Danthron Inhibits Murine WEHI-3 Cells *In Vivo*, and Enhances Macrophage Phagocytosis and Natural Killer Cell Cytotoxic Activity in Leukemic Mice

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Abstract. *Danthron has been shown to induce apoptotic cell death, and inhibit migration and invasion of human gastric or brain cancer cells in vitro. However, there is no report addressing whether danthron affects murine leukemia cells or immune responses in vivo. Herein, this study focused on the in-vivo effects of danthron on WEHI-3 leukemia in mice and immune responses in vivo. The results indicated that danthron reduced spleen weight and increased the percentage of cells with CD3 and CD19 markers, indicating that differentiation of the precursors of T- and B-cells was promoted in the leukemic mice. The results also showed that danthron promoted the activity of phagocytosis by macrophages isolated from the peritoneal cavity but had no effect on peripheral blood mononuclear cells. Danthron also promoted natural killer cell cytocytic activity at an effector and target cell ratio of 100:1 in comparison with leukemic animals in vivo. Taken together, these results demonstrated that application of danthron might affect WEHI-3 leukemia in mice and modulate immune responses in vivo.*

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Worldwide, leukemia is one of the major causes of death by cancer (1). In the United States, about 3,250 individuals under 20 years old are diagnosed with leukemia each year (2). In Taiwan, approximately 4 persons per 100,000 die per year according to the 2009 report of the Department of Health, Taiwan, R.O.C. (3). Leukemia is the thirteenth most common malignancy in Taiwan (4). However, the best strategies for treatment of human leukemia have not yet been established.

Numerous studies have shown that the increased consumption of a plant-based diet led to a reduced risk and development of cancer such as colon cancer (5, 6), and as shown in an animal study (7). Danthron (1,8-dihydroxyanthraquinone) is an anthraquinone from *Rheum palmatum* L. (Polygonaceae) (8-11), a plant with several biological activities, including induction of apoptosis and DNA damage, and inhibition of DNA-repair gene expression (12-14). However, there is no report regarding the effects of danthron on WEHI-3 cells in animals *in vivo*. It is reported that daily oral administration of danthron induced melanosis

coli in guinea pig large intestines and caused a transient, dose-related wave of apoptosis in colonic surface epithelial cells (15). Danthron treatment may also reduce neurotoxicity related to beta-amyloid protein in primary cortical cultures (16). Recently, our reports have shown that danthron induced apoptosis and DNA damage through the mitochondria-dependent or caspase cascade-mediated signaling pathways in human gastric cancer SNU-1 cells (14) and brain glioblastoma multiforme GBM 8401 cells (12). We also showed that danthron suppressed the migration and invasion of GBM 8401 cells through the inhibition of mRNA expression of focal adhesion kinase, Rho kinase-1 and metalloproteinase-9 (8). However, there are no reports to address the effects of danthron on WEHI-3 cells *in vivo*.

In the present study, we focused on the effect of danthron on WEHI-3 cell leukemia and immune responses in BALB/c mice *in vivo*.

Materials and Methods

Materials and reagents. Danthron, dimethyl sulfoxide (DMSO) and propidium iodide (PI) were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). RPMI-1640 medium, fetal bovine serum (FBS), penicillin-streptomycin and L-glutamine were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA).

WEHI-3 murine leukemia cells. The murine myelomonocytic leukemia WEHI-3 cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine in 75-cm^2 tissue culture flasks and grown at 37°C under a humidified 5% CO_2 atmosphere (17).

Male BALB/c mice. Male BALB/c mice at the age of 8 weeks and at a weight of 22-28 g were purchased from the Laboratory Animal Center, College of Medicine, National Taiwan University (Taipei, Taiwan).

Establishment of leukemic mice and danthron treatment. Fifty BALB/c mice were randomly divided into 5 groups of 10 animals (17). Group I was the control (normal mice). Group II was olive oil treatment only. Group III was intraperitoneally (*i.p.*) injected with WEHI-3 cells (1×10^5 cells/mice) and then treated with 100 μl olive oil. Groups IV and V were injected with WEHI-3 cells (1×10^5 cells/mice) and then treated with danthron (30 and 60 mg/kg, respectively) in olive oil. All animals were orally given the above doses daily for up to 2 weeks before being weighed. Danthron was dissolved in olive oil (Sigma-Aldrich Corp.) for treating mice in Groups IV and V.

Spleen tissues. At the end of the experiment, each mouse from each group was weighed before blood was collected. Spleen samples were obtained and weighed individually (18).

