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Immune activation and radioprotection by *Echinacea purpurea* (American herb)

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SUMMARY

The effect of immune activation by *Echinacea purpurea* was investigated by measuring total immunoglobulin (Ig) G, IgM. and the radioprotective effect of immune activation by *Echinacea purpurea* was investigated by measuring T lymphocyte subsets in the peripheral blood of mice following whole body irradiation. *Echinacea purpurea* activated macrophages to stimulate IFN-γ production in association with the secondary activation of T lymphocytes, resulting in a decrease in IgG and IgM production. Cytokines released from macrophages in mouse peripheral blood after *Echinacea purpurea* administration activated helper T cells to proliferate. In addition, activated macrophages in association with the secondary T lymphocyte activation increased IFN-γ production and stimulated proliferation of cytotoxic T cells and suppressor T cells, indicating the activation of cell-mediated immune responses.

Key words: *Echinacea purpure*; Immunology; Radiation protection; T lymphocyte

INTRODUCTION

Echinacea has been used for many years by North American indigenous people as a panacea for a variety of diseases including infections, trauma, inflammation, and fever (Moerman, 1979). Echinacea is a perennial plant of the purple barren chrysanthemum family, which is indigenous to limited areas of the western and central dessert areas of North America and is currently grown worldwide as an ornamental plant, and for use as a food and medicinal agent. The U.S. Food and Drug Administration (FDA) recognized it as a food, while the Commission E (Task Force E of the Federal Bureau of Health of Germany) approved it as medicine. In North America, where it originated, it is very popular as a medicinal agent that can activate immune system.

To date, the effect of Echinacea has been investigated in a variety of fields since its introduction in the early 1970s by Mayer, a German physician. Antiviral properties (Beuscher *et al.*, 1995; Kwarek *et al.*, 1996), antibacterial properties (Brown, 1986), and immune activation (Bauer *et al.*, 1988; Bukovsky *et al.*, 1993; Beuscher

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et al., 1995) have since been reported. Echinacea's active constituents include polysaccharides (Agner *et al.*, 1985), caffeic acid derivatives, flavonoids, polyacetylene, and glycoproteins (Jacobson, 1967; Becker *et al.*, 1982; Bauer *et al.*, 1989). There are nine kinds of Echinacea, among which *Echinacea purpurea* (*Echinacea purpurea*), *E. pallida*, and *E. angustifolia* have been utilized for their medicinal effects (Bauer *et al.*, 2002).

Radiation is used in a variety of fields in research, medicine and industry, and although it can have many useful and beneficial effects, it can also be potentially harmful to the human body. As result, various methods for minimizing the toxic effects of radiation have been actively investigated. Previous studies developed radioprotective agents to protect bodies from indirect effect of radiation by eliminating free radicals produced in response to radiation (Georgieva *et al.*, 2002; Andreassen *et al.*, 2003), and immunostimulants to inhibit immune suppression (Jorgensen *et al.*, 2003; Yang *et al.*, 2003). However, the toxicity and side effects of such agents have been critical problems. Certain medicinal herbs and their active constituents may be useful as radioprotective agents, and may be less toxic than their non-herbal counterparts.

We focused on hematocytes as a marker to evaluate the protective effect of Echinacea against irradiation and examined blood cell counts over time and antioxidant activity in the peripheral blood. We found that Echinacea administration inhibited the reduction of white blood cell counts, especially lymphocytes and monocytes after exposure to radiation. Therefore, in this study, we investigated the radioprotective effect of Echinacea purpurea by measuring total immunoglobulin (Ig) G, IgM, and T lymphocyte subsets in the peripheral blood.

MATERIALS AND METHODS

Echinacea purpurea

Whole parts of *Echinacea purpurea* were harvested in the blooming season (July) and squeezed to extract juice, which was dried and lyophilized for experiments. Dried *Echinacea purpurea* was resuspended in physiologic saline, and administered to mice intraperitoneally at 360 mg/kg every other day. *Echinacea purpurea* was given at least three weeks before experiments and was administered until the end of the experiments.

Experimental animals

The experimental animals for the studies on IgG and IgM in peripheral blood were six-week old male C3H/HeNCrj mice (average body weight, 18-24 g) purchased from Charles River, Japan. For the studies on T lymphocytes, three-week old male C57BL/6crSlc mice (body weight, 8-13 g on average) purchased from Japan SLC Inc. were used. The mice were housed under conventional conditions (room temperature, 22 ± 3 ℃; humidity, 60 %) with commonly available chow (CA-1, CLEA Japan Inc.) and tap water. For adaptation to the new environment, mice were acclimated for one week before beginning the experiments.

Radiation

Mice were systemically irradiated (2 Gy) using an x-ray generator designed for animal use (Phillips, Inc.). A plastic jig used to restrain and rotate the mice at a constant speed so that they could be irradiated evenly. The irradiation conditions were 200 kV, 0.35 Gy/min with a filter for 0.1 mmCu + 1 mm Al.

Blood collection

Whole blood was collected from the mouse

heart by puncturing with a 23-G needle under anesthesia, mixed with heparin, and either centrifuged (15 min at 1500 rpm) to separate serum for the immunoglobulin studies, or suspended 1:1 in PBS and then processed as described below for the T lymphocyte studies.

Measurement of total IgG and IgM in mouse peripheral blood

Mice were divided into two groups: the control group, to which physiologic saline was given, and the IgG and IgM groups, to which *Echinacea purpurea* was given. Each group comprised 10 mice.

Total serum IgG and IgM were measured by enzyme-linked immunosorbent assay (ELISA) with a mouse IgG ELISA quantification kit and a mouse IgM ELISA quantification kit (Bethyl Laboratories Inc., Montgomery, TX).

In a 96-well microplate, 100 μl of the solid-phase antibody (affinity purified goat anti-mouse IgG-Fc or IgM antibody) diluted 100-fold with coating buffer (0.05 M sodium carbonate, pH 9.6) was dispensed into each well and incubated at room temperature for 60 min. After incubation, the coating buffer was discarded and the wells were rinsed two times with washing buffer (50 mM Tris, 0.14 M NaCl, Tween 20; pH 8.0). Then, 200 μl of postcoat solution (50 mM Tris, 0.14 M NaCl, 1 % BSA; pH 8.0) was poured into each well to solidify the solid-phase antibody. After incubation at room temperature for 30 min, the postcoat solution was discarded and the wells were rinsed twice with washing buffer. Serum (100 μl) was diluted 50-fold with the sample diluting solution (50 mM Tris, 0.14 M NaCl, 1 % BSA, 0.05 % Tween 20; pH 8.0) or known concentrations of standard serum (mouse IgG or IgM reference serum) were dispensed into the wells and incubated at room temperature for 60 min. After incubation, the serum was discarded and the wells were washed four times with washing buffer. In each well, 100 μl of enzyme-labeled antibody (HRP-conjugated goat anti-mouse IgG-Fc or HRP-conjugated goat anti-mouse IgM antibodies), diluted at 1:120,000 with conjugate diluting solution (same composition as sample diluting solution), was dispensed for enzymatic reaction, and after incubation for 60 min, the enzyme solution was decanted and the wells were washed four times.

After rinsing, 100 μl of enzyme substrate solution (TMB; 3, 3, 5, 5-tetramethyl benzidine) was dispensed and incubated for 15 min to develop color. After 100 μl of stop solution (2 M H_2SO_4) was dispensed into each well, OD was measured on a MPR A4 microplate reader (Toyosohtatsu, Japan) at a reference wavelength of 450 nm. Total serum IgG and IgM concentrations were measured after a standard curve with the ODs of standard sera was drawn.

For statistical analysis, because the concentrations of total serum IgG and IgM showed normal distribution, the parametric *t*-test was used to compare the two groups.

Measurement of CD3-, CD4-, and CD8-positive T lymphocytes in peripheral blood

Lymphocytes were separated by the gravity centrifugation method. Lymphocyte separating solution (5 mL; sodium hypaque, Ficoll 400; specific gravity, 1.0875 ± 0.0005 at 25 ℃) was added into a 15 ml sample tube, on to which 5 ml of cell suspension was carefully loaded. After centrifugation at room temperature (15-20 ℃) at 500 g for 20 min, plasma in the supernatant was collected to extract lymphocyte subsets. After addition of PBS (pH 7.2, without Ca^{2+} or Mg^{2+}) supplemented with 10 % inactivated FBS (heat-inactivated at 56 ℃ for 30 min), and red blood cell lysing solution, the

mixture was centrifuged at room temperature at 400 g for 10 min. The supernatant was collected and the cells were resuspended and washed twice in PBS containing FBS. Lymphocytes were resuspended in PBS prior to analysis.

