune and hemopoietic cells. Arabinogalactan stimulates macrophages to secrete a myriad of cytokines, many of which are powerful NK enhancers. Healthy, young adult mice were injected daily, intraperitoneally with arabinogalactan (500ug in 0.1ml pH7.2 PBS) for 7 or 14 days. Control mice were given the PBS vehicle only, following the above injection regimen. Animals from both groups were sampled 24 hours following the last injection and the immune and hemopoietic cell populations were assessed quantitatively in the spleen and bone marrow. At both one and two weeks of arabinogalactan administration, lymphoid cells in the bone marrow were significantly decreased compared to control animals not receiving arabinogalactan. Further, bone marrow NK cells were significantly decreased after one week of arabinogalactan administration but were significantly increased (more than 2-fold) in the spleen two weeks after beginning arabinogalactan administration. The results indicate that arabinogalactan, although purported to be one of the numerous possible immunostimulating compounds found within Echinacea *purpurea*, appears either directly or indirectly responsible for the significant decline in lymphoid cells and NK cells, in the bone marrow, after as little as one week of administration. A cascade of induced cytokines in the *in*

vivo presence of this polysaccharide, some of which are immunoinhibitory and some immunoenhancing, appear, at least in the short term (1 week), to favor the inhibitory ones. That lymphocyte proliferation-inducing cytokines are inhibited profoundly is suggested by the observation that the absolute numbers of lymphoid cells in the bone marrow, the generating center of B and NK lymphocytes, remains significantly subnormal.

Introduction

The natural killer (NK) cell enhancing properties of a commercially available extract of the herb Echinacea *purpurea* have recently been demonstrated in our lab (Sun et al. 1999; Currier et al., submitted; Currier & Miller, 2000, in press, submitted). Furthermore, a growing body of evidence exists identifying the possible immunostimulating compounds within the plant product (Roesler et al., 1991a, b; Steinmuller et al., 1993; Muller-Jakic et al., 1994; Bauer et al., 1996). We wanted to determine the *in vivo* effects of one such compound, the complex carbohydrate arabinogalactan, on NK cells. No controlled, *in vivo* study exists, demonstrating the effects of administration of arabinogalactan on these cells. In the present study we aimed to determine the effect, *in vivo*, of administration of arabinogalactan not only on NK cells, but on a range of other immune and hemopoietic cell populations, in the spleen and bone marrow of mice. The hypothesis giving rise to this study derives from the fact that macrophages have been shown to release numerous cytokines upon stimulation with arabinogalactan *in vitro* (Stimpel et al., 1984; Luettig et al., 1989) and *in vivo* (Rininger et al., 2000). The cytokine profile produced by macrophages is known to contain many factors that are exquisite NK cell enhancers, IFN-B2 and TNF-a for example (Hauer & Anderer, 1993; Luettig et al., 1989; Kelly, 1999; Stein et al., 1999; Rininger et al., 2000). It was hypothesized therefore that administration of arabinogalactan for one or two weeks, using the administration protocol employed with *in vivo* administration of whole Echinacea *purpurea* root extract, would stimulate macrophages to

produce NK cell enhancing cytokines which in turn would result in increased NK cell numbers.

Results

After one week of administration of arabinogalactan the absolute number of NK cells was significantly decreased in the bone marrow to levels 14 times less than those found in control animals receiving only the vehicle injections (Fig. 1). By contrast, NK cell numbers in the spleen were at control levels after one week of arabinogalactan administration compared to control animals.

Table 1 indicates that after one week of arabinogalactan administration the total non-NK lymphoid cells in the bone marrow were, like NK cells, also significantly decreased (more than 3-fold) with respect to controls, while all other hemopoietic cell lineages in the bone marrow and spleen remained unchanged.

Fig. 2 indicates that NK cell numbers were significantly *increased* (more than 2-fold) in the spleen after two weeks of arabinogalactan administration compared to control mice, receiving only the vehicle. However, NK cells were at control levels in the bone marrow (Fig. 2) at this time.

After two weeks of administration of arabinogalactan, lymphoid cells were still significantly decreased (1.7 fold) in the bone marrow while all other hemopoietic cell lineages remained at control levels in that organ (Table 2). In the spleen, after two weeks of administration, mature granulocytes and cells of the monocytoid lineage were both significantly decreased relative to control mice, while all other hemopoietic cell lineages remained at control levels in that organ (Table 2).

Table 1.

The effect of 1 week Arabinogalactan (Arabino.) administration to young, adult male mice on the population sizes of the hemopoietic and immune cell lineages in the bone marrow and spleen.

Treatment	Total Lymphoid Cells ($\times 10^6$)	Mature Granulocytes ($\times 10^6$)	Precursor Myeloid Cells ($\times 10^6$)	Nucleated Erythroid Cells ($\times 10^6$)	Monocytoid ($\times 10^6$)
Bone Marrow (per femur)					
Arabino.[a]	0.286±0.067[c]*	2.98±0.33	0.504±0.052	4.14±0.50	0.047±0.005
Vehicle[b]	0.999±0.137	2.99±0.51	0.511±0.115	2.97±0.38	0.081±0.026
Spleen					
Arabino.	56.07±5.26	4.96±0.80	0.580±0.093	16.94±1.79	0.068±0.045
Vehicle	57.42±1.94	4.71±1.78	0.546±0.125	18.57±1.61	0.102±0.043

[a]given daily injection (500ug in 0.1 ml 7.2 PBS) via lateral tail vein for 7 days
[b]0.1 ml 7.2 PBS, without arabinogalactan
[c]Mean s.e.:5-6 mice
[d]determined from differential counts of 2,000 total cells in each organ from each mouse, on stained cytospot preparations and converted, via the known total numbers of nucleated cells/organ (enumerated by means of an electronic cell counter), to absolute numbers of cells in each morphologically identifiable series.
*$p<0.05$

Table 2.

The effect of 2 week Arabinogalactan (Arabino.) administration to young, adult male mice on the population sizes of the hemopoietic and immune cell lineages in the bone marrow and spleen.

Treatment	Lymphoid cells (x10⁶)	Mature granulocytes (x10⁶)	Progenitor Myeloid cells (x10⁶)	Nucleated Erythroid Cells (x10⁶)	Monocytoid (x10⁶)
Bone Marrow (per femur)					
Arabino.[a]	0.299±0.042[c]*	3.26±0.38	0.476±0.052	3.87±0.33	0.037±0.007
Vehicle[b]	0.515±0.053	2.50±0.32	0.389±0.049	4.16±0.46	0.046±0.015
Spleen					
Arabino.	58.25±3.80	2.48±0.42*	0.620±0.149	17.83±2.04	0.119±0.052*
Vehicle	52.84±4.81	4.40±0.39	1.32±0.29	25.14±3.48	0.605±0.088

[a]given daily injection (500ug in 0.1 ml 7.2 PBS) via lateral tail vein for 14 days
[b]0.1 ml 7.2 PBS, without arabinogalactan
[c]Mean s.e.:6-7 mice
[d]determined from differential counts of 2,000 total cells in each organ from each mouse, on stained cytospot preparations and converted, via the known total numbers of nucleated cells/organ (enumerated by means of an electronic cell counter), to absolute numbers of cells in each morphologically identifiable series.
*$p<0.05$

Fig.1.

Total number (mean±s.e.) of NK (ASGM-1+) cells (lymphocyte morphology) in the bone marrow and spleen of control diet- and arabinogalactan-fed (1 week), young adult DBA/2 male mice. ( ) = number of mice. *p<0.05. Control mice (open bars), arabinogalactan administered mice (closed bars).

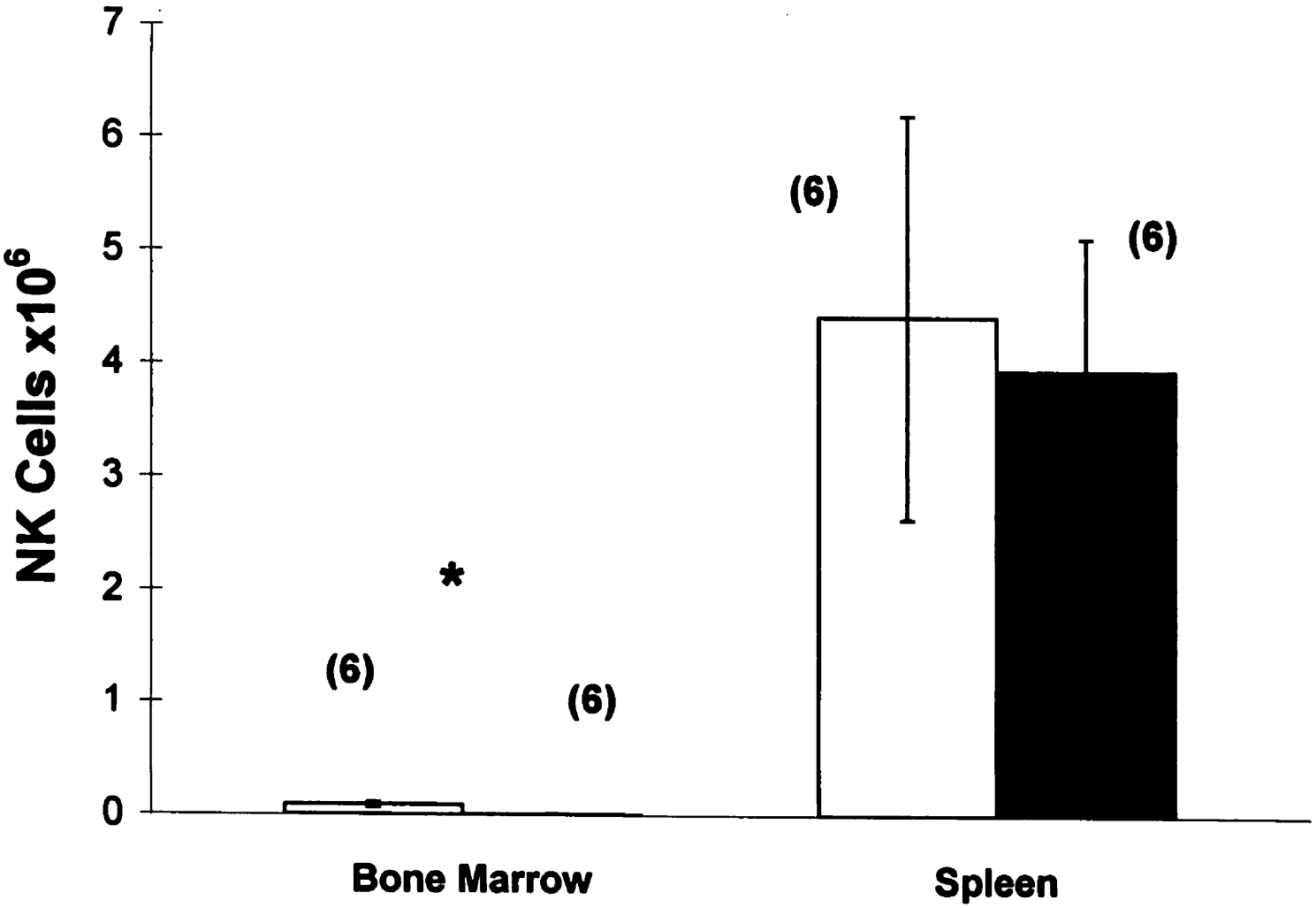
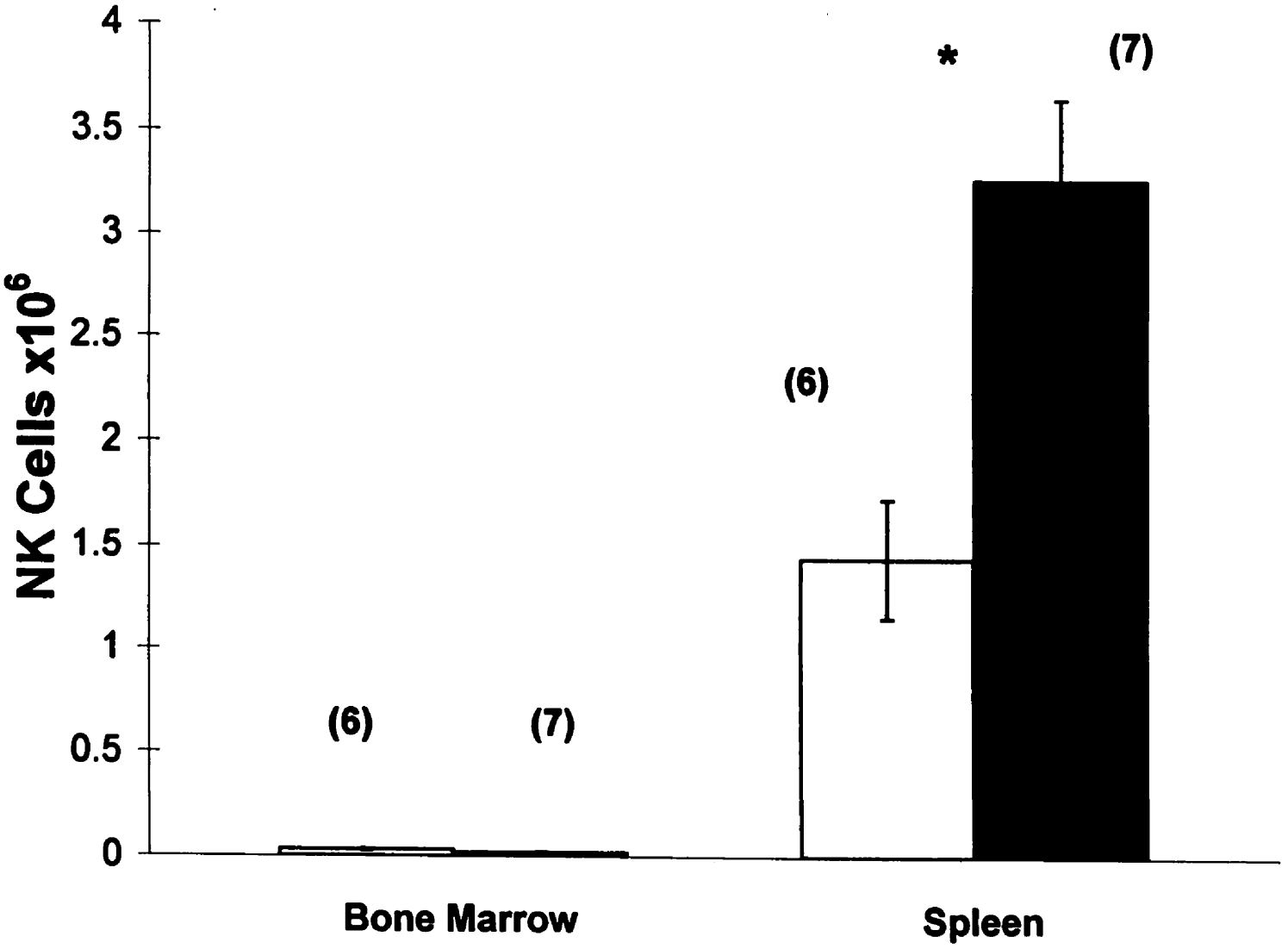

NK Cells x10^6
7
6
5
4
3
2
1
0
*
(6)
(6)
(6)
(6)
Bone Marrow
Spleen

Fig.2.

Total number (mean±s.e.) of NK (ASGM-1+) cells (lymphocyte morphology) in the bone marrow and spleen of control diet- and arabinogalactan-fed (2 weeks), young adult DBA/2 male mice. ( ) = number of mice. *$p<0.05$. Control mice (open bars), arabinogalactan administered mice (closed bars).


NK Cells x10^6
4
3.5
3
2.5
2
1.5
1
0.5
0
(6)
(7)
(6)
*
(7)
Bone Marrow
Spleen

Discussion

The results suggest that arabinogalactan, although purported to be one of the numerous possible immunostimulating compounds found within Echinacea *purpurea*, is at least in the present protocol, in some way directly or indirectly responsible for the striking decline in lymphoid cells and NK cells, in the bone marrow, after as little as one week of administration. The majority of lymphoid cells in the bone marrow are of the B cell lineage and their decrease in numbers could be due to the inhibition of their proliferation and/or differentiation within the bone marrow, their organ of birth. Evidence shows that some of the cytokines produced and released by macrophages upon stimulation with arabinogalactan, are in fact inhibitory to B cells, i.e., IFN-B2 and TNF-a (Reynolds et al., 1987; Mongini et al., 1988; Luettig et al., 1989; Hauer & Anderer, 1993; Abed et al., 1994; Rininger et al., 2000). Further, it could be reasoned that the same deleterious effect isn't obvious in the splenic lymphoid cell numbers perhaps because the large T cell lymphocyte population in this organ may mask any decline in B cell lymphocyte numbers.

Since NK cells were also significantly decreased in the bone marrow after one week it can be suggested that arabinogalactan is also inhibiting their production in their organ of birth, as seen with lymphoid cells. Whether this is through an indirect cytokine-mediated inhibitory mechanism via macrophages, is unclear. Nevertheless the effect is transient; control levels of NK cells being found in the BM by 2 weeks after beginning arabinogalactan feeding and at that same time period, statistically significant supernormal levels of NK cells (again absolute numbers) were found in the spleen. Another possible alternative

explanation, for this sudden increase in NK cell numbers in the spleen, may be the shift of their production to some organ other than the bone marrow as a result of the apparent inhibition within the bone marrow. Such a phenomena has been shown before (Biron et al., 1983; Miller & Shatz, 1991) under abnormal physiological conditions.

The present study has provided the first systematic analysis, under controlled laboratory conditions, of the effects of the complex carbohydrate, arabinogalactan, *in vivo*, on the immune and hemopoietic cell lineages in the bone marrow and spleen, in mice. One unexpected observation was the sustained subnormal levels (absolute numbers) of B cells in their bone marrow production site. Moreover, the initially negative, but transient, reduction in NK cells suggests that compounds isolated from whole root extract of Echinacea *purpurea* appear to not be as immunostimulatory as whole Echinacea *purpurea* itself. There is evidence that indeed this may be true (Voaden & Jacobson, 1972; Rininger & Kickner, 2000).

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INTRODUCTION TO CHAPTER 7

The work in Chapter 7 extends our findings in Chapters 3-5 in which the effects of administration of MLT and/or E. *purpurea* were studied in healthy, young adult mice. I again utilized our standard erythroleukemic cell line (see Chapters 1 and 2) and examined the effects of administration of MLT and/or E. *purpurea* in leukemic mice. It was hypothesized that the significantly increased NK cell numbers, resulting from administration of these compounds to healthy mice, would also exist in leukemic mice, having therefore obvious advantage in eradicating the tumor cells and prolonging life span.

CHAPTER 7: E. *purpurea* and melatonin augment natural killer cells in leukemic mice and prolong life span.

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Abstract

We recently showed that daily, dietary administration of E. *purpurea* root extract for as little as one week resulted in significant elevations of natural killer (NK) cells (immune cells which are cytolytic to virus-containing cells and many tumor cells). Such boosting of this fundamental immune cell population suggests a prophylactic role for this herb in normal animals. Based on this evidence, we aimed in the present work to assess the role of dietary administration of this herbal extract to mice bearing leukemia, a type of tumor well known to be a target for NK cells. A commercially available root extract of E. purpurea, which we have already shown to be highly effective in mice, was administered daily for 50 days from the onset of leukemia (day 0). Control leukemic mice received no extract. Other leukemic mice received the NK-enhancing neurohormone, melatonin, administered precisely as above. In all treatment and control categories, some mice were sampled at 9 days after tumor onset, others were sampled at 3 months and still others were left to assess treatment effect on life span. At 9 days (intermediate stage leukemia; death ensuing by day 17-18), E. *purpurea*-treated mice had a 2.5-fold increase in the absolute numbers of NK cells in their spleens. By 3 months after leukemia onset, E. *purpurea*-treated mice still had 2-3 times the *normal* numbers of NK cells in their spleens. No leukemic, untreated (control) mice remained alive at 3 months, hence the comparison with normal animals. Moreover, at 3 months post tumor onset all the major hemopoietic and immune cell lineages in their bone marrow birth site, were recorded at normal numbers, in E. *purpurea*-consuming, leukemic mice. The survival advantage provided by administering these leukemic mice with E.

purpurea was highly significant vs. untreated, leukemic mice when analyzed by Kaplan-Meier Survival Statistics. The present study has provided the first systematic analysis, under controlled laboratory conditions, of the effect(s) of the botanical, E. *purpurea*, *in vivo*, in leukemic hosts. The profoundly positive effects of this herb in disease abatement, observed in this study, suggests a therapeutic potential of E. *purpurea,* at least with respect to leukemia, if not other tumors as well.

Introduction

We have recently demonstrated (Sun et al., 1999), that Echinacea *purpurea*-derived phytochemicals, act as stimulants only to those cells responsible for nonspecific immunity, i.e., natural killer (NK) cells, well-established killers of virus-containing cells and tumor cells. NK cells have long been recognized as the first line of defense against virus infected/tumor cells (Keissling et al., 1975; Riccardi et al., 1981; Biron & Welsh, 1982; Lala et al., 1986; Christopher et al., 1991). We have shown that daily, dietary administration of E. *purpurea* root extract for as little as one week, resulted in significant elevations of NK cells in the organ of their birth (bone marrow), as well as in the primary organ of their function (spleen), indicating a prophylactic role for this herb in normal animals (Sun et al., 1999). Even after 2 weeks of daily administration of E. *purpurea*, all hemopoietic and immune cells, with the exception of NK cells, which remained elevated, were at levels precisely comparable to control (Sun et al., 1999). Extracts of the roots and other parts of various Echinacea species (*purpurea*, *angustifolia*), have come to prominence over the past decade for their reported (usually anecdotal) medicinal value including abatement and/or elimination of such pathologies as inflammations, viral/bacterial infections, AIDS, and even tumors (Stimpel et al., 1984; Tragni et al., 1985; Lersch et al., 1990, 1992; Roesler et al., 1991a, b; Steinmuller et al., 1993; Hill et al., 1996).

Two mechanisms appear to be responsible for the beneficial effects imbued by Echinacea species-derived phytochemicals. One of the naturally occurring "drugs" from Echinacea species plants which has already been

identified, is the polysaccharide, arabinogalactan, a 75,000 MW molecule known to stimulate monocytes and macrophages (*in vitro*) and shown to be cytotoxic for microorganisms (Luettig et al., 1989). Arabinogalactan also stimulates macrophages (Stimpel et al., 1984; Leuttig et al., 1989; Wagner & Jurcic, 1991; Fry et al., 1998) to secrete the cytokines TNF-a, interferons, and Il-1 - all potent *enhancers* of NK cells. Secondly, certain alkamides contained within Echinacea species plants are inhibitors of cyclooxygenase and 5-lipoxygenase (Wagner et al., 1989; Muller-Jakic, 1994), key enzymes in the production of prostaglandins, the latter being potent *suppressors* of NK cells (Fulton & Heppner, 1985; Lala et al., 1986). We have already demonstrated that the prostaglandin inhibitor, indomethacin, results in NK cell stimulation in leukemic mice (Christopher et al., 1991; Miller et al., 1992; Dussault & Miller, 1993). Thus, while the polysaccharide (arabinogalactan) results in the production of NK *stimulators*, other Echinacea species products (alkamides) release NK cells from their natural endogenous *inhibitors,* i.e., prostaglandins.

Melatonin, on the other hand, is a neuroimmunomodulator, a pineal gland hormone and biogenic indoleamine (N-acetyl-5 methoxytryptamine), long known to be a chronomodulator in biological systems. The hormone acts to functionally synchronize eukaryotes with the photoperiod. Endogenous melatonin production follows a strict circadian rhythm, being maximally produced during the dark periods of the 24-hr cycle, and broken down soon after production, having species-dependent (hamster, rat, donkey, monkey, human), and even age-dependent half lives in the blood ranging from 7.5 to 59 minutes (Mallo et al., 1990; Pang et al., 1990; Cavallo and Ritschel, 1996; Yellon, 1996; Brown et al.,

1997; Yeleswaram et al., 1997). We have recently demonstrated (Currier et al., 2000), that exogenously administered melatonin selectively enhances the population size of NK cells when given daily in the diet for 1 week or for 2 weeks to healthy, young adult mice, suggesting a potentially prophylactic role for this agent - a phenomenon not dissimilar from that which we observed employing E. *purpurea* (Sun et al., 1999). With respect to the mechanism responsible for the influence of melatonin on NK cells, there is evidence in mammals indicating that melatonin *in vivo*, enhances the lytic functions of mature NK cells (Poon et al., 1994). However, it is completely unknown whether this results directly from receptor-ligand-type interactions between melatonin and NK cell surface receptors, or indirectly, via melatonin-stimulated, T-helper cell-enhanced production of the cytokine, IL-2. That the latter event is most probable derives from the fact that T-helper cells bear receptors for melatonin and IL-2 is an exquisite stimulant of NK cell numbers and function (Christopher et al., 1991; Naume and Espevik, 1991).

Thus, the selective, positive influence of *in vivo* administered E. *purpurea,* or melatonin, on cells mediating non-specific immunity, i.e., NK cells, suggests a plausible mechanism behind the numerous, anecdotal claims over the years, that these agents may have a role in tumor abatement in humans, although the mode of action(s) has remained unknown and, to date, unstudied.

The literature contains only minimal information concerning life span increment, or the status of normal hemopoietic or immune cells in tumor-bearing hosts given a botanical. For example, Nanba et al. (1987), have administered orally to tumor-bearing mice, fruit bodies from the plant Lentinus *edodes*, with

positive results. However, nothing is known of NK cell population kinetics/dynamics, *in vivo*, even though these cells represent the first line of defense in tumor combat, in leukemic hosts (or in hosts bearing other tumors). It was the aim of the present study to administer Echinacea species-derived phytocompounds, under controlled laboratory conditions including use of (i) animals of identical species (strain), age and sex, (ii) tumor of precisely known stage of development, (iii) therapeutic agents (E. *purpurea*, melatonin) of regulated dose and exposure time, and (iv) standardized housing conditions for all treated and control animals. The hypothesis giving rise to this study, derives from the fact that the leukemias and lymphomas are targets of NK cells. Moreover, the leukemia of this study is virus-derived. Hence, any agent that enhances the immune mechanism directed at virus-harboring cells, i.e., NK cell-mediated immunity, may be expected to be ameliorative in leukemia-stricken animals and possibly humans.

Results

Fig. 1 indicates that daily, dietary administration of either MLT or E.p., beginning at the time of tumor onset (day 0), and for 9 days thereafter, resulted in a 2.5-fold increase in the absolute numbers of splenic NK cells. Co-administration of the two agents for the 9-day period, by contrast, had no significant effect on splenic NK cell population numbers (Fig. 1). In the bone marrow, administration of either agent, or both together for 9 days after tumor onset, had no significant effect on the NK cell population size, relative to control, in that organ (Fig. 2).

By 3 months after tumor onset, no control mice remained alive (Figs. 1, 2). In the spleen (Fig. 1), the NK cell population sizes of E.p.-treated, and MLT-treated mice, ranging from 7 - 10 x 10^6 cells/ spleen are super-normal for mice of this age. We have previously found the normal numbers of approximately 3 x 10^6/spleen (Mahoney et al., 1998). However, the spleen-localized NK cells in E.p.-treated, and MLT-treated mice, were significantly more numerous ($p < 0.04$ and $p < 0.01$, respectively) than in mice treated with both MLT+E.p. The NK cells of the bone marrow at 3 months after tumor onset were not significantly different in mice treated with either MLT, or E.p., or with both agents (Fig. 2).

Fig. 3 indicates that life span of tumor-bearing mice was significantly improved by administering either MLT or E.p. or both agents simultaneously from the early stages of tumor onset. Whereas all untreated, leukemic mice were dead by 27 days after tumor onset, approximately one third of MLT-treated, and similar proportions of E.p.-treated, tumor-bearing mice survived not only until 3 months but beyond, indicating long-term survival and/or cure. The best survival

rates, i.e., almost 50% of leukemic mice, were obtained when both agents were co-administered (Fig. 3).

With respect to the other hemopoietic cell lineages in the spleen, there was, at 9 days after tumor onset, no effect of MLT, or E.p. administered separately, on any major cell population (Table 1). There was, however, at this time, a significant reduction in the population sizes of non-NK lymphoid cells and erythroid cells, relative to control, when both agents were co-administered. (Table 1). By 3 months after tumor onset, there was no significant change in the numbers of cells in any of the major hemopoietic lineages in the spleen, when both agents were co-administered vs. either agent alone (Table 1).

In the bone marrow of tumor-bearing mice, a significant reduction in the cell numbers in most but not all, of the hemopoietic lineages was observed at 9 days after tumor onset, when either E.p. alone, or melatonin alone, was administered, but not when both were given together, relative to untreated controls (Table 2). At 3 months after tumor onset, exposure to either agent, or both agents simultaneously, resulted in the numbers of cells in the bone marrow (Table 2), in each of the major hemopoietic lineages, being virtually indistinguishable from our established findings in *normal* mice of this age (Miller & Osmond, 1974; 1975; Mahoney et al., 1998). Thus, in the presence of E.p. and/or MLT, resumption of normal hemopoiesis and lymphopoiesis in these treated, long-term survivors of leukemia, has occurred.

Table 1.

Numbers of hemopoietic cells in the spleens of tumor-bearing[a] mice treated with melatonin, E. *purpurea*, or both, at 9 days or 3 months after tumor onset

Duration[b]	Cell type[c]	Control (X10⁶)[d]	Melatonin[e] (X10⁶)	E. *purpurea*[f] (X10⁶)	Melatonin + E. *purpurea* (X10⁶)
9 days	Lymphoid	137.95±15.7[g]	132.11±3.36	146.08±9.96	82.72±3.77*
	Myeloid	11.32± 2.5	15.67±2.64	11.78±2.49	6.83±0.84
	Erythroid	23.97± 2.6	22.76±2.72	22.53±3.91	14.81±1.55*
	Monocytoid	0.34± 0.1	0.16±0.16	0.30±0.21	0.61±0.13
3 months	Lymphoid	-[h]	36.60±3.45	33.57±2.75	41.95±2.99
	Myeloid	-	2.96±0.55	4.51±1.45	5.83±1.36
	Erythroid	-	6.95±2.03	5.53±1.28	8.03±1.85
	Monocytoid	-	0.06±0.05	0	0.03±0.01

[a] Mice received intravenously, 3 x 10^6 FLV-induced erythroleukemia cells at 10 wk of age.
[b] Mice were killed at 9 days or 3 months after tumor onset.
[c] Cells were categorized into 4 major types and recorded individually on hematologically stained cytospots, as a percentage of 2000 total cells counted. From the known total cellularity/spleen, the proportions of each cell type were converted to absolute numbers of cells/spleen/mouse.
[d] At tumor onset (day 0), mice were fed finely ground, standardized chow, fresh daily.
[e] At tumor onset, melatonin was ground into the chow (above) such that each mouse consumed 0.0142 mg/day, for 9 days or 50 days (terminating 40 days before the 3 month sampling).
[f] At tumor onset, E. *purpurea* root, commercially prepared as a powered extract, was homogenized into the chow (above) such that each mouse consumed 0.45 mg/day for 9 days or 50 days (terminating 40 days before the 3 month sampling).
[g] Mean ±standard error
[h] No untreated mice survived until 3 months.
* $p < 0.005 - 0.007$ vs. corresponding control.

Table 2.

Numbers of hemopoietic cells in the bone marrow (per femur) of tumor bearing[a] mice treated with melatonin, E. *purpurea*, or both, 9 days or 3 months after tumor onset.

9 days	Lymphoid	2.83±0.29[g]	2.31±0.22	1.52±0.08*	2.26±0.22
	Myeloid	3.18±0.39	2.29±0.25	1.58±0.24*	2.58±0.32
	Erythroid	1.33±0.21	0.72±0.08*	0.61±0.06*	1.16±0.15
	Monocytoid	0.08±0.02	0.002±0.002	0.002±0.002	0.09±0.01
3 months	Lymphoid	-[h]	2.42±0.14	2.43±0.16	4.06±0.40
	Myeloid	-	3.76±0.50	3.44±0.33	4.87±0.66
	Erythroid	-	1.57±0.15	1.36±0.03	2.51±0.21
	Monocytoid	-	0.003±0.003	0.011±0.008	0.09±0.01

[a] Mice received intravenously, 3 x 10^6 FLV-induced erythroleukemia cells at 10 wk of age.
[b] Mice were sampled at 9 days or 3 months after tumor onset.
[c] Cells were categorized into 4 major types and recorded individually on hematologically stained cytospots, as a percentage of 2000 total cells counted. From the known total cellularity per femur, the proportions of each cell type were converted to absolute numbers of cells/femur/mouse.
[d] At tumor onset (day 0), mice were fed finely ground, standardized chow, fresh daily.
[e] At tumor onset, melatonin was ground into the chow (above) such that each mouse consumed 0.0142 mg/day, for 9 days or 50 days (terminating 40 days before the 3 month sampling).
[f] At tumor onset, E. *purpurea* root, commercially prepared as a powered extract, was homogenized into the chow (above) such that each mouse consumed 0.45 mg/day for 9 days or 50 days (terminating 40 days before the 3 month sampling).
[g] Mean ± standard error
[h] No untreated mice survived until 3 months.
* $p < 0.05$ - 0.001 vs. corresponding control

Fig. 1.

Numbers of NK cells in the spleens of tumor-bearing mice treated with melatonin (MLT), E.p. (E. *purpurea*), or both, for 9 days or 3 months after tumor onset. $p<0.000007$: MLT or E.p. vs. control. $p<0.04$ vs. MLT+E.p. ( ) = number of mice. cross = all mice dead.

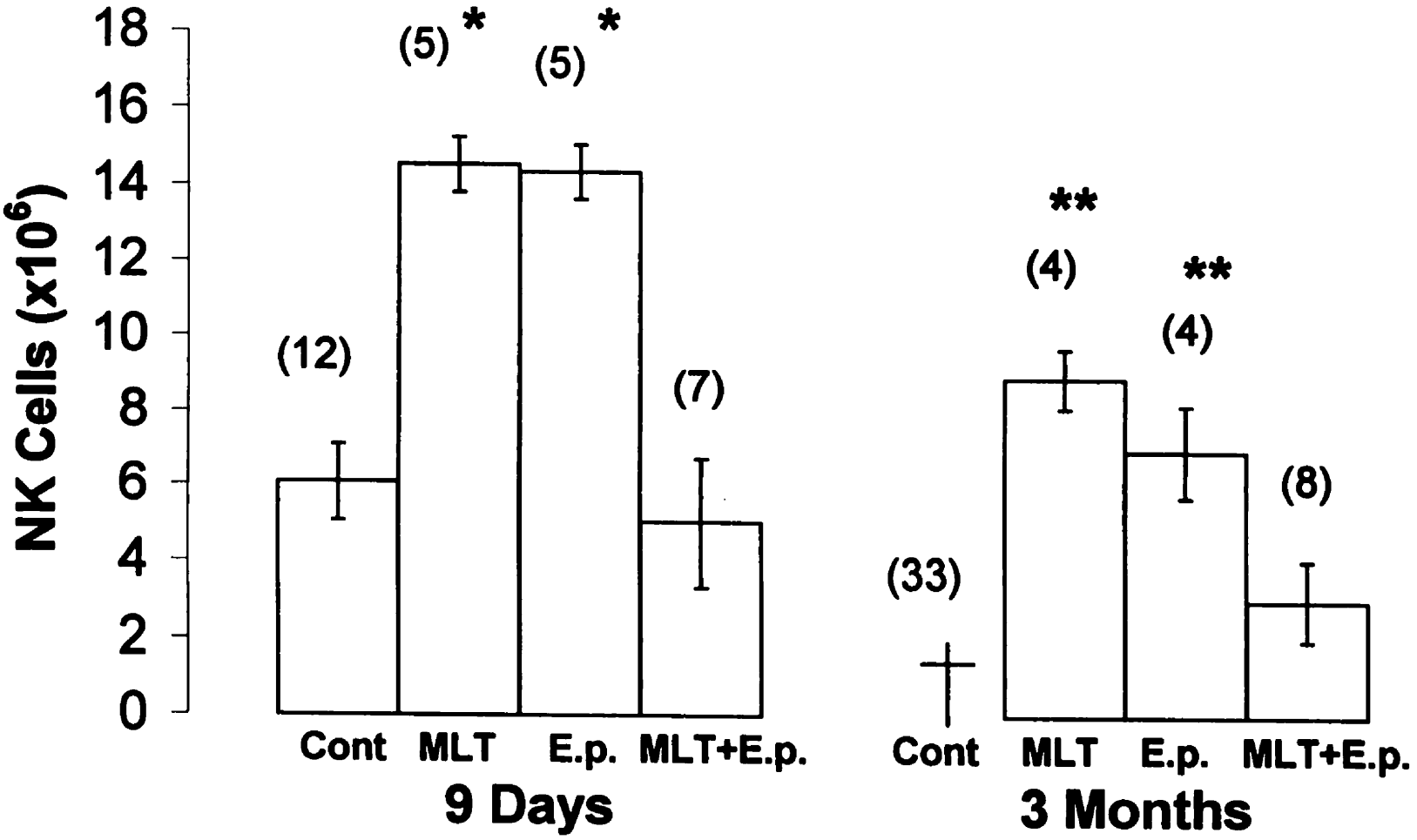

NK Cells (x10^6)
18
16
14
12
10
8
6
4
2
0
(5) *
(5) *
(12)
(7)
Cont
MLT
E.p.
MLT+E.p.
9 Days
**
(4)
**
(4)
(33)
(8)
Cont
MLT
E.p.
MLT+E.p.
3 Months

Fig. 2.

Numbers of NK cells in the bone marrow (one femur) of tumor-bearing mice treated with melatonin (MLT), or E.p. (E. purpurea), or both, for 9 days or 3 months after tumor onset. ( ) = number of mice. cross = all mice dead.

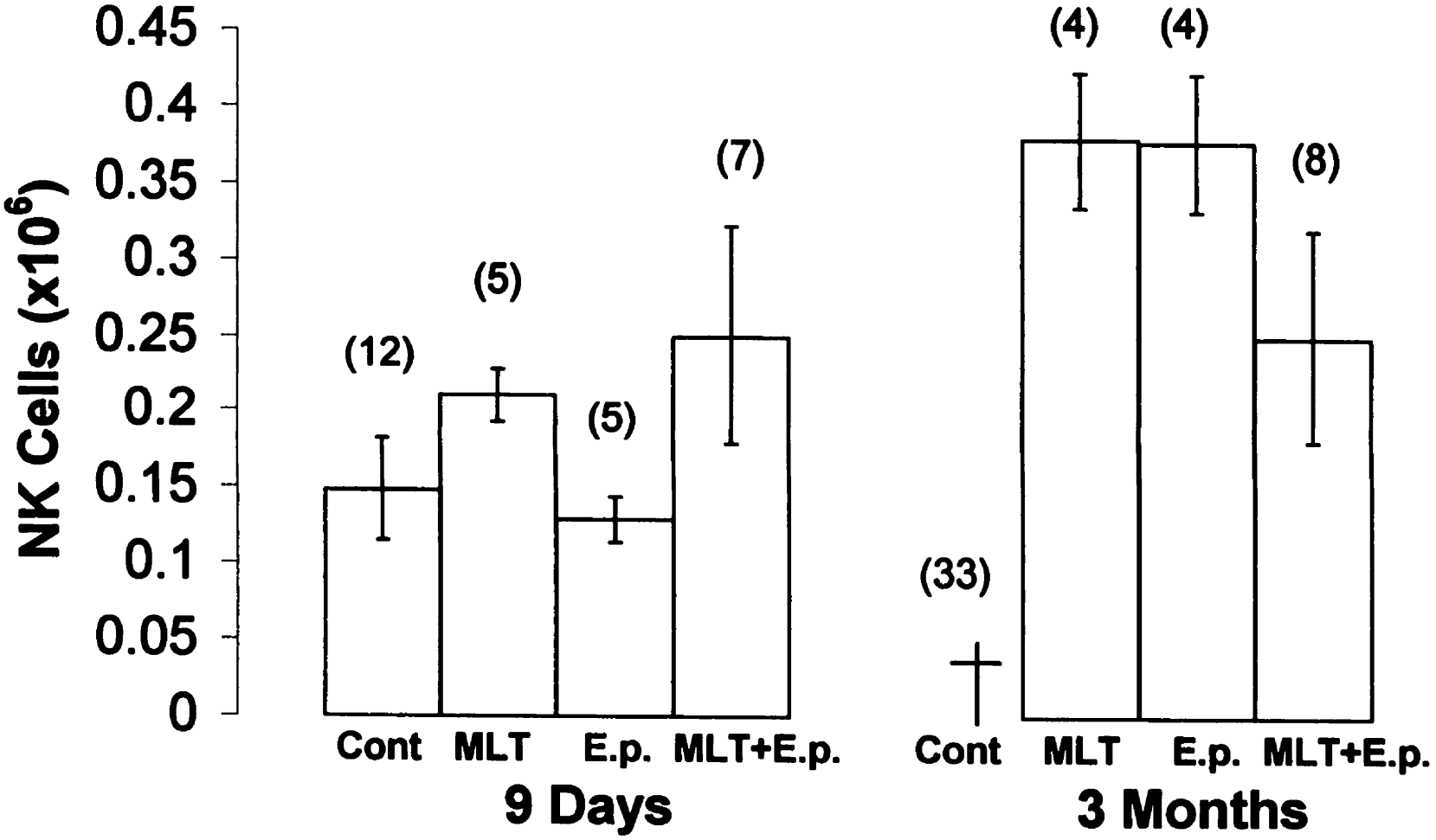
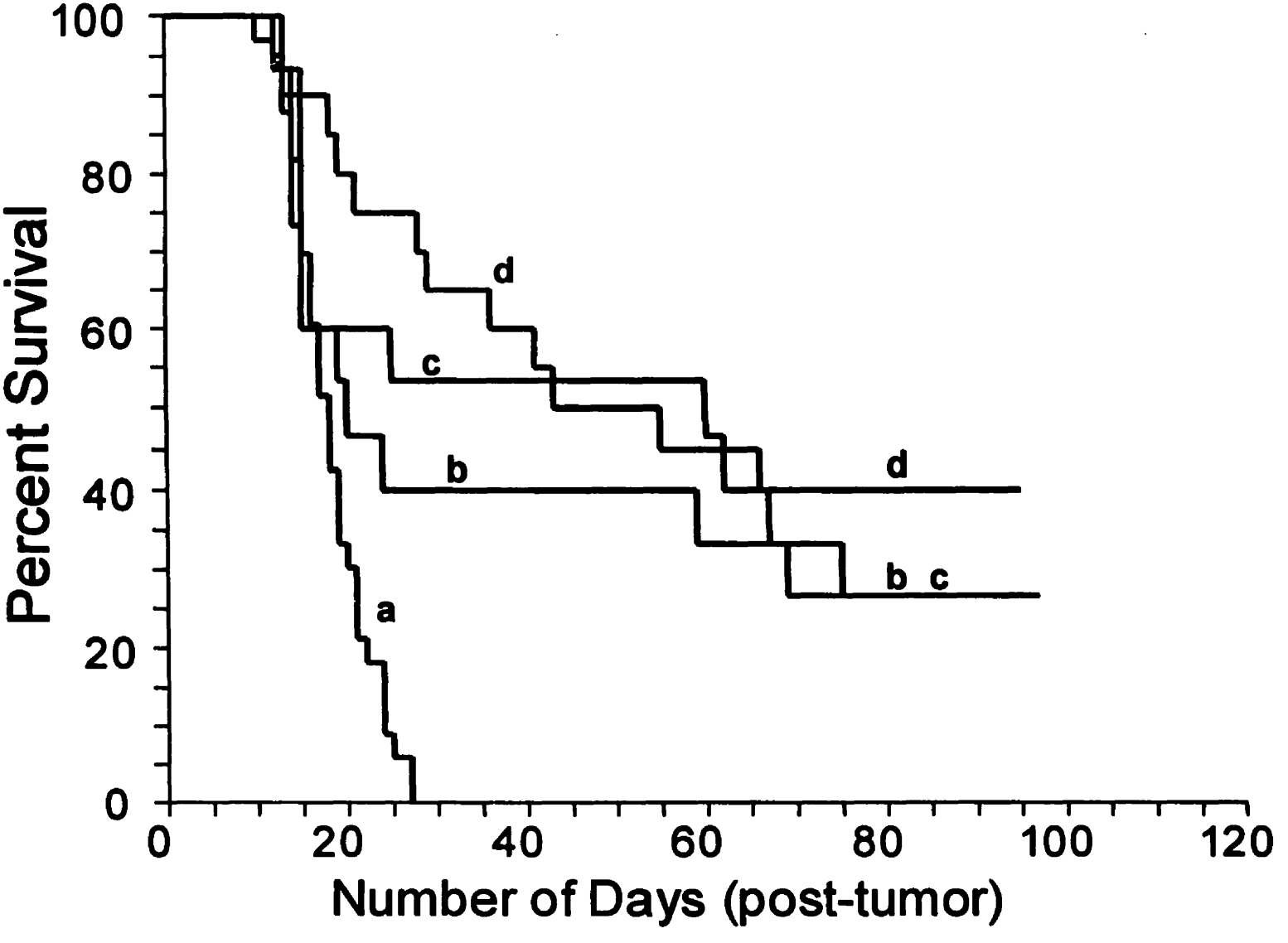

NK Cells (x10^6)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
(12)
(5)
(5)
(7)
Cont
MLT
E.p.
MLT+E.p.
9 Days
(33)
(4)
(4)
(8)
Cont
MLT
E.p.
MLT+E.p.
3 Months

Fig. 3.

Survival incidence of tumor-bearing mice treated with melatonin (MLT) (**c**), or E.p. (**b**), or both (**d**), for 50 days after tumor onset (day 0) (untreated controls (**a**)). Data was analyzed by Kaplan-Meier Statistics. Survival advantage provided by MLT or E. *purpurea* individually was statistically significant vs. control ($p < 0.0068$ and $p < 0.022$, respectively), although it was even greater when both agents were simultaneously administered ($p < 0.00035$ vs. control). Each of the 4 groups had 45 animals at time 0; all mice in the control group succumbed as indicated; of the mice in the MLT or E.p.-treated groups, 20 lived to and beyond 3 months; of the mice in the MLT+E.p.-treated group, 27 lived to and beyond 3 months.


Percent Survival
100
80
60
40
20
0
0
20
40
60
80
100
120
Number of Days (post-tumor)
d
c
b
a
d
b c

Discussion

The present study has provided a systematic analysis, under controlled laboratory conditions, of tumor amelioration *in vivo*, employing non-conventional therapeutic agents, i.e., melatonin and phytocompounds of the herb, E. *purpurea*. At 9 days after tumor onset, a stage which we have previously well established as being intermediate in the development of this leukemia, we have shown that dietary E. *purpurea*, a naturally occurring botanical which is commercially available, inexpensive, and non-toxic at any dose (Mengs et al., 1991; Lersch et al., 1992; Melchart et al., 1995), significantly prolonged life span to levels even higher than those which we have already observed using the cytokine, interleukin-2, the prostaglandin inhibitor, indomethacin, and the interferon inducer poly I:C (Christopher et al., 1991; Dussault & Miller, 1993; Currier & Miller, 1998). Indomethacin is beset with side effects, including hemorrhage while the naturally occurring molecules, interleukin -2 and interferon, are the antithesis of E. *purpurea* in being toxic when exogenously administered, even at low doses over time (Djeu et al., 1979; Rosenstein et al., 1986; West et al., 1987; Kohler et al., 1989) and must be regulated in a patient-specific, dose/frequency manner. In recombinant form the latter are, moreover, financially prohibitive, and not commercially available. Melatonin, on the other hand, the second agent used in the present study, is, like the cytokines (interleukin-2, interferon), a naturally occurring molecule, present *in vivo* in minute quantities. Exogenously administered, it is subject to potential dose/frequency problems of feedback inhibition, and thus, can interfere with the endogenous, photoregulatory

rhythyms. However, melatonin is considerably less expensive, and commercially readily available.

In tandem with the survival advantage provided by E. *purpurea* (and melatonin) was the fact that the immune cells whose numbers were significantly augmented were those acting at the first line of defense in combat against developing neoplasms, i.e., NK cells. Moreover, it has been well demonstrated that absolute increases in NK cell numbers, such as observed in the present study, does indeed reflect an increase in the *functional* armament available, i.e., for tumor combat (Keissling et al., 1975; Kasai et al., 1981; Biron & Welsh, 1982; Itoh et al., 1982; Hefeneider et al., 1983; Koo et al., 1986; Lotzova et al., 1986; Kalland, 1987). Interestingly, in all treated tumor-bearing hosts, at both time intervals studied, i.e., 9 days and 3 months, the bone marrow revealed no significant change in NK cell numbers regardless of treatment (E. *purpurea* alone, melatonin alone, or both together). The spleen (the destiny of the vast majority of bone marrow-generated NK cells) contained - at 9 days and 3 months - significantly fewer NK cells when E.p. + MLT were given, but not when either agent was given alone. It is probable that a trafficking shift of many of the newly generated, bone marrow-derived NK cells, under double agent treatment (E.p. + MLT), but not single agent treatment (E.p. or MLT), to organs other than the spleen, has occurred. Such a phenomenon has been demonstrated in other sustained, non-physiological situations (Biron et al., 1983). However, why such a shuttling of many newly generated, bone marrow derived NK cells to organs other than the spleen should occur in the presence of both agents, but not each alone, is unclear. It is certain, nevertheless, that in the presence of both agents

together *in vivo*, new NK cell production in the bone marrow birth site has not been impaired (NK cell numbers statistically unchanged regardless of treatment) indicating no "overdose" (toxic) effect from the presence of both agents together. Moreover, we have already demonstrated a phenomenon similar to this, i.e., reduced splenic NK cells when indomethacin and interleukin-2 (both powerful NK stimulants like E. *purpurea* and melatonin) were co-administered, but not when each was given alone (Christopher et al., 199l; Dussault and Miller, 1993).

Many of the other non-NK cell populations in the spleen and the bone marrow after 9 days of treatment have been reduced when E. *purpurea*, or melatonin, or both together, are given vs. untreated leukemic mice. This may reflect early adjustments/interplay among several complex non-physiological phenomena involving the presence of growing tumor, and one or both of two exogenously introduced agents (E. *purpurea*, melatonin). Furthermore, at 3 months post tumor onset, no tumor (erythroleukemic) cells were microscopically found. Moreover, scans of the cytospots of several thousand hemopoeitic and immune cells in both organs in all mice under study revealed that the absolute numbers of nucleated erythroid precursors observed in the bone marrow were comparable to that found in *normal* mice in the age range of these long term survivors, i.e., 5-6 months (Miller & Osmond, 1974; 1975; Mahoney et al., 1998). These observations, thus, signal the absence of erythroleukemic blast re-growth.

The tumor employed in this study (erythroleukemia) is unresponsive to any of the immunostimulants generated (directly/indirectly) by E. *purpurea* or melatonin, and in fact this leukemia will proliferate only in response to internal,

autocrine mechanisms (Lacombe et al., 1987; Stage-Maroquin et al., 1996), involving epo and epoR. The consistently and significantly elevated levels of NK cells in E. *purpurea*-treated, and melatonin-treated mice may well have been adequate to eliminate the disease, especially beginning in the early stages after tumor onset.

Although survival has been significantly improved by the particular protocol of agent administration implemented in this study, it is still not 100%. Manipulations of the administration protocol may be all that is necessary to further augment life span, i.e., higher doses and more frequent exposures of either or both agents. Moreover, studies are underway using inoculation methods, administering, individually and/or collectively, 2 known NK cell immunostimulants existing within the many components of E. *purpurea* (the polysaccharide, arabinogalactan, and the alkamide, 1,8-pentadecadiene).

Thus, a novel and unconventional approach to tumor therapy has been employed in the present study, in which a botanical compound (E. *purpurea*), and/or an endogenously occurring neurohormone (melatonin), was administered through a diet to leukemic mice. Using only the protocol described earlier, as the only means of "therapy" for our intermediate-stage, leukemic mice, 3 important conditions indicative of tumor abatement and potential host cure, have occurred: (i) microscopic (as well as clinical) absence of tumor re-growth, (ii) re-establishment by 3 months of normal levels of hemopoiesis in the bone marrow generating center for all blood borne red and white cells, and (iii) significantly prolonged life span. Most conventional chemotherapy is beset with 2 significant drawbacks. Firstly, as synthesized chemical concoctions, they are foreign to,

and highly toxic to, normal, living tissue as well as the targeted tumor cells, especially with sustained use, resulting in reduced quality - and ultimately quantity - of life. Secondly, most chemotherapies are tailored for tumor cell destruction and/or inhibition of their capacity to proliferate, rather that being directed toward enhancing of the naturally occurring, lethal hit, tumor lytic mechanisms already in place, i.e., the various cells comprising the specific and non-specific immune (NK) systems. It is with the latter mechanism of tumor combat that the present work is concerned.

In summary, this work has demonstrated, under controlled laboratory conditions, that phytocompounds from the naturally occurring herb, E. *purpurea*, may be profoundly valuable tools in leukemia combat. We have already demonstrated the prophylactic potential of E. *purpurea* in significantly elevating NK cells in normal, healthy mice (Sun et al., 1999). Clearly, the therapeutic potential of E. *purpurea*, at least, if not other botanicals as well, suggests that they could have a formal and fundamental role to play in modern anti-tumor therapy.

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INTRODUCTION TO CHAPTER 8

In Chapter 8 I employed a novel protocol attempting to immunize mice with non-viable cells of our standard erythroleukemic cell line. Immunization against tumor antigens is a technique for which experimental and clinical evidence is growing. In addition, I planned to also administer E. *purpurea* daily to these immunized mice, following viable tumor cell injection 5 weeks after immunization. The hypothesis was that "boosting" against the tumor cells + therapy via phytochemicals would afford an even greater survival advantage and immunoenhancement than any of the therapeutic treatments previously investigated in my work.

CHAPTER 8: The effect of immunization with/without nutriceutical feeding in erythroleukemic mice.

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Abstract

Tumor amelioration via vaccination/immunization is a practice for which considerable experimental and clinical support is growing. Moreover, combination therapies have proven to be even more advantageous than treatment of the afflicted host with one agent alone. In the present work, we hypothesized that immunization with killed tumor (leukemia) cells prior to the onset of overt leukemia (induced via injection of viable tumor cells), followed by an additional method for tumor abatement, i.e., dietary administration of the immunoenhancing phytocompound, E. *purpurea,* would be substantially more beneficial in the therapeutic regimen than immunization alone. A commercially available extract of E. *purpurea* root, already proven potent, was administered via the chow, for periods of 9 days or 3 months after the onset of leukemia to mice that had been injected (immunized) 5 weeks earlier with killed leukemia cells. Immunized mice (± E. *purpurea*) had significantly prolonged life spans vs. unimmunized mice, with an even greater proportion of hosts surviving long-term in the E. *purpurea*-fed group. Natural killer (NK) cells, the mediators of non-specific immunity and well-demonstrated mediators of tumor cytolysis, were very significantly elevated in immunized, leukemic mice receiving E. *purpurea* in their diet vs. those receiving untreated chow. Early in tumor development (9 days), cells mediating *specific* immunity (T, B lymphocytes) were 10-12 times higher in absolute numbers in the spleens in *all* immunized, leukemic mice vs. unimmunized, leukemic mice at the same stage of tumor progression. The results demonstrate that combination therapy, involving specific tumor cell immunization, followed by daily phytotherapy (dietary E. *purpurea*), led to an

even greater level of immunoenhancement and life span prolongation than immunization alone.

Introduction

Vaccination and immunization against developing tumors of assorted types is a recent concept for which there is, nevertheless, considerable experimental documentation of success, employing a wide variety of methodological approaches. Anti-tumor immunotherapy consists of inducing immunity to tumor cell antigens, and thereby triggering a cytolytic response, ideally and ultimately, eliminating the tumor. Unfortunately, most tumor cells are either non- or only weakly immunogenic, successfully evading detection by all immune cells. Attempts at creating immune responses to growing tumors are aimed at increasing the immunogenicity of the tumor cells themselves, or modifying them in other ways so that they will indeed help the cells of the immune system to find and destroy them. Collectively, results at tumor immunization have derived from a wide variety of protocols, including genetic engineering of tumor cells with/without viral modification (Schirrmacher et al., 1998; 1999; Simons et al., 1999; Carr-Brendel et al., 1999; Charles et al., 2000). These methods manipulate tumor cells (i) such that the can lead to the production of immunostimulants, directly or indirectly, (ii) and/or render weakly immunogenic tumor cells highly immunogenic and, thus, visible to cytolytic immune cells, (iii) and/or otherwise genetically manipulate the tumor cells to become attractive targets for immune cells. Other genetic modifications of tumor cells can even entice tumor cells to commit suicide (Ramesh et al., 1999). Other approaches involve vaccinating with modified specific, tumor-derived/associated peptides or proteins to trigger a subsequent strong cytolytic immune response to the specific peptide-expressing tumor cell (Dyall et al., 1998; Davila & Celis,

2000; White et al., 2000). Another method consists of inducing mice to make their own antibodies to the products of an over expressed gene which is the cause of their cancer (Erez-Alon et al., 1998), while still another technique consists of utilizing the ability of antigen-processing, or dendritic, cells (accessory cells in the immune system), to take up and modify tumor specific antigens (Tarte & Klein, 1999; Morse & Lyerly, 2000). Upon injection of these tumor-antigen-bearing dendritic cells, key cells in the immune system become stimulated, leading to a strong anti-tumor reaction.

Yet another approach to eliciting an anti-tumor immune response *in vivo* consists of injecting tumor cells or their extract, after killing by sonication, or heat (Li et al., 1998; Okamoto et al., 2000). Combination therapy, in which vaccination/immunization occurs along with *in vivo* hyperthermia, or cyclophosphamide injection, has been proven more advantageous in tumor amelioration than vaccination/immunization alone (Li et al., 1998; Okamoto et al., 2000)

In the present work we hypothesized that immunization with killed tumor (leukemia) cells prior to the onset of leukemia (injection of viable tumor cells), combined subsequently, with a secondary method of tumor amelioration, i.e., dietary administration of the immunoenhancing phytocompound, E. *purpurea*, would be substantially more beneficial in tumor therapy than the immunization event alone. Indeed, our results showed that phytotherapy after specific tumor cell immunization, led to an even greater level of immunoenhancement and life span prolongation than immunization alone.

Results

Fig. 1 indicates that immunization with killed erythroleukemia cells 5 wk prior to administration of the equivalent number of live tumor cells, significantly improved survival over mice given only live tumor cells - the mortality in the latter group representing that which we have consistently found (Christopher et al., 1991; Currier & Miller, in press). In mice which were given killed tumor cells, followed 5 wk later by viable tumor cells *and* dietary E. *purpurea,* survival advantage by the addition of the phytocompound was not initially significantly influenced over that provided by killed tumor cell injection alone. However, at and beyond 3 mo., survival in immunized, E. *purpurea*-fed mice was almost twice that of mice given only the immunizing dose of killed tumor cells (Fig. 1).

Fig. 2 indicates that at 9 days after the addition of dietary E. *purpurea* to mice immunized and given viable tumor cells, the absolute numbers of NK cells in both the spleen and the bone marrow declined to levels approximately one half and two thirds, respectively, of those of immunized mice given viable tumor cells but no E. *purpurea* in the chow. Continued feeding, however, with E. *purpurea* to immunized mice, resulted, by 3 mo post-injection of viable tumor cells, in highly significant elevations of NK cell numbers in both the spleen and bone marrow, relative to the levels found in immunized mice given normal chow (Fig. 3).

Tables 1 and 2 indicate that at both 9 days and 3 months, respectively, E. *purpurea* feeding to immunized, leukemia mice had virtually no influence on the absolute numbers of the various hemopoietic cell lineages in the spleen or bone marrow. Only bone marrow monocytes at 9 days (Table 1) and splenic erythroid

cells at 3 months (Table 2) deviated from immunized mice not administered E.p. Our previous observations in normal mice (Sun, et al., 1999) showed that dietary E. *purpurea* similarly had no short or longer-term influence on the non-NK cell populations of these organs.

Table 1.

Effect, in immunized mice, of dietary E. *purpurea* on the non-NK, hemopoietic cell subpopulations in the bone marrow and spleen during the early[a] stages of leukemia development.

Treatment	Non-NK Lymphoid (x10⁶)	Precursor Myeloid (x10⁶)	Mature Granulocytes (x10⁶)	Nucleated Erythroid (x10⁶)	Monocytes (x10⁶)
Bone Marrow (per femur)					
KC[c]+VC[d]+E[e]	4.54±0.49[f]	0.943±0.133	2.49±0.30	3.12±0.42	0.077±0.018*
KC+VC	4.60±0.30	1.16±0.14	2.50±0.24	2.16±0.21	0.227±0.038
Spleen					
KC+VC+E	1481.41±101.49	12.04±3.14	76.04±7.19	496.49±81.07	7.42 ±4.01
KC+VC	1226.14±127.74	26.73±5.87	67.43±8.52	733.63±126.71	9.74±2.50

[a] 9 days after tumor onset (day 0).
[b] Differential counts on hematologically stained, microscope slide preparations were conducted on 2000 total hemopoietic cells/organ/mouse in all experimental and control mice. The percentages of cells in each category were then converted, via the previously determined total cellularity in each organ (femur, spleen), to the absolute numbers (x 10^6) of cells in each subpopulation of hemopoietic cells/organ/mouse.
[c] killed cells (KC) were produced by lethally irradiating erythroleukemic cells with 9.2Gy; 115rads/min for 8 min. Subsequently, 3 x 10^6 killed cells were injected to mice via the lateral tail vein.
[d] viable erythroleukemic cells (VC) (3 x 10^6) were injected into mice 5 weeks after KC.
[e] E. *purpurea* (E) was administered in the diet beginning at tumor onset - 0.45 mg/mouse/day.
[f] Mean±s.e.: 7 mice
*$p < 0.05$

Table 2.

Effect, in immunized mice, of dietary E. *purpurea* on the non- NK, hemopoietic cell subpopulations in the bone marrow and spleen during long term[a] leukemia development

Treatment	Blastic Lymphoid ($\times 10^6$)	Blastic Myeloid ($\times 10^6$)	Mature granulocytes ($\times 10^6$)	Nucleated Erythroid ($\times 10^6$)	Monocytes ($\times 10^6$)
Bone Marrow (per femur)					
KC[c]+VC[d]+E[e]	3.71±0.16[f]	0.668±0.143	3.50±0.53	2.00±0.19	0.052±0.017
KC+VC	3.72±0.29	0.755±0.100	3.56±0.25	2.02±0.17	0.109±0.021
Spleen					
KC+VC+E	62.47±2.22	0.239±0.135	4.95±1.15	23.18±2.87*	0.113±0.061
KC+VC	44.82±10.09	0.059±0.032	5.81±1.70	10.39±2.38	0.087±0.069

[a] 3 months after tumor onset (day 0).
[b] Differential counts on hematologically stained, microscope slide preparations were conducted on 2000 total hemopoietic cells/organ/mouse in all experimental and control mice. The percentages of cells in each category were then converted, via the previously determined total cellulity in each organ (femur, spleen), to the absolute numbers ($\times 10^6$) of cells in each subpopulation of hemopoietic cells/organ/mouse.
[c] killed cells (KC) were produced by lethally irradiating erythroleukemic cells with 9.2Gy; 115rads/min for 8 min. Subsequently 3×10^6 killed cells were injected to mice via the lateral tail vein.
[d] viable erythroleukemic cells (VC) (3×10^6) were injected into mice 5 weeks after KC.
[e] E. *purpurea* (E) was administered in the diet beginning at tumor onset - 0.45 mg/mouse/day.
[f] Mean±s.e.: 7 mice.
* $p < 0.05$

Fig. 1.

Longevity of mice receiving only viable tumor cells (VC), or, injected 5 wk earlier with killed tumor cells (KC) + VC, or, injected with KC + VC, and given E. *purpurea* (E) daily (0.45mg/day/mouse) in the chow. $p < 0.0000005$:VC *vs* KC+VC; $p < 0.0000005$: VC *vs* KC+VC+E.

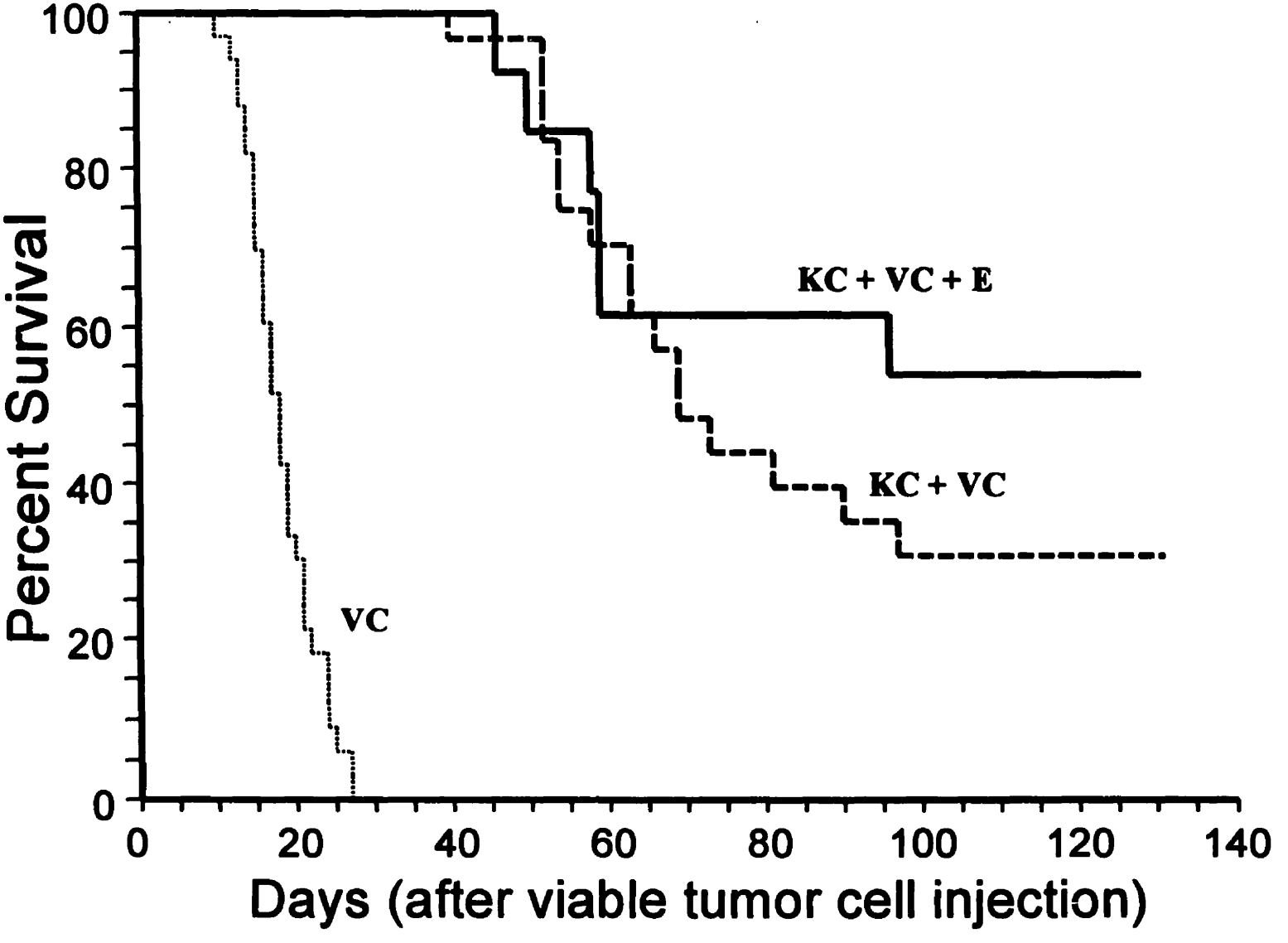

100
80
60
40
20
0
Percent Survival
KC + VC + E
KC + VC
VC
0
20
40
60
80
100
120
140
Days (after viable tumor cell injection)

Fig. 2.

Absolute numbers of NK cells in the bone marrow and spleen at 9 days after tumor onset (day 0). All mice had been given KC 5 weeks prior to tumor onset. Daily dietary administration of E. *purpurea* (E.p.) for day 0 - 9 resulted in lower absolute numbers of NK cells in both organs. The numbers of NK cells in the bone marrow (BM) of mice consuming E.p. were 66.8% ($p < 0.01$) of those in mice not consuming E.p. The numbers of NK cells in the spleens of mice consuming E.p. were 54.8 % of those in mice consuming untreated chow. ( ) = number of mice; Mean ± s.e. Control diet mice (open bars), E.p. fed mice (closed bars).

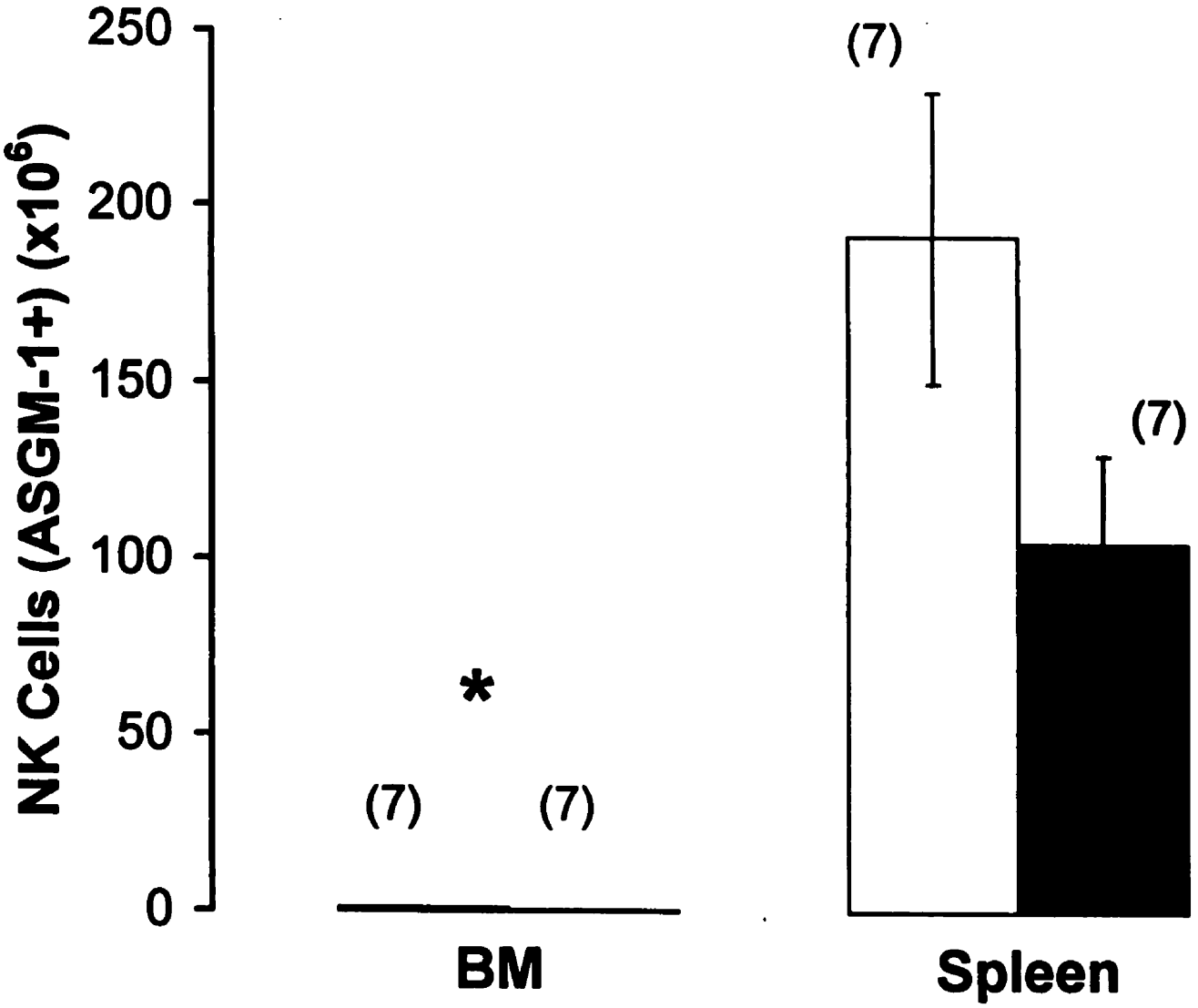
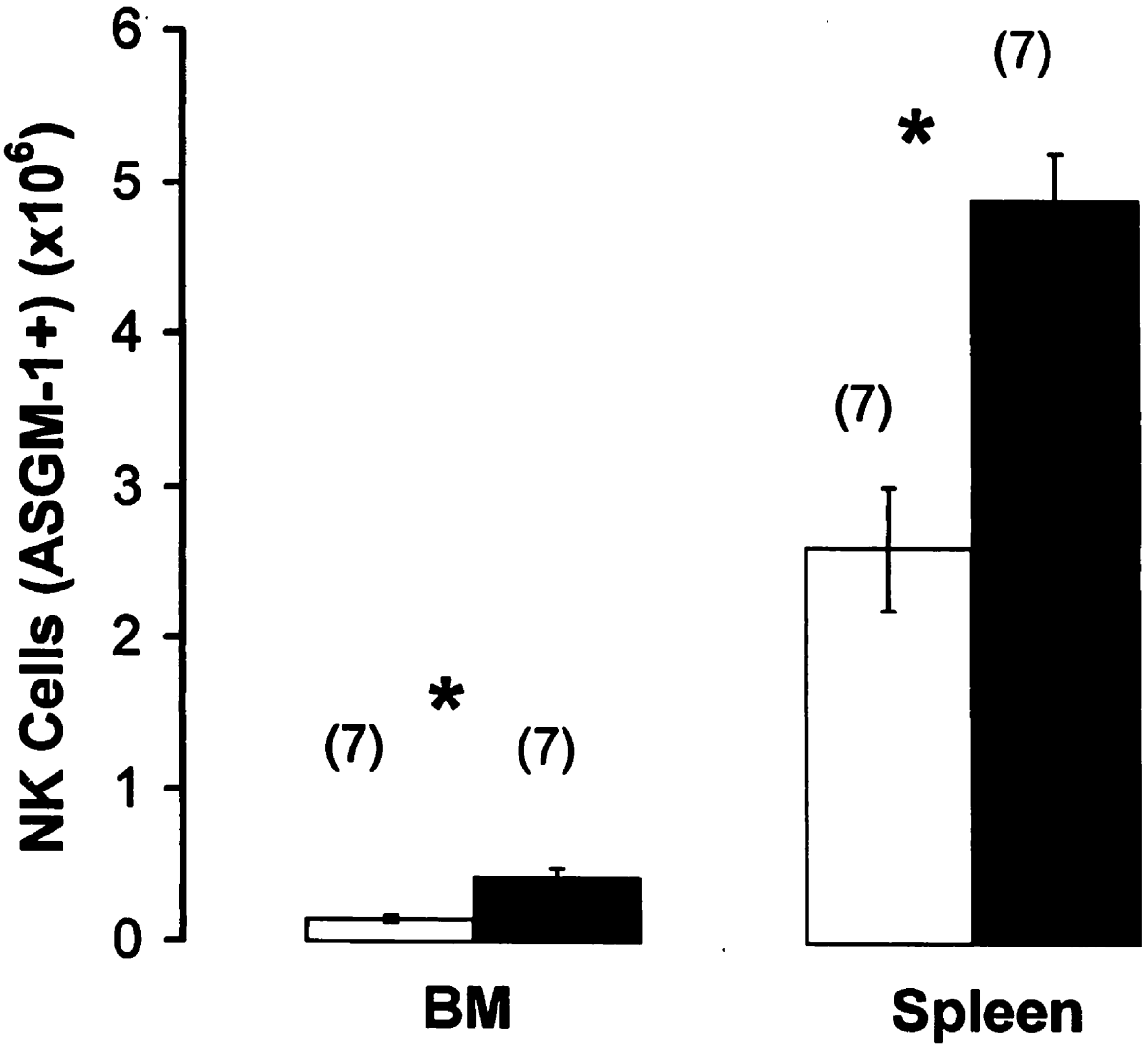

250
200
150
100
50
0
NK Cells (ASGM-1+) (x10^6)
*
(7)
(7)
(7)
(7)
BM
Spleen

Fig. 3.

Absolute numbers of NK cells in the bone marrow and spleen at 3 months after tumor onset (day 0). All mice had been given KC 5 weeks prior to tumor onset. Daily dietary administration of E. *purpurea* (E. p.) for 3 months from tumor onset resulted in elevated numbers of NK cells in both organs. The numbers of NK cells in the bone marrow (BM) of mice consuming E. p. rose to 290% of those in mice not consuming E. p. ($p < 0.003$), while the numbers of NK cells in the spleens of mice consuming E. p. rose 188% ($p < 0.001$) relative to those of mice consuming untreated chow. ( ) = number of mice; Mean± s.e. Control diet mice (open bars), E.p. fed mice (closed bars).


NK Cells (ASGM-1+) (x10^6)
6
5
4
3
2
1
0
(7)
*
(7)
(7)
*
(7)
BM
Spleen

Discussion

The survival advantage offered by immunizing mice prior to administration of viable tumor cells necessarily indicates that the cells responding to the immunizing process were non-NK immune cells, i.e., T/B lymphocytes. NK cells are short-lived, rapidly renewing cells with no long-lived, memory component to the lineage (Miller, 1982; Zoller et al., 1982; Biron et al., 1983; Hurme, 1984; Pollack & Rosse, 1987; Rahal et al., 1991), and are, thus, incapable of responding in immunization processes. The 5 week interval employed in the present study, between administration of killed tumor cells to elicit immunization, and the injection of viable leukemia cells to instigate the active tumor, was well within the time range needed to activate naive T (±B) lymphocytes and increase, thus, the specific, tumor-cell-antigen-sensitive, reactive immune cell pool.

Survival advantage imbued by the phytocompound appeared only in the later stages in immunized, leukemic mice, and paralleled the highly significant elevation in NK cell numbers also found in these mice. Soon after (9 days) tumor cell injection to immunized hosts, with and without E. *purpurea* feeding, the numbers of non-NK lymphocytes, i.e., T and B cells in the spleen were 10-12-fold those in the spleens of unimmunized, diet-untreated, leukemic mice (Christopher et al., 1991; Miller et al., 1992). This non-NK lymphocyte increment indicates a strong immune response by non-NK lymphocytes, to the presence of the killed tumor cells. This phenomenon is responsible for the very significantly increased levels of survival in immunized, leukemic mice vs. unimmunized, leukemic mice. Strong support for this T/B cell-mediated immune reaction in the killed-tumor-cell-injected mice derives from our earlier observations in untreated,

leukemic mice (Christopher et al., 1991; Miller et al., 1992). In those studies, we demonstrated that the bone marrow, a site not involved in clonal expansion to foreign antigens, contains numbers of non-NK lymphocytes, putative T/B cells, which do not differ significantly from those in the bone marrow of the immunized, leukemic mice in the present study. In keeping with the role of T/B lymphocytes as mediators of tumor-cell-antigen immunity, the present data reveal that only the lymphocytes cells of the spleen (site of clonal proliferation in response to foreign antigens) demonstrate this manifold increase in non-NK lymphocyte numbers. T cell-mediated immunity to erythroleukemic cells has been shown (Isaak et al., 1989; Schultz et al., 1990; Kaido et al., 1993). Moreover, we have previously demonstrated (Miller et al., 1992) that Lyt-2+ cells, i.e., T lymphocytes, in the spleen reacted promptly to the presence of erythroleukemia in the host, so that early in tumor growth (7 days) their absolute numbers were significantly ($p < 0.001$) higher relative to those of *normal* mice of identical age, strain and gender (Miller et al., 1992).

Splenic NK cell numbers, during the early period after tumor onset (9 days) in immunized mice, increased even more profoundly to 40-60 times those found in unimmunized, leukemic mice (Miller et al., 1992; Currier and Miller, in press), supporting the well established fact that erythroleukemia antigens are powerful stimulants for NK cells (Hagner, 1984; Lust et al., 1984; Afifi et al., 1986; Iorio et al., 1986; 1989; Thurlow et al., 1986; Neri et al., 1989). In the bone marrow, however, NK cells increased only 2-fold, again demonstrating the resistance of bone marrow based cells (T, B, or NK) to the systemic presence of foreign antigens. The stability of the bone marrow, in this regard, is a

phenomenon which appears to exist not only in the presence of systemic foreign antigens, but, as we have previously shown, even in the presence of an NK cell-stimulating pharmaceutical (Miller, 1992).

The fall in NK cell numbers observed only at 9 days in immunized, leukemic mice fed E. *purpurea* vs. those fed untreated chow may relate to the fact that E. *purpurea* stimulates monocytes to produce the cytokine, interferon, the latter, in turn, being a powerful stimulant of NK cells. In the present study, in immunized, E. *purpurea*-fed-mice, the bone marrow (generating site of monocytes) contains significantly fewer numbers of monocytes at day 9 of tumor development, than does the bone marrow of immunized mice fed untreated chow. Consequently, the low NK cell numbers in E. *purpurea*-fed mice at day 9 of tumor development may be an indirect result of this transiently low number of monocytes.

Finally, irrespective of the early (day 9) disturbances in cell numbers within some hemopoietic/immune cell lineages, by 3 months after tumor onset, these populations in both organs, have returned to within the *normal* ranges (absolute numbers) already demonstrated by us (Christopher et al., 1991; Miller, 1992; Currier and Miller, 1998; Whyte and Miller, 1998; Sun et al., 1999). This was the case for immunized mice fed either untreated, or E. *purpurea*-containing chow. However, in mice fed E. *purpurea*, a further survival advantage may have been imbued by the sustained, significant elevation in absolute numbers of NK cells observed at 3 mo post-tumor onset. The number of E. *purpurea*-fed, immunized, leukemic mice, which survived to and beyond 3 months was approximately twice that of immunized, leukemic mice fed untreated chow.

Given the well-demonstrated anti-leukemia role of NK cells, the parallelism of these 2 phenomena may be more than coincidental.

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INTRODUCTION TO CHAPTER 9

In previous chapters I focused on the effects of MLT and/or E. *purpurea* administration on NK cell numbers in healthy and leukemic, *young adult* mice. The age related decline in NK cell numbers in laboratory mice is well documented. This decline may be due to a) decrease in NK cell production in the bone marrow b) inhibition of NK cell function mediated by a suppressive factor or c) impairment in target cell binding. Previous work in our laboratory showed that administration of IL-2 or indomethacin failed to reverse this defect in NK cell activity in elderly mice, regardless of cause. Leukemias, lymphomas and infections are higher in frequency in elderly animals than in young adults. In Chapter 9 I wanted to examine the effect of administration of the phytochemical E. *purpurea* on aged mice. My hypothesis was that it might be possible, using all of our standard techniques, to rejuvenate the NK cell system in these elderly mice. This study dramatically showed that whereas nothing previously employed in our lab could augment NK cells in elderly animals, the herb E. *purpurea* was able not only to increase NK cell production but also to resume their functional activity in parallel to that of young adult mice.

CHAPTER 9: Natural killer cells from aging mice treated with extracts from E. *purpurea* are quantitatively and functionally rejuvenated.

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Abstract

A growing body of anecdotal evidence in young and adult humans suggests that certain phytochemicals have the capacity to ameliorate tumors and reduce infections, especially those mediated by virus, *in vivo*. These indications prompted us, therefore, to investigate the potentially immunostimulating effect of one such phytocompound, E. *purpurea*, on natural killer (NK) cells since these cells are active in spontaneous, non-specific immunity against neoplasms and virus-mediated infections. We elected to study aging mice, since, at this stage of life, like humans, the above-mentioned afflictions increase in frequency. We had previously found that neither the cytokine, interleukin-2, nor the pharmacological agent, indomethacin, both potent stimulators of NK cell numbers/function in younger adult mice, was effective in stimulating NK cells in elderly mice. The present study was designed to assess the numbers/production of NK cells in the spleen and bone marrow of aging, normal mice, after *in vivo* dietary administration of E. *purpurea* (14 days), or, after injection of thyroxin, a stimulant of NK cell function (10 days). Immunoperoxidase labeling techniques, coupled with hematologic tetrachrome staining were used to identify NK cells in both the spleen (primary site of NK cell function) and bone marrow (site of NK cell generation). Double immunofluorescence staining, employing propidium iodide, was used to assess NK cell lytic function. Our results revealed that E. *purpurea*, but not thyroxin, had the capacity to increase NK cell numbers, in aging mice, reflecting increased new NK cell production in their bone marrow generation site, leading to an increase in the absolute numbers of NK cells in the spleen, their primary destiny. The E. *purpurea*-mediated increase in NK cell numbers was

indeed paralleled by an increase in their anti-tumor, lytic functional capacity. Collectively, the data indicate that E. *purpurea*, at least, and possibly other plant compounds, appear to contain phytochemicals capable of stimulating *de novo* production of NK cells, as well as augmenting their cytolytic function, in animals of advanced age.

Introduction

It has been well established that NK cell-mediated immunity, which acts as the first line of defense against developing tumors and virus-infected cells (Kasai et al., 1980; Biron and Welsh, 1982; Hanna, 1985), declines with advancing age in laboratory animals (Albright and Albright, 1983; Ghoneum et al., 1991), and, although more variable, in humans as well (Krishnaraj, 1992; Kutza and Murasko, 1994). We have recently established the mechanism for the decline in NK cell-mediated, anti-tumor activity in aged mice (Dussault and Miller, 1994), and found that it results from at least two phenomena: (i) reduced new cell production in the NK lineage in their bone marrow birth site, and, (ii) reduced efficiency of mature NK cells to bind to their target cells (tumor, or virus-infected) - an essential event prior to NK cell-mediated target destruction. We further found that NK cells from elderly animals respond to neither the drug indomethacin, nor the cytokine IL-2, which, in young adult animals profoundly stimulate population growth and/or function of NK cells (Dussault and Miller, 1994; 1995).

It was established more than a decade ago, that age-related changes in thyroid hormone levels correlated well with the decline in various immune functions (Fabris et al., 1982; 1986). Thyroid hormone production is reduced in aging mice (Provinciali et al., 1991) and conversely, *in vivo* administration of the thyroid hormone, thyroxin, to older mice results in regrowth of the thymus and recovery of T cell-mediated immune responses (Fabris et al., 1982; 1986; Hirokawa et al., 1994). *In vivo* administration of thyroxin significantly increases the NK cell-mediated functional activity of old male mice, while having minimal or

no effect on functional NK responses in juvenile mice (Provinciali et al., 1991). Moreover, we have recently shown (Mahoney et al., 1998) that although 6 month old female mice had significantly more NK cells than did their male counterparts, 10 days of thyroxin injection *in vivo*, in both sexes, resulted in males and females achieving comparable, significant elevations in their NK cell numbers. These observations collectively formed part of the basis for our present quantitative and functional studies on NK cells in aged mice.

Secondly, a growing body of anecdotal evidence in young and adult humans suggests that phytochemicals may have the capacity to ameliorate tumors and reduce virus infections (Melchart et al., 1995; Bauer, 1996; See et al., 1997). These observations prompted our systematic investigation of the potentially immunostimulating role of phytocompounds in E. *purpurea*, in aging mice, under controlled conditions. Indeed, we have recently shown in young, healthy mice, that E. *purpurea* significantly stimulates NK cell numbers over the short and long term *in vivo* (Sun et al., 1999). It is known the E. *purpurea* contains specific molecules such as arabinogalactan, isobutylamides, tannins, flavonoids, germacrene, humulene, and borneol. Some of these are known NK cell stimulants, acting either directly to enhance NK cells (arabinogalactan), or indirectly, by releasing NK cells from their prostaglandin inhibitors. Isobutylamides, for example, inhibit the production of 5-lipoxygenase and cyclooxygenase, key enzymes in the production of prostaglandins. Moreover, E. *purpurea* has the advantage in young adult mice, of stimulating new NK cell *production* in the bone marrow birth site, leading thus, to the possibility that it

may be the single agent, identified to date, to potentially do so in aged mouse bone marrow as well.

Our present results have revealed that, indeed, E. *purpurea*, but not thyroxin (nor the cytokine, IL-2 nor the drug, indomethacin), had the capacity to increase both the absolute numbers and the functional activity of NK cells, in aged mice, indicating that E. *purpurea*-bearing compounds, and possibly other phytochemicals, may represent specific groups of compounds uniquely capable of stimulating non-specific (NK cell-mediated) immunity in the elderly.

Results

Fig. 1 indicates that 10 days of thyroxin exposure had no positive effect on new NK cell *production* in the bone marrow (evidenced by lack of increase in NK cell numbers in this, the organ of their generation). Consequently, there was no increase in NK cell numbers in the spleen; the organ that is the predominant recipient of all newly generated NK cells. In the bone marrow of all normal, healthy aging mice, new NK cell production falls to negligible levels and, thyroxin was not able to revive NK cell production/numbers (Fig. 1). NK cell cytolytic *function,* nevertheless, was significantly ($p<0.05$) elevated at least at the higher E:T ratios in the presence of thyroxin (Fig. 2) relative to control. The proportional changes, relative to the corresponding control, across the 5 E:T ratios (Fig. 2) after thyroxin administration, were recorded at -33.1%, 9.5%, 44.8%, 42.8% and 7.8%, respectively.

The influence of thyroxin on other immune and hemopoietic cell lineages in both the spleen and bone marrow was insignificant. That is, after thyroxin administration (10 days, as above) to these aged mice, non-NK lymphocytes, myeloid cells, nucleated erythroid cells and monocytes in the spleens of 6 mice averaged 89.7%, 8.6%, 0.9% and 0.7%, respectively, vs. 89.8%, 5.8%, 3.3% and 1.1%, respectively, in the spleens of sham-injected (control) mice. The same lack of influence was also observed in the femoral bone marrow of the thyroxin-injected aged mice wherein non-NK lymphocytes, myeloid cells, nucleated erythroid cells and monocytes averaged 5.2%, 94.2%, 2.6% and 0.01%, respectively, vs. 6.0%, 89.4%, 4.5% and 0.03%, respectively, in the sham-injected mice.

However, in the presence of E. *purpurea*, administered in the diet, NK cells responded positively both in number and function. NK cells increased, in the aging bone marrow, from almost undetectable levels ($0 \pm 0 \times 10^6$) in control aging animals to significantly increased ($p<0.004$) numbers ($0.18 \pm 0.03 \times 10^6$) after 14 days of E. *purpurea*-feeding (Fig. 3). In the spleen as well, NK cells were 30% more numerous ($3.9 \pm 0.56 \times 10^6$) in E. *purpurea*-fed mice than in control ($2.8 \pm 0.3 \times 10^6$) (Fig. 3).

No positive influence was found, however, on the absolute numbers of any other hemopoietic or immune cell population after 2 wk of ingesting E. *purpurea*, in either the spleen or the bone marrow, in accordance with our previous observations in young, adult mice (Sun et al., 1999), wherein we found that all these non-NK cell populations remained steadfastly at control levels after 14 days of daily feeding of E. *purpurea*. Thus, in the present study, non-NK lymphocytes, myeloid cells, nucleated erythroid cells and monocytes in the spleens of these aged, herb-fed mice, averaged 83.1%, 7.4%, 8.7% and 0.6%, respectively, vs. 80.0%, 8.3%, 10.0% and 1.6%, respectively in mice consuming untreated (control) diet. Moreover, the bone marrow of these herb-fed mice averaged a population distribution for non-NK lymphocytes, myeloid cells, nucleated erythroid cells and monocytes of 21.6%, 48.6%, 27.9%, and 1.8%, respectively, while the distribution of these cell lineages in control diet-fed mice averaged 34.6%, 51.5%, 10.6% and 3.7%, respectively.

Just 14 days of consuming the phytocompound by these aging animals has returned NK cell *numbers* to levels similar to those in young, adult mice, as regularly observed by us and others (Keissling et al., 1975; Koo et al., 1982;

Lotzova et al., 1986; Christopher et al., 1991). Fig. 4 indicates a consistent and statistically significant ($p<0.03$ - 0.001) elevation in NK cytolytic activity at every E:T ratio (1:1 - 25:1) assessed (130.9%, 62.3%, 140.0%, 92.9% and 155.9%, respectively), suggesting that the increase in absolute numbers of NK cells seen under the influence of the phytocompound (Fig. 3), may represent useful, functional cells. That this is indeed the case is defined in the quantitative analysis. That is, the total absolute level of NK cell activity (TNKA) in the spleens of E. *purpurea*-fed mice is 20% higher than control levels (TNKA = 1.2), while the spleens of thyroxin-treated mice demonstrated a total absolute level of NK cell activity (TNKA) of only 2/3 that of the corresponding sham-injected controls (TNKA = 0.68), indicating not only an absence of benefit of thyroxin on the production/numbers/absolute activity of the NK cell population in the aged, but rather a clearly negative effect of this hormone.

Fig. 1.

Absolute number of NK cells, detected by the presence of the surface molecule, ASGM-1, in the bone marrow and spleen of aged DBA/2 mice injected bi-daily for 10 days (i.p., 0.2µg in 0.1µl PBS) with the thyroid hormone, thyroxin (closed bars). Control mice received the vehicle only (open bars). Mean ± s.e.; ( )=number of mice.

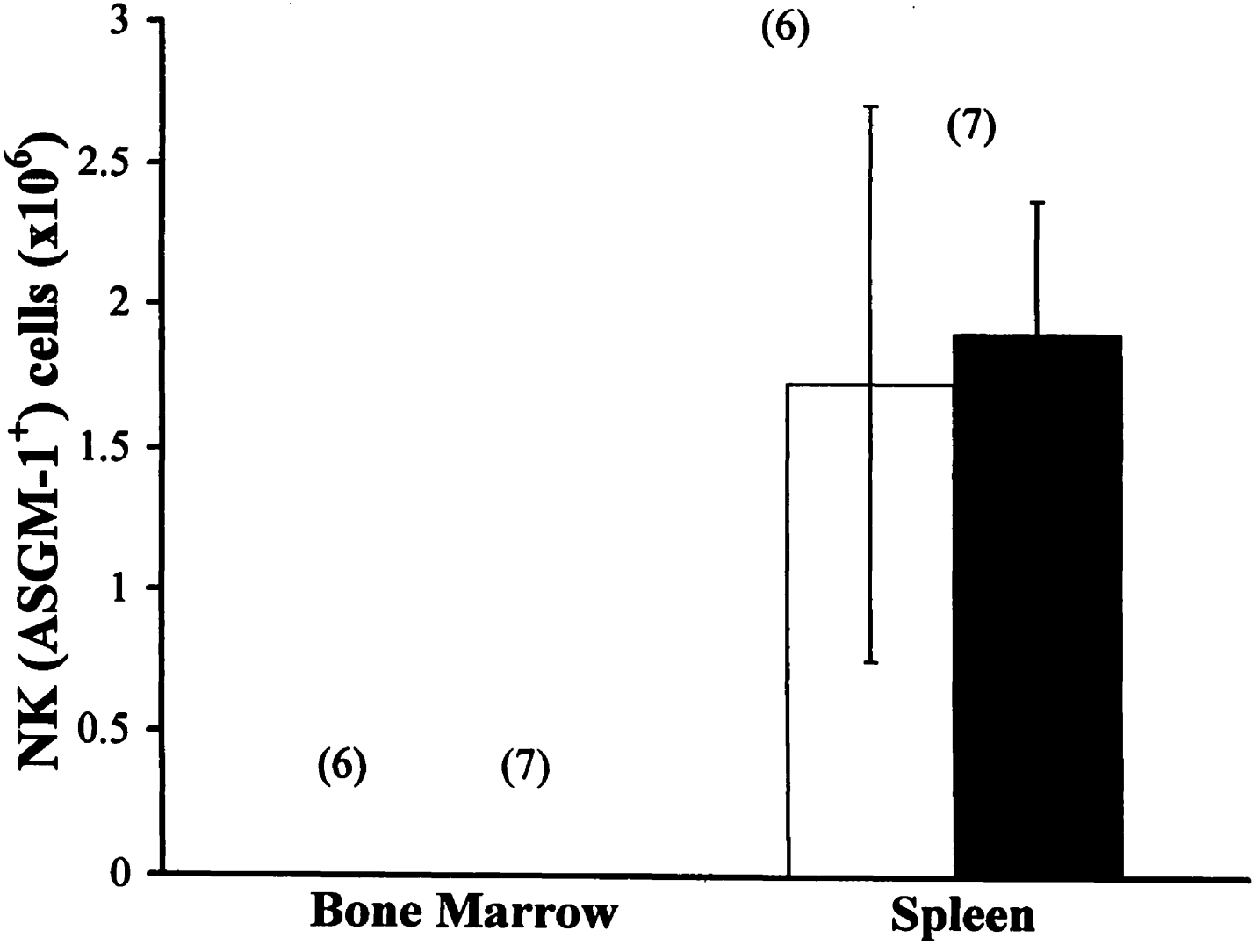

NK (ASGM-1+) cells (x10^6)
3
2.5
2
1.5
1
0.5
0
(6)
(7)
(6)
(7)
Bone Marrow
Spleen

Fig. 2.

Cytotoxicity levels recorded at several E:T ratios, as a percentage of labeled tumor target cells killed by NK cells as effectors. Thyroxin was injected bi-daily for 10 days, as above (sampling on day 11), while control mice received vehicle only. Mean±s.e.: 6 mice (thyroxin-injected); 5 mice (control).

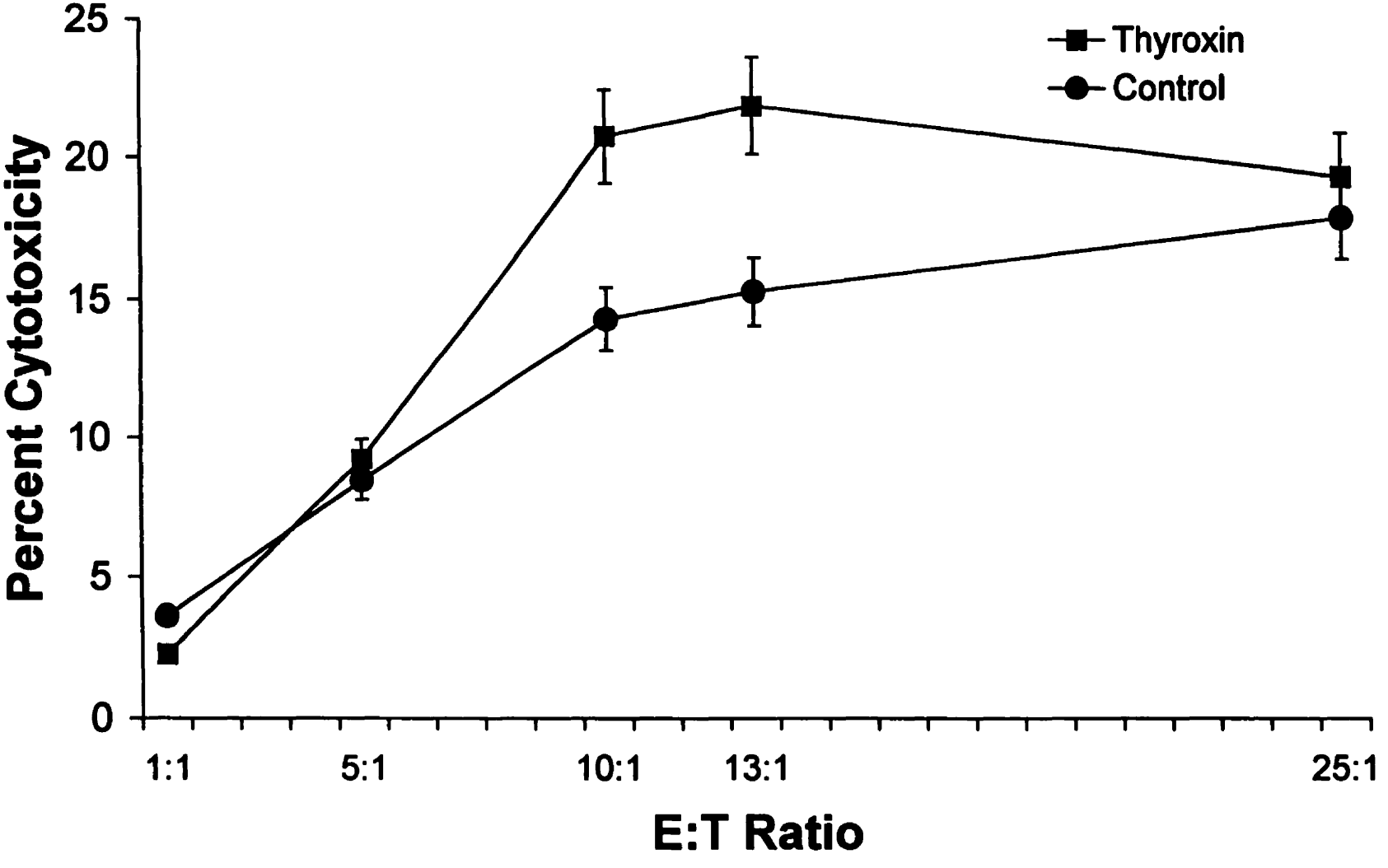

Thyroxin
Control
Percent Cytotoxicity
25
20
15
10
5
0
1:1
5:1
10:1
13:1
25:1
E:T Ratio

Fig. 3.

Absolute number of NK cells, detected by the presence of the surface molecule, ASGM-1, in the bone marrow and spleen of aged DBA/2 mice given E. *purpurea* root extract (closed bars), ground fresh daily into the chow (0.45mg/mouse/day) for 14 days. Control mice received untreated chow only (open bars). Mean±s.e.; ( )=number of mice. *$p<0.004$.

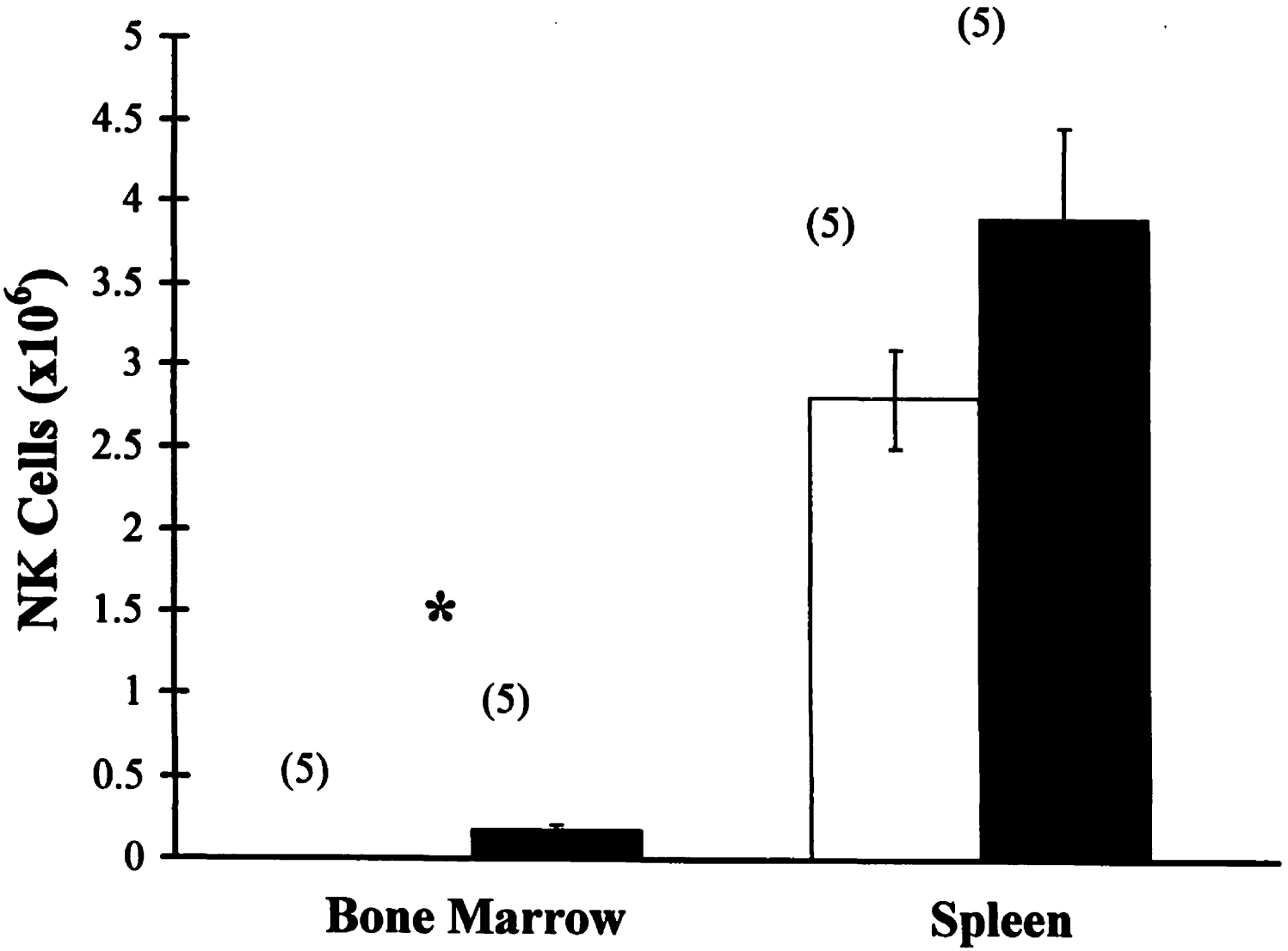
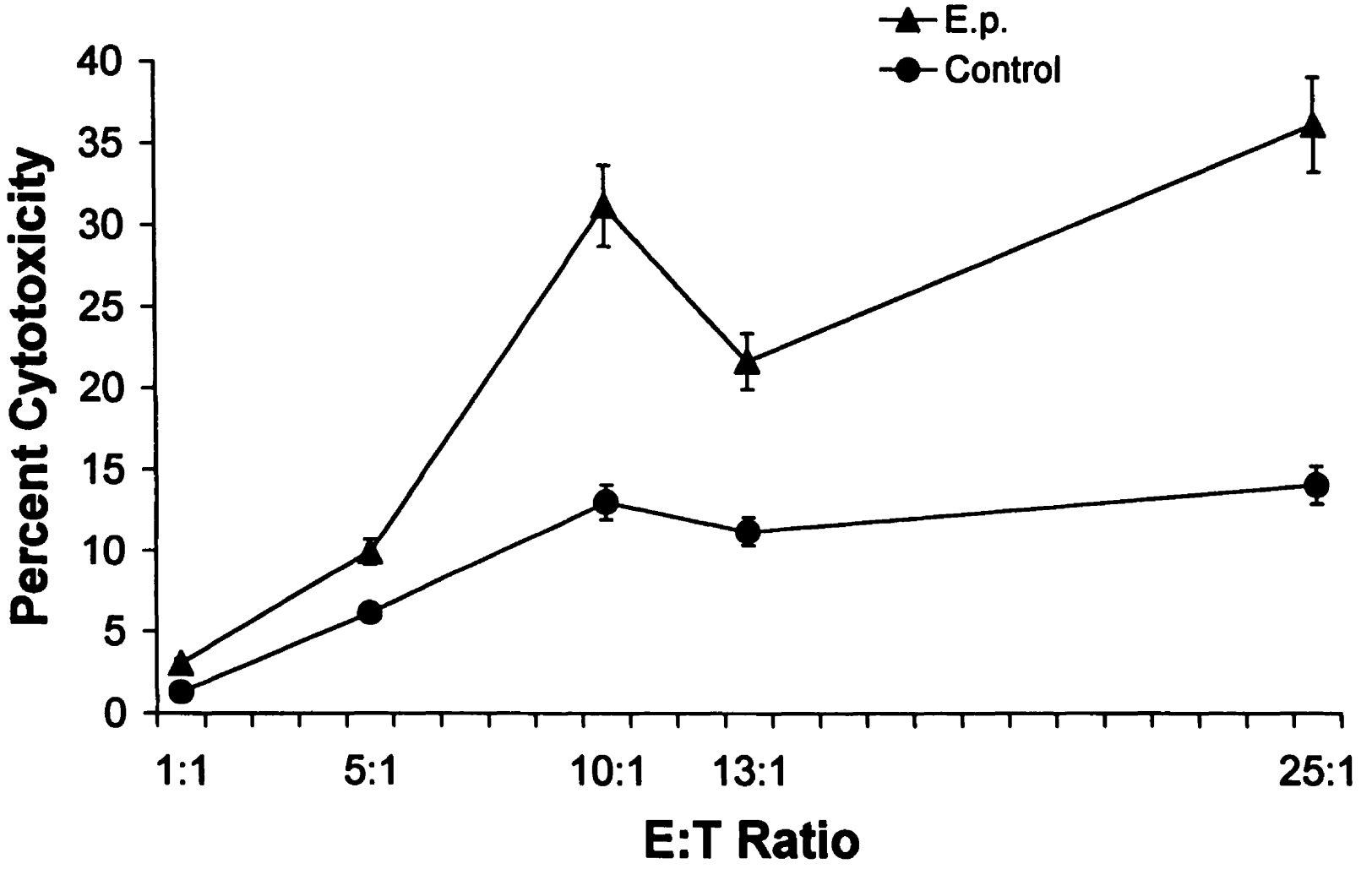

NK Cells (x10^6)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
(5)
*
(5)
(5)
(5)
Bone Marrow
Spleen

Fig. 4.

Cytotoxicity levels recorded at several E:T ratios, as a percentage of labeled tumor target cells killed by NK cells as effectors. E. *purpurea* was administered fresh daily in the diet for 14 days, as above (sampling on day 15). Control mice received the ground chow as vehicle for E. *purpurea*. Mean±s.e.: 6 mice (E. *purpurea*-fed); 5 mice (control).


E.p.
Control
Percent Cytotoxicity
40
35
30
25
20
15
10
5
0
1:1
5:1
10:1
13:1
25:1
E:T Ratio

Discussion

We have shown that thyroxin in aged mice was moderately able to stimulate the *function* of NK cells, in accordance with other studies (Sharma et al., 1982; Provinciali et al., 1991). This work has shown, however, that *de novo*, new NK cell *production* from their normal bone marrow birth site, is not the mechanism behind this increase in functional activity. The mode of action of thyroxin appears be one of modulating the intra-cellular cytolytic machinery by increasing the sensitivity of pre-existing NK cells normally responsive to interferon. Such a phenomenon has already been demonstrated (Gelato et al., 1975; Cognini et al., 1983). Interferon has long been known to potently stimulate the lytic activity (function) of NK cells (Gidlund et al., 1978; Djeu et al., 1979).

An observation emerging from the results concerns the lower numbers of NK cells in control mouse spleens, together with considerable within-column variability in the sham-injected control (thyroxin vehicle) mice vs. the sham-diet-fed control (no E. *purpurea*) mice. These control data should have been theoretically identical, given that all mice were of the same sex, age, strain and housed identically. We have previously shown, however, a considerable negative sham effect (Miller & Osmond, 1975; Dussault & Miller, 1993; 1994), brought on by the daily handling (stressing) of mice for injection purposes (drug or vehicle). Such handling results in erratic reductions in spleen/bone marrow immune cells, relative to completely unmanipulated mice. The latter are best represented, in the present study, by those mice that were simply fed the experimental agent (E. *purpurea*), with no handling involved. These data

reinforce the need for using sham controls, vs. normal animals as controls, for all procedures involving rodent handling.

With respect to NK cells, the present analysis now formally provides both quantitative and functional evidence leading to the significant conclusion that E. *purpurea*, as used in the present protocol at least, appears to be the singular agent, identified to date, capable of stimulating *de novo* NK cell production/numbers in aging animals, significantly increasing, thus, the *numbers* of NK cells in the organs of NK cell birth (bone marrow) and primary function (spleen), to the levels present in young adulthood (Keissling et al., 1975; Koo et al., 1982; Lotzova et al., 1986; Christopher et al., 1991). Trafficking of NK cells is unidirectional, via the blood, from their bone marrow birth site to the peripheral organs, the spleen being, virtually exclusively, their ultimate destiny and site of function (Keissling et al., 1975; Haller & Wigzell, 1977; Miller, 1982; Pollack & Rosse, 1987; Miller & Shatz, 1991). Unlike T and B lymphocytes, mediators of specific immunity, NK cells do not recirculate, possess no immunological memory nor clone in response to antigen (Haller & Wigzell, 1977; Seaman et al., 1978; Zoller et al., 1982). NK cells renew exponentially, approximately every 24 hr (Miller, 1982). The spleen is the benefactor of this phytocompound-driven, increased NK cell production in the bone marrow. Our previous attempts aimed at stimulating NK cells by means of cytokines and drugs, in aging animals, have proven ineffective (Dussault & Miller, 1994; 1995) in altering NK cell numbers, production or function, in either the bone marrow or the spleen. Such lack of exogenous influence suggests that there has been no major, inter-organ trafficking disruption involving other organs, which may have resulted from such

cytokines/drugs, or in the present case, from the presence of thyroxin or E. *purpurea*. In elderly humans, similarly, exogenous administration of various cytokines results in little or no stimulatory influence on a variety of immune parameters (Kawakami & Bloom, 1988; Lerner et al., 1989; Kutza & Murasko, 1994).

Given the commercial availability of E. *purpurea*, on an over-the-counter basis in most Western countries, it would be feasible to assess, *in vivo*, whether or not aging humans as well, possess the same E. *purpurea*-mediated, NK cell-specific, immunoenhancing mechanisms.

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SUMMARY & CONCLUSIONS

In summary, I have demonstrated the effects of administration of several different compounds, including poly I:C, TNF-a, MLT, and E. *purpurea*, to both healthy and leukemic, young adult mice, as well as to healthy elderly mice, in an attempt to boost NK cell numbers. It is advantageous that any immunostimulating agent intended for use as a tumor therapy, be free from complications and side effects that reduce the quantity and quality of life of the treated host. Many cytokine and hormone therapies are beset with harmful side effects, and ideally should be discouraged in favour of less toxic, but equally effective, alternative therapies. For this reason, the most impressive results within this project derive from administration of a commercially available extract of the plant Echinacea *purpurea*, demonstrating an ability to enhance NK cell numbers and life span in leukemic, young adult mice, to those levels provided by the aforementioned hormones and cytokines. These results, coupled with studies in which E. *purpurea* has been shown to be non-toxic in a wide range of doses, leads to the implication that it is valuable as a powerful and effective tumor therapy agent. Further, results in this study demonstrate that administration of E. *purpurea* to elderly mice, increased NK cell numbers and activity, to levels comparably found in healthy, young adult mice. These results, coupled with the fact that elderly mice experience increased frequencies of infections and leukemias, suggest possible prophylactic and curative roles for E. *purpurea* in these mice.

In conclusion, the entire project has shown the NK enhancing effects and life span prolongation in leukemic mice, of cytokines, hormones and herbal extracts. The major contribution of this thesis has been to demonstrate that non-toxic, readily available, and relatively inexpensive phytocompounds, herbal products, could perhaps be as effective in leukemia amelioration than are conventional chemotherapeutic methods – the latter being toxic, expensive and usually devastating to quality of life.

ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

In Chapter 1 I expanded on existing scientific literature which showed that the powerful IFN inducer poly I:C is an exquisite NK cell enhancer *in vivo*. The protocol involved using this agent therapeutically in leukemic mice, with irradiation and bone marrow transplants, to examine the effects of poly I:C on (i) NK cells and (ii) survival potential in these leukemic mice. My results showed that NK cell numbers were augmented in mice given poly I:C before and after BMT, and their life span was increased over control mice not receiving poly I:C.

Chapter 2 extended the protocol from Chapter 1 by adding to the poly I:C therapy, TNF-a, also a known powerful NK stimulant. This combination therapy in young adult, leukemic mice, in addition to the irradiation and BMT protocol, provided an added advantage over those mice receiving only poly I:C. The results indicate that in this case these 2 stimulants are indeed better than one, in both augmenting NK cells, to levels greater than those achieved by poly I:C alone, and in even further extending the life span of these double-treated leukemic hosts.

In Chapter 3 I investigated the role of the neurohormone melatonin in augmenting NK cell numbers. Some *in vitro* studies implicate melatonin as a NK enhancer. Yet, very little *in vivo* evidence exists for its effect on NK cells. Evidence is steadily growing for the relationships between the neuroendocrine system and the immune system. My work in Chapter 3 showed the absolute (quantitative) effects of melatonin, on immune and hemopoietic cell lineages of young adult mice, for one or two weeks of oral administration of the agent. At both time intervals, NK cell numbers, as well as another cell type mediating natural resistance, cells of the monocyte/macrophage lineage, were significantly increased in the spleen and the bone marrow over controls not receiving MLT in their diet.

MLT is a hormone and beset with limitations, as are the cytokines IFN (induced by poly I:C – Chapter 1) and TNF-a (Chapter 2), and inherently its administration to mice could be subject to complications. In Chapter 4 I then investigated the plant product Echinacea *purpurea*, for which no evidence exists of its effects *in vivo* on those cells mediating natural resistance, NK cells. Further, this herb has been shown to be completely non-toxic in a wide range of doses. The results showed that E. purpurea is in fact a powerful NK cell enhancer *in vivo* in normal healthy mice, giving rise to the possibility of prophylactic potential for this herb.

In Chapter 5 I utilized the results from Chapters 3 and 4 in which I found that both MLT and E.p. are potent enhancers of NK cells. I investigated their effects when administered <u>together</u>, on young adult mice, the hypothesis being that both co-administered may be more potent in enhancing NK cells than either

one alone. The results showed that co-adminstration of the two immunostimulants did not result in an enhancement of NK cells, but quite the opposite. NK cells in those mice administered both MLT and E.p. had significantly *decreased* NK cells compared to those mice receiving MLT alone.

One of the known components of E. *purpurea* is the complex carbohydrate, arabinogalactan. In Chapter 6 I investigated the possibility that arabinogalactan may be an enhancer of NK cells through its already known action on NK "support" cells, i.e., those of the monocyte/macrophage lineage. My results demonstrated for the first time, that NK cells are significantly *decreased* after one or two weeks of arabinogalactan administration *in vivo*.

No *in vivo* evidence exists for the effects of MLT and/or E. *purpurea* administration in leukemic hosts. The results from Chapter 7 indicate that leukemic mice administered E.p., had significantly augmented NK cell numbers and increased life span compared to untreated leukemic (control), or normal mice. Leukemic mice administered both MLT and E.p. had no significant change in NK cell numbers, but showed the greatest increase in life span. This study provided the first controlled analysis of the effects of MLT and/or E.p. in leukemic mice.

Chapter 8 examined the effect of tumor-immunization, a process for which clinical evidence is steadily growing. Yet, no conclusive *in vivo* study in laboratory mice exists, employing combination therapy with an established enhancer of those cells mediating natural resistance, namely NK cells. This study provided the first systematic, laboratory-controlled analysis of the effect of tumor-immunization, coupled with the administration of what I have now

demonstrated to be a potent NK enhancer, E. *purpurea*. The results indicate that, in fact, immunization coupled with administration of E. *purpurea*, is more advantageous then immunization alone. Leukemic mice given the combination therapy had significantly greater NK cells and life span than mice given the tumor-immunization alone

In Chapter 9 I extended the results from observations with E.p. in normal and leukemic young adult mice, and examined their effects in *aged* mice. I wanted to determine the effects of E.p. administration to elderly mice, on NK cell numbers and function. The *in vivo* effects of E. *purpurea* on aged mice have never been investigated, and the results indicated that E. *purpurea* had the capacity to increase NK cell numbers and function after two weeks of administration. This is highly significant since neither the growth hormone thyroxin nor the cytokine interleukin-2 nor the drug indomethacin have ever had any significant positive influence in aged mice.

In summary, the entire study has led for the first time to the formal elucidation of herbs as having prophylactic and therapeutic values.

SUGGESTIONS FOR FURTHER WORK

There are two areas of knowledge that would need to be expanded, relating to work in this thesis. Firstly, the further investigation of the dissected components of Echinacea *purpurea,* to identify additional immunostimulating chemicals, is necessary. The scientific literature lacks complete information on the *in vivo* effects of the compounds dissected from E.p., on NK cells. One such immunostimulatory compound could be a selected member of the alkamide family of chemicals found within E.p. Members of the alkamide family have been shown to inhibit the production of the enzymes necessary for prostaglandin formation *in vitro.* Prostaglandin is an NK cell inhibitor. Therefore, *in vivo* administration of alkamides may remove NK cells from endogenous inhibitors and thus enhance natural resistance.

Secondly, the further investigation of the effects of E. *purpurea* (and its individual components) in aged, and infant, leukemic mice is necessary. Leukemia occurs more frequently in infant and elderly mice due to a decreased production or function of cells mediating natural resistance. My work in Chapter 9 indicates that this function can be restored in aged mice with the administration of E. *purpurea*, and therefore may be effective in combating leukemia in mice of these age extremes and increase life span.