Blood sample collection and immunofluorescence staining. Blood samples (about 1 ml) from each mouse of the different groups were collected after the completion of the experiments. The blood samples were immediately added to 1× Pharm Lyse™ lysing buffer (BD

Biosciences, San Jose, CA, USA) for lysing of the red blood cells and then were centrifuged at 1500 rpm at 4°C for 15 min for isolating white blood cells. The isolated cells were examined for cell markers, including CD3, CD11b, CD19 and Mac-3 based on the staining with antibodies anti-CD3-fluorescein isothiocyanate (FITC), CD11b-FITC, CD19-phycoerythrin (PE) and Mac-3-PE (BD Pharmingen Inc, San Diego, CA USA) and then levels of the cell markers were determined by flow cytometry (Becton Dickinson, FACS Calibur™, Franklin Lakes, NJ, USA) as previously described (18, 19).

Quantification of phagocytic activity of macrophages from each group of animals. Cells were isolated from peripheral blood mononuclear cells (PBMCs) and the peritoneal cavity of each group and incubated with FITC-labeled *Escherichia coli* (20 μl) for 1 h at 37°C, in compliance with the manufacturer's instruction (PHAGOTEST kit, ORPEGEN Pharma Gesellschaft für biotechnologische, Heidelberg, Germany). The reaction was stopped by the addition of quenching solution (100 μl). At the end of phagocytosis, monocytes and macrophages were washed and DNA was stained according to the manufacturer's protocol. Approximately 5×10^4 cells/tube from each treatment were then analyzed by flow cytometry. Fluorescence data were collected on 10,000 cells and analyzed using BD CELLQUEST Pro software (18, 20).

Quantification of cytotoxic activity of natural killer (NK) cells. Approximately 1×10^5 splenocytes in 1 ml medium from each spleen of each mouse were cultured in each well of a 96-well plate. Approximately 2.5×10^7 YAC-1 cells, an optimal target for mouse NK cells, with serum-free RPMI-1640 medium in 15-ml tubes, and PKH-67/Dil.C buffer (Sigma-Aldrich Corp.) were added to the cells in each well, then mixed at 25°C for 2 min before 2 ml (PBS) was added to the mixtures for 1 min. Subsequently, ~ 4 ml RPMI-1640 medium were added and cells incubated for 10 min followed by centrifugation at 1,200 rpm and 25°C. Approximately 2.5×10^6 YAC-1 cells were placed into 96-well plates in 100 μl before the addition of the splenocytes to the well and incubation 6 h and the NK cell cytotoxicity was determined by a PI exclusion method and flow cytometry as previously described (20, 21).

Statistical analysis. The results are expressed as the mean±S.D. (n=10) and the difference between control and experimental groups was analyzed by one-way ANOVA and Student's *t*-test. $P<0.05$ was considered statistically significant.

Results

Danthron affected WEHI-3-induced leukemia in BALB/c mice. Representative images of the animals (Figure 1A) after intraperitoneal injection with WEHI-3 cells for 2 weeks and then treatment with 30 and 60 mg/kg of danthron showed leukemia-induced splenic tumors in Figure 1A and B. The size of spleen from each treatment indicated that danthron reduced the splenic tumor size (Figure 1B) and splenic weights (Figure 1C) when compared with untreated leukemic mice. These effects were a dose-dependent response to treatment.

Danthron affected the whole-blood cell surface markers from BALB/c mice after injection with WEHI-3 cells. The results of cell markers of white blood cells from BALB/c mice after
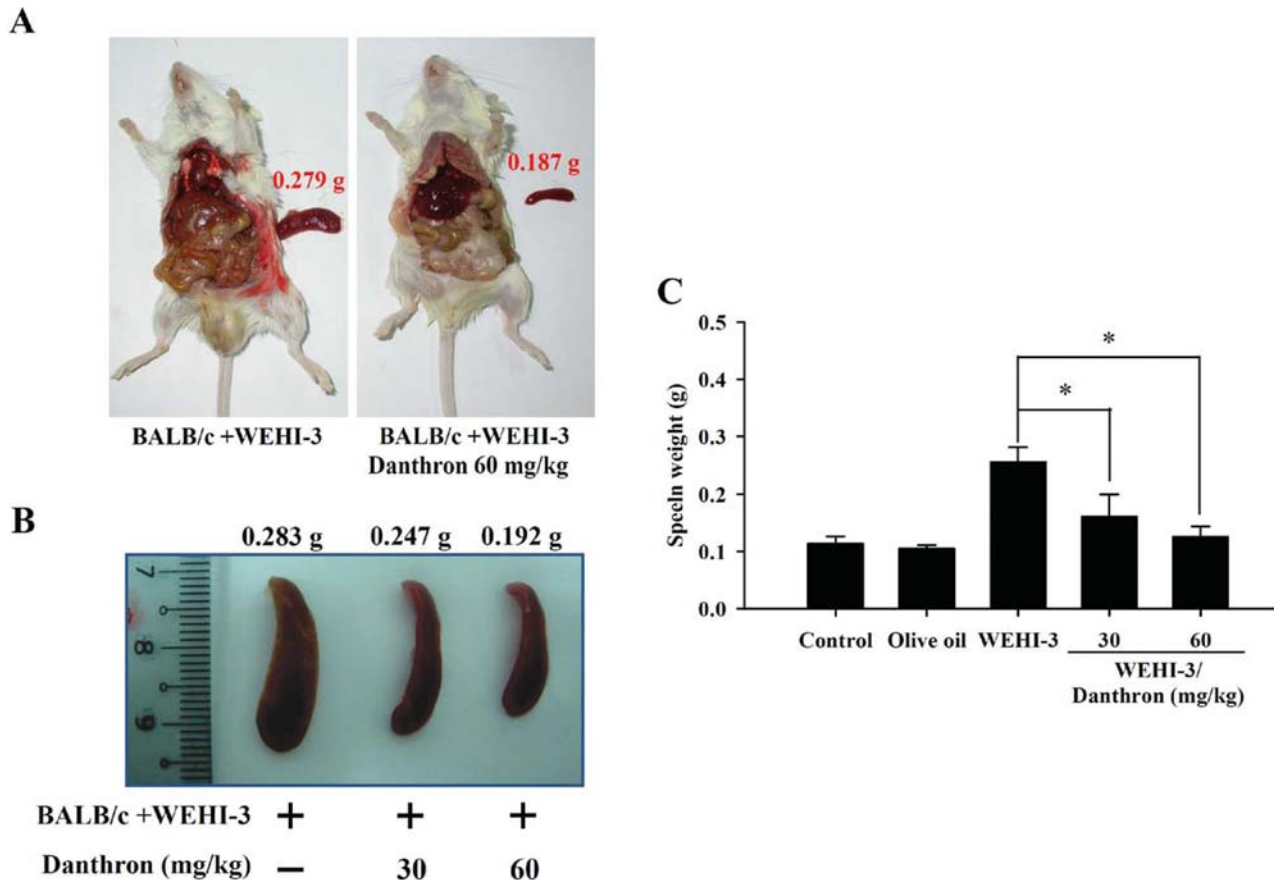
Figure 1. *Representative images of BALB/c mice after injection with WEHI-3 cells (1×10^5 cells/mice) and treatment with danthron for 2 weeks. Animals from each group were intraperitoneally injected with WEHI-3 cells in RPMI-1640 medium for 2 weeks and/or co-treated with 30 and 60 mg/kg of danthron. Animals were sacrificed and then blood was collected. Representative images (A) and weights of spleen (B and C) are shown. Each point is the mean±S.D. (n=10). *p<0.05 significantly different.*

treatment with danthron in olive oil are presented in Figure 2. The data indicate that danthron increased the levels of CD3 (Figure 2A) and CD19 (Figure 2B), but did not significantly affect the levels of CD11b (Figure 2C) and Mac-3 (Figure 2D) in the leukemic mice. It can be seen that danthron induced significant differences in T-cell (CD3) and B-cell (CD19) markers between WEHI-3 only-treated and danthron-treated groups (*p*<0.05).

Danthron affected phagocytosis in leukemic BALB/c mice. The results from flow cytometric analysis indicated that treatment with 30 and 60 mg/kg of danthron did not affect phagocytosis by macrophages from PBMCs (Figure 3A), but did promote that by cells from the peritoneal cavity (Figure 3B) of mice.

Danthron affected the activity of NK cells from leukemic BALB/c mice. The data from flow cytometric analysis showed that the YAC-1 target cells were killed by NK cells

isolated from spleens of the mice after treatment with 60 mg/kg of danthron at a target cell ratio of 100:1 (Figure 4), the high dose (60 mg/kg) of danthron treatment showed significant increase in cytotoxicity compared to the leukemic control and low-dose danthron treatment. However, the low dose of treatment did significantly affect NK cytotoxicity (Figure 4).

Discussion

The reasons for using WEHI-3 cell leukemia model to examine the effects of danthron in BALB/c mice *in vivo* are i) there are no reports to show danthron affecting leukemia cells *in vivo*; ii) murine host systems are of low cost; iii) establishment of cancer is easy, and this model has accepted experimental endpoints (22, 23); iv) it is well accepted that the murine WEHI-3 leukemia cