Biogerontology (2005) 6:157–163
DOI 10.1007/s10522-005-7951-8

Research article

Enhancement of natural killer cells and increased survival of aging mice fed daily *Echinacea* root extract from youth

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Received 11 November 2004; accepted in revised form 14 February 2005

Key words: aged, *Echinacea*, hemopoiesis, immunity, life span

Abstract

In spite of *Echinacea*-based products being among the best-selling herbs in the world to date, to allay assorted ailments, the debate is still on-going with respect to the efficacy of ingesting the herb intermittently, continuously, or only at the beginning of an affliction. We sought, therefore, to find out if mice, receiving dietary *Echinacea* daily, throughout life, from youth until late middle-age, demonstrated any longevity/survival differences, and/or any differences in their various populations of immune/hemopoietic cells. Sustained and/or high levels of these cells are crucial for longevity. Some mice were maintained on a regular chow diet to which was added *Echinacea purpurea* daily (2 mg/mouse), from puberty (7 week) until just beyond 13 months of age (late middle-age in mice). Control mice, identically housed and maintained, received identical chow without the herb. Mice consuming untreated diet had a 79% survival by 10 months of age, while those consuming *Echinacea* daily in the diet were still 100% alive by 10 months. At approximately 13 months of age, mice consuming untreated diet had a 46% survival rate while those consuming *Echinacea*, were 74% alive at this time. Moreover, the key immune cells, acting as the first line of defense against developing neoplasms in mice and humans, i.e., natural killer (NK) cells, were significantly elevated in absolute number both in their bone marrow production site, as well as in the major organ to which they traffic and function, i.e., the spleen. The cells of the myeloid/granulocyte lineages remained steadfastly at control levels in both the bone marrow and spleen in *Echinacea*-consuming mice. Thus, it appears that regular intake of *Echinacea* may indeed be beneficial/prophylactic, if only for the reason that it maintains in an elevated state, NK cells, prime elements in immunosurveillance against spontaneous-developing tumors, a phenomenon which increases in frequency with progressive aging.

Introduction

That many phytochemicals, i.e., plant/herb derivatives, have the capacity to alleviate a wide variety of pathological conditions accounts for the explosive growth in the nutraceutical industry within the last decade. We have already studied one such product, i.e., root extract of the plant *Echinacea purpurea* (Sun et al. 1999) and found that daily dietary administration, throughout 14 days, to young adult mice, had the capacity to stimulate a specific kind of immune cell, the natural killer (NK) cell, which is responsible for combating virus-mediated afflictions as well as certain tumors.

We elected subsequently, to assess the role of *E. purpurea* in mice during late middle-age, a period in life in both animals and humans, where NK cells are moderately to profoundly reduced. Correspondingly, and more than co-incidentally,

⋆ This project was supported by a grant from the Cancer Research Society, Inc.

it is also a time when there is a great increase in the frequency of developing tumors of assorted types. We had previously shown that the mechanism for such age-related reduction in NK cells was (i) a reduction in production of new NK cells in the bone marrow, (ii) a defect in the ability of NK cells to bind tumor cells, an event which must precede NK cell-mediate lysis of the cell (Dussault and Miller 1994). Moreover, these agents had no effect on leukemia abatement in elderly mice, since sustained administration of either/both resulted in no change in the absolute numbers of leukemia cells counted in either the bone marrow or the spleen (Dussault and Miller 1996). We had also demonstrated that neither the cytokine, IL−2, nor the drug, indomethacin which are both powerful stimulants of NK cell production/function in young adult mice, were completely unable to stimulate these parameters in elderly mice (Dussault and Miller 1994). By contrast, we subsequently demonstrated, that by administering to aged mice (15–16 months), daily dietary *E. purpurea* for 14 days, we were able to rejuvenate their NK cell populations such that at the conclusion of 14 days, NK cell absolute numbers, NK cell lytic functions, and NK cell production, thereby generating more new NK cells, in these old mice, had returned to the level of that of the young adult mouse (Currier and Miller 2000).

Based on this collective body of knowledge, we hypothesized that frequent dosing with this immuno-enhancing herb, from youth to late adulthood, may be prophylactic. That is, sustained elevated levels of those specific immune cells, known to be most active against tumors, may be adequate to allay the progressive increase in frequency of spontaneously developing neoplasms known to occur in animals and humans with advancing age. Thus, we administered, via the diet daily, *E. purpurea*, to mice from puberty until late middle-age. Indeed, we found that not only were age-related deaths significantly delayed in time in *Echinacea*-consuming mice, but the numbers of healthy, active *Echinacea*-consuming mice, about to enter old age, was almost twice that of mice consuming the standard diet.

Materials and methods

Mice

BALB/cByJ male mice were purchased at 5 week of age from Jackson Laboratories (Bar Harbor, Maine, USA). Male mice of this strain have a life span of 18–20 months with a maximum life span of approximately 2 years (Myers 1978; Jackson Laboratories, Bar Harbor, ME). Upon arrival in the McGill University Animal Care Facility, all mice were housed three per cage, under specifically pathogen-free conditions in autoclaved micro-isolator cages (Allentown Caging, Inc., New Jersey, USA) and provided autoclaved food and water *ad libidum*. Temperature and humidity were constant throughout as was a fixed 12 h light/dark cycle. All mice were allowed a 10 day adjustment period after arrival in the facility prior to their use in any experimental protocol. From the commencement of this study (age: 7 week), until its conclusion (13 month) all mice under experimental and control protocols were identically treated in terms of their husbandry/living conditions.

The dietary additive – Echinacea purpurea

A commercially available extract of *Echinacea purpurea* root was obtained from Santé Naturelle (AG) Ltée, LaPrairie, QC, Canada. From dose-response analysis previously done in our laboratory, this agent, in the dose used for dietary administration, has been proven non-toxic (no abnormal findings in several clinical parameters), and effective as a stimulant of non-specific immunity mediated primarily by NK cells (Sun et al. 1999; Currier and Miller 2001, 2002). Standard mouse chow (LabChow, Agribrands, Inc.), ± *Echinacea*, was provided fresh daily. From youth until old age, mice regularly consumed approximately 6 g total chow/day with/without 2 mg *Echinacea purpurea*. A dose of this herb, many times greater than this, has nevertheless been demonstrated to be non-toxic (Weiss 1988; Mengs et al. 1991; Ernst 2002). Finally, given the

long term nature of this study, the logical assumption was that any minor variations in food and/or herb consumption per day, among mice in any one group, or between control (untreated chow), and experimental (*Echinacea*-containing chow) groups, would have been canceled, or averaged over the long period of this study (7 week 13 month).

Mouse sampling

When mice were just beyond 13 month of age, they were euthanized by asphyxiation in a CO_2 chamber, and their spleen and both femurs (bone marrow source) were removed, placed in ice-cold MEM (Minimal Essential Medium) pH 7.4 containing 10% millipore-filtered fetal bovine serum (FBS). The organs were prepared for single cell analysis by methods in standard use in our laboratory (Dussault and Miller 1994; Miller and Kearney 1997; Mahoney et al. 1998; Whyte and Miller 1998; Sun et al. 1999). Ultimately, from the washed, single cell suspensions of hemopoietic cells, from both organs, the total numbers of viable, hemopoietic cells per organ per mouse were obtained by means of a hemocytometer.

Immunoperoxidase labelling of natural killer cells

NK cells were identified by means of their ASGM−1 surface marker, by methods in standard use in our laboratory (Miller et al. 1992; Dussault and Miller 1993, 1995). The ASGM−1 surface molecule is present on 100% of mature and maturing NK cells (Kasai et al. 1980; Young et al. 1980; Beck et al. 1982). NK cells are progressively tagged, by a primary antibody when cells are in suspension followed by a secondary antibody, also administered to the NK cells while they are in suspension. Spleen and bone marrow suspensions are then cytospotted such that all cells lie in a monolayer on glass slides, which are then subjected to the final NK cell tag, i.e., a chromogenic agent (diaminobenzidine), thus enabling their ready distinction from other lymphocytes (T and B). The microscope slides, containing now discernable NK cells, are dried, and are finally counterstained with a hematologic tetrachrome stain (Wright–Giemsa). Collectively, the combination of stains reveals the identity, not only of NK cells, but also of all the other lineages of hemopoietic and immune cells including their precursor (proliferating) and mature forms.

Differential analysis of hemopoietic cells

In both organs (spleen and bone marrow) cells were, thus, morphologically identified and recorded as belonging to one of five distinct categories: NK cells, lymphocytes, granulocytic cells (mature and precursors), nucleated erythroid cells, monocytes by means of well-established methods in use in our laboratory (references above). Differential counts of 1000–2000 cells/organ/mouse were performed and the percentages of each cell type were determined. The resulting percentages obtained for each cell type/organ/mouse, were then converted, via the known total organ cellularity (hemocytometer-obtained), to the absolute number of cells/organ/mouse.

Statistical analysis

Student's *t*-test (two-tailed) was used to compare the differences for each organ between the corresponding means of *Echinacea*-consuming and control mice. Values of $P < 0.05$ were considered statistically significant. Significant differences in survival data were ascertained by means of the Kaplan–Meier Survival Analysis Statistics, Mann–Whitney *U*-test, and the VassarStats Program of trend analysis.

Results

Normal mice, consuming daily *Echinacea* throughout life were profoundly affected with respect to many of their immune/hemopoietic cells as well as their life expectancy.

The numbers of bone marrow-based lymphocytes, myeloid/granulocytic cells and monocytes, in *Echinacea*-consuming mice, remained at or near the numbers found in control mice, consuming untreated, regular (R) chow (Figure 1a). However, the absolute numbers of nucleated erythroid cells (precursors of blood-borne red cells responsible for O_2/CO_2 exchange) were significantly increased in the bone marrow of *Echina-*

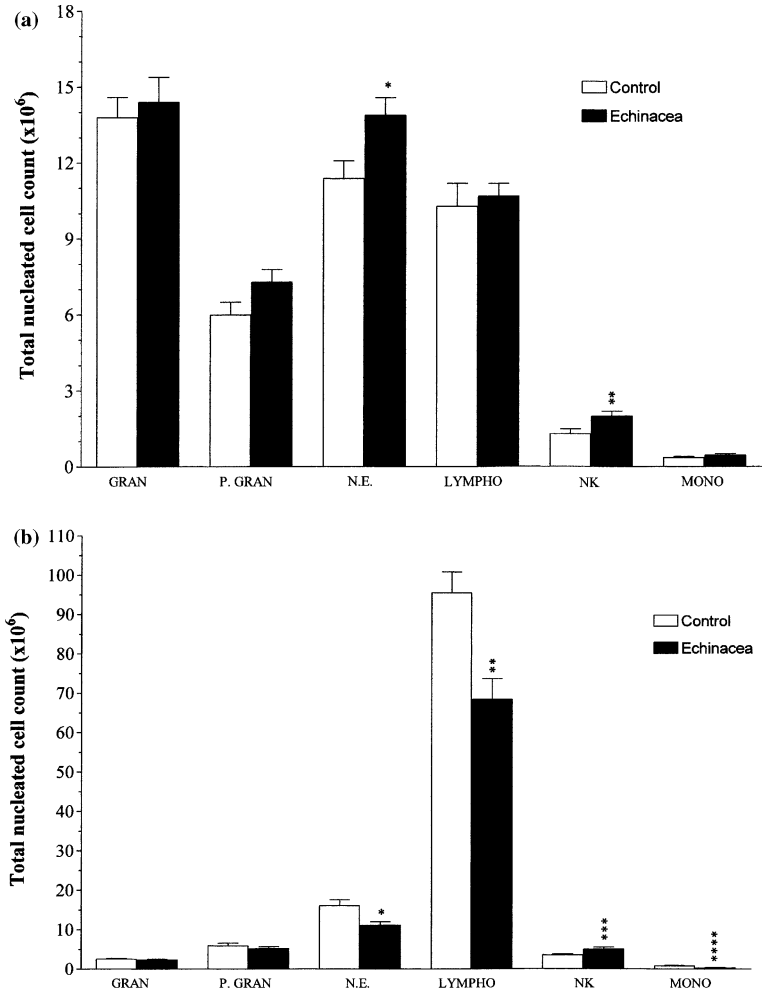

Figure 1. (a) Total numbers of hemopoietic and immune cells in the bone marrow of mice, with and without daily dietary *Echinacea* from youth (7 week) until later middle-age (>13 month). Mean ± SE. Control: $n = 17$; *Echinacea*: $n = 19$. $^{*}P < 0.02$, $^{**}P < 0.01$. (b) Total numbers of hemopoietic and immune cells in the spleens of mice, with and without daily dietary *Echinacea* from youth (7 week) until late middle-age (>13 month). Mean ± SE. Control: $n = 17$; *Echinacea*: $n = 19$. $^{*}P < 0.01$, $^{**}P < 0.007$, $^{***}P < 0.004$, $^{****}P < 0.001$.

cea-consuming mice (Figure 1a). By contrast, lymphocytes, monocytes and nucleated erythroid cells were significantly reduced in the spleens of mice on *Echinacea*-containing diets (E) vs. mice consuming untreated, regular (R) chow (Figure 1b). As in the bone marrow, the absolute numbers of myeloid/granulocytic cells remained unchanged from control levels in the spleen (Figure 1b). NK cells were the only cells to be significantly enhanced in numbers in both their bone marrow production site as well as in the spleen, the site to which mature, NK cells traffic and function (Figure 1a and b). Finally, Figure 2 reveals that mice consuming *Echinacea* from youth until late middle-age (>13 month), showed a delayed onset in age-related deaths, and moreover, the number of mice alive, healthy and about to enter the final segment of their life (old age), was almost twice that of mice consuming regular, untreated chow.

Discussion

This study has shown, for the first time in any species, that daily dietary exposure to *Echinacea*,

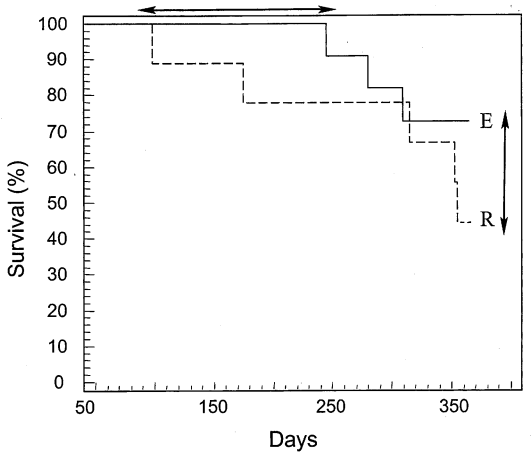

Figure 2. Life span alterations in mice consuming daily dietary *Echinacea* ($n = 30$) vs. mice consuming control (standard, untreated) chow ($n = 30$). In the former group, deaths due to aging/'natural causes' were not observed until 4 months after the first deaths were observed in the control group. At >13 months of age, 74% of all the 'starting mice' were still alive in the *Echinacea*-consuming group vs. 46% in the control group.

throughout a large proportion of the life span (puberty through late middle-age), produces a significant and sustained enhancement of tumor-lytic NK cells. Indeed, it is the unique role of *Echinacea* as an immuno-stimulant, which gives this herb it's considerable medicinal value. One known action of *Echinacea* is to inhibit (via the contained alkylamides), two enzymes fundamental to prostaglandin production, i.e., cyclooxygenase and lipooxygenase, and consequently, the ultimate levels of prostaglandins (Wagner et al. 1989; Muller-Jakic et al. 1994; Raso et al. 2002). Prostaglandins are well demonstrated suppressors/inhibitors of NK cells (Kendall and Targan 1980; de Tracey and Adkinson 1980; Rappaport and Dodge 1982; Lala et al. 1986). Moreover, a decrease in prostaglandins also favors the IL−1-mediated stimulation of monocytes–macrophages (Kendall and Targan 1980; Lala et al. 1986), which then secrete interferons and other direct stimulants of NK cells. *Echinacea, in vivo*, results in an increase in other cytokines which favor NK cell production/lytic function, i.e., TNF-alpha, IFN-beta (Leuttig et al. 1989; Roesler et al. 1991; Barak et al. 2002). A second molecular mechanism by which *Echinacea* exerts an immune stimulatory function, is via its contained family of polysaccharrides known as arabinogalactans. Macrophage uptake of these sugars, also results in release of specific cytokines (IL−1, TNF-alpha, IFN-beta 2) which directly stimulate NK cells.

In aging, normal mice of another strain (DBA/2), we found that only 2 weeks of daily dietary administration of *Echinacea*, to these mice when they were 15–16 months of age, resulted in a rejuvenation of NK cell production, NK cell numbers and an increase in NK cell-mediated, tumor-lytic function – all comparable to levels seen in the young adult mouse (Currier and Miller 2000). Given that NK cells renew rapidly and have a short functional life span (Miller 1982; Zoller et al. 1982; Pollack and Rosse 1987; Miller and Shatz 1991), it is not surprising that only 2 weeks of daily *Echinacea* consumption, would have revved up these cells (Currier and Miller 2000). Moreover, it has been well demonstrated that increase in NK cell numbers parallels the tumor-lytic functional activity in the host (Keissling et al. 1975; Hanna and Burton 1981; Biron et al. 1983; Lala et al. 1985; Christopher et al. 1991; Miller et al. 1992; Brittenden et al. 1996).

The absolute numbers of nucleated erythroid cells in the bone marrow of mice fed the *Echinacea*-containing diet was increased, and represents an enhanced production of these cells. However, the increase in absolute numbers of these cells by the bone marrow was almost precisely canceled out by the downward shift in the number of these cells found in the spleen. Nothing is known conclusively about the mechanism by which *Echinacea* influences erythropoiesis, however some conclusions may be drawn from what is known. For instance, TNF-alpha, interferons, and IL−1 are inhibitory to erythropoiesis (Feelders et al. 1998; Goicoechea et al. 1998; Allen et al. 1999). These are precisely the cytokines which are produced by monocytes–macrophages following exposure to *Echinacea*. By contrast, it has been shown that these cytokines can stimulate the earliest erythropoiesis progenitors (BFU-E), while inhibiting the growth of later progenitors (CFU-E) (Trey and Kushner 1995; Means 1999; Barany 2001). Furthermore, the stromal microenvironment which governs erythropoiesis in the bone marrow and spleen, may also be susceptible to

the presence of enhanced levels or types of circulating cytokines in the presence of dietary *Echinacea*. Irrespective of the dynamics of interplay (negative/positive feedback) between the two organs with regard to erythropoiesis, it may well be that the normal, whole-body, steady state levels of nucleated erythroid cells in *Echinacea*-consuming mice have been maintained, since the arithmetic total of nucleated erythroid cells from the bone marrow and spleen in these mice, is very similar to the arithmetic total of these cells from the bone marrow and spleen of mice consuming untreated chow.

In the present study, wherein no animals had died by 'middle age' (10 month) in the *Echinacea*-consuming group, from 'natural' (age-related) causes (undetected spontaneous tumors, etc.), it appears probable that the sustained high levels of NK cells up to this time at least, were adequate to ward off any early – but clinically undetectable – life-threatening neoplasms, which increase in frequency in all mammals with increasing age. Thus, life-long consumption of this herb appears to have prophylactic advantage in this regard. Nevertheless, survival advantage cannot be indisputably related to tumor paucity in the *Echinacea*-consuming mice, since other disease conditions of aging, often gene-based, also increase with age in both humans and mice.

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