Flow cytometry reagents for lymphocyte subset measurement were added into the lymphocyte suspension in PBS, and the mixture was stained for immunofluorescence for about 30 min at 4 ℃ in a dark room. After the reaction, the solution was rinsed three times with PBS, and CD3, CD4, and CD8 subsets were analyzed by a FACS Caliber flow cytometer (Becton Dickinson).

To analyze T lymphocyte subsets, Multicolor Flow cytometry (FCS) System (Santa Cruz Biotechnology Inc.) was employed and CD3-, CD4-, and CD8-positive T lymphocytes in the peripheral blood were counted by three-color flow cytometry using anti CD3-PE-Cy5.5, anti CD4-FITC, and anti CD8-PE.

RESULTS

Total IgG and IgM in mouse peripheral blood

A standard curve was generated based on the optical density (OD) of mouse IgG standard solutions of known concentrations, and total IgG in mouse serum was measured as shown in Table 1. Compared with the control group, total serum IgG was significantly reduced in the *Echinacea purpurea* group ($p<0.05$), indicating that *Echinacea purpurea* suppressed IgG production.

Likewise, a standard curve based on the ODs of mouse IgM standard sera of known concentrations was used to quantify IgM in mouse serum, as shown in Table 2.

ELISA revealed that the amount of IgM in mouse serum was significantly lower in the *Echinacea purpurea* group as compared with the control group ($p<0.05$), indicating that *Echinacea purpurea* suppressed IgM production.

Table1. Effect of *Echinacea purpurea* on total IgG in mouse serum

Groups	Total IgG (ng/ml) Mean±SE
Control	366±26.2
Echinacea purpurea	*294±36.4

* Significant difference ($p<0.05$) between Control group and *Echinacea purpurea* group by Wilcoxon test.

Table 2. Effect of *Echinacea purpurea* on IgM in mouse serum

Groups	IgM (ng/ml) Mean±SEM
Control	720±62.4
Echinacea purpurea	*600±50.6

* Significant difference ($p<0.05$) between Control group and Echinacea purpurea group by Wilcoxon test.

Analysis of T lymphocyte subsets in mouse peripheral blood

On the cytogram, the lymphocyte fraction was gated, so that CD4-positive ($CD3^+CD4^+$) and CD8-positive ($CD3^+CD8^+$) cells were counted.

First, CD4-positive cells were counted by flow cytometry for comparison. As shown in Fig. 1, the number of CD4-positive cells was increased (by 93 %) in only the *E. purpura* group. Likewise, the number CD4-positive cells increased by 40 % in the *Echinacea purpurea* with irradiation group compared with the irradiation alone group. This indicates that *Echinacea purpurea* administration increased CD4-postive cells, i.e., helper T cells in the peripheral blood.

CD8-positive cells were also counted by flow cytometry. As shown in Fig. 2, CD8-positive cells were increased by 201 % in the

Echinacea purpurea group compared with the control group. Compared with the irradiation alone group, CD8-positive cells were decreased by 33 % in the Echinacea purpurea with irradiation group. This indicated that *Echinacea purpurea* administration increased CD8-positve cells, i.e., suppresser T cells and killer T cells in the peripheral blood. However, in the *Echinacea purpurea* with irradiation group, irradiation decreased the number of CD8-positive cells.

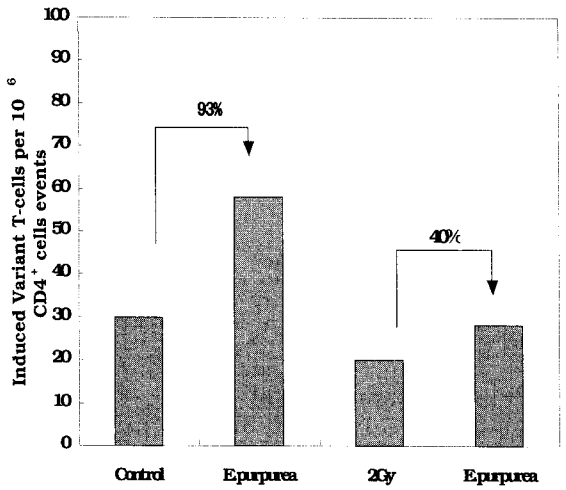

Fig. 1. Comparison of the induced frequency of $CD4^+$in C57BL/6Crslc.

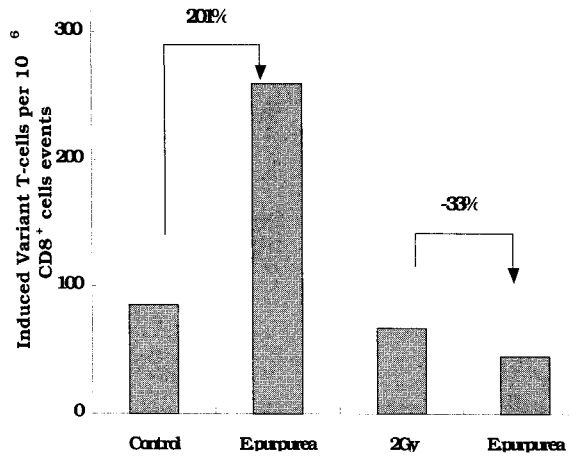

Fig. 2. Comparison of the induced frequency of $CD8^+$in C57BL/6Crslc.

DISCUSSION

Total IgG and IgM in mouse peripheral blood

When irradiation causes death, infection is often the direct cause. Vulnerability to infection in subjects with weakened immune systems is largely due to the impairment of lymphocytes. Since lymphocytes are extremely sensitive to irradiation, irradiation remarkably weakens the immune system. In this study, therefore, we examined the effect of *Echinacea purpurea* on the production of IgG and IgM, which are responsible for the primary and secondary immune responses.

The production of IgG and IgM after *Echinacea purpurea* administration was examined. *Echinacea purpurea* administration decreased the production of IgG and IgM in mouse peripheral blood. Rehman *et al.* reported that Echinacea administration for six weeks increased IgG production in the early to middle term in rats (Rehman *et al.*, 1999). However, in contrast, *Echinacea purpurea* decreased IgG production in the present study. Echinacea has interferon (IFN)-like activity to induce and activate macrophages and T lymphocytes, which may explain the difference between the two results (Rininger *et al.*, 2000). Activated macrophages secrete cytokines (IL-1, IL-6, IL-12, and TNF-α), and activate T cells. Furthermore, activated T cells secrete IFN-γ which inhibits differentiation to antibody-producing B cells. Hayashi *et al.* reported that Echinacea increased IFN-γ (Hayashi *et al.*, 2001). Therefore, the cell-mediated immune response is activated, while the humoral immune response is in turn suppressed (Abbas *et al.*, 1996; Mosmann and Sad, 1996). As observed in this study, IFN-like activity of *Echinacea purpurea* stimulated the cell-mediated immune response of macrophages and T cells, while it suppressed the humoral immune response and reduced antibody production.

Analysis of T lymphocyte subsets in mouse peripheral blood

Echinacea purpurea administration significantly decreased total IgG and IgM in mouse peripheral blood. *Echinacea purpurea* stimulated the cell-mediated immune response by increasing the levels of IFN-γ, which are associated with activation of macrophages and T cells, while impairing the humoral immune response. To analyze T lymphocyte subsets that play a central role in cell-mediated immune responses in mouse peripheral blood after Echinacea purpurea administration, CD4 and CD8 cell surface antigens were examined by flow cytometry and the effect of *Echinacea purpurea* on cell-mediated immune response was examined.

Cell-specific markers, designated as CD markers, exist on the surface of lymphocytes, and are used to sort out the hallmark or characteristics, and function of protein in the process of differentiation of lymphocytes produced in the bone marrow to a_variety of cells. CD3 is exclusively found in T cells. CD4-positive ($CD3^+$ $CD4^+$) cells are helper T cells, while CD8-positive ($CD3^+CD8^+$) cells are cytotoxic T cells and suppresser T cells. T lymphocyte subsets can be examined by measuring these CD markers.

In this study, CD4-positive T lymphocytes were counted in mouse peripheral blood in each group. It was observed that *Echinacea purpurea* increased helper T cells in mouse peripheral blood. It seemed that *Echinacea purpurea* activated macrophages (Mosmann and Sad, 1996; Rininger *et al.*, 2000; Hayashi *et al.*, 2001) and subsequently stimulated T cells. Rininger *et al.* (2000) and Burger *et al.* (1997) reported that Echinacea administration activated macrophages to increase the production of cytokines (IL-1, IL-8, IL-12, and TNF-α). IL-1 and IL-12 can promote the proliferation of helper T cells. This also suggests that cytokines (IL-1, IL-6, IL-12, and TNF-α) released from macrophages by *Echinacea purpurea* administration activated helper T cells to proliferate.

CD-8 positive cells in mouse peripheral blood were examined in each group of mice. It was observed that *Echinacea purpurea* increased the number of cytotoxic T cells and suppressor T cells. The mechanism likely involved an increase in INF-levels following treatment with *Echinacea purpurea*. The increase in IFN-γ levels, in addition to the suppression of antibody production mentioned previously, activates cell-mediated immune responses, such as proliferation and activation of type I helper T (Th1) cells. Given that cytotoxic T cells and suppressor T cells were activated to proliferate in this study, *Echinacea purpurea* administration stimulated the proliferation of Th1 cells via the increase of IFN-γ and the activation of the cell-mediated immune response of cytotoxic T cells. Evidently, the reduction of antibody production observed in previous studies was caused by the stimulation of the cell-mediated immune response due to an increase in IFN-γ and the suppression of the humoral immune response. Since suppressor T cells inhibit antibody production, the increase in suppressor T cells may decrease antibody production. Unfortunately, it was not confirmed whether cytotoxic T cells or suppressor T cells were increased. Therefore, it is necessary to further clarify the T lymphocyte subsets involved and the cytokine production profiles more thoroughly in order to elucidate the effect of *Echinacea purpurea* on the immune system. CD8-positive cells decreased in the *Echinacea purpurea* with irradiation group more than in the irradiation alone group. It may be possible to render this study more reliable by repeated experiments.

CONCLUSION

Echinacea purpurea has been used since

ancient times by native Americans as a medicinal herb, and recently its therapeutic efficacy has been evaluated in a number scientific studies. In this study, we focused on immune stimulation by *Echinacea purpurea*, and examined changes in the effect of irradiation after *Echinacea purpurea* administration, as well as the radioprotective effect of Echinacea purpurea, by observing its effect on the immune system.

We recently performed experiments to examine the radioprotective effect of *Echinacea purpurea* in hematocytes (see study 2 in_this issue). The free radical scavenging effect of antioxidants contained in *Echinacea purpurea*, such as echinacosides and caffeic acid, inhibited the reduction of white blood cell counts (lymphocytes, and monocytes) after irradiation. It was confirmed that the antioxidants contained in *Echinacea purpurea* increased the antioxidant activity of mouse peripheral blood.

In this study, the effect of *Echinacea purpurea* on the immune system was examined. *Echinacea purpurea* decreased IgG and IgM production by increasing the production of IFN-γ production associated with secondary T lymphocyte stimulation by macrophages.

With respect to T lymphocyte subsets in mouse peripheral blood, *Echinacea purpurea* administration stimulated macrophages to release cytokines, which in turn stimulated helper T cells to proliferate. A stimulated cell-mediated immune response was indicated because the proliferation of cytotoxic T cells and suppressor T cells was stimulated by the increase in IFN-γ associated with the secondary T lymphocyte activation by macrophages.

Taken together, our results showed that *Echinacea purpurea* eliminated free radicals produced by irradiation with its radical scavenging effect, reduced cytotoxicity due to oxidization, such as that caused by lipid peroxides, suppressed the decrease of white blood cell counts after irradiation, and prevented the impairment of immunity. It also activated macrophages and T cells and stimulated cytokine production (e.g., IFN-γ), heightened cell-mediated immune responses, prevented the weakening of the immune response associated with irradiation, and presumably bolstered the ability to protect against infection.

In summary, *Echinacea purpurea* evidently is an effective radioprotective agent with low cytotoxicity and side effects.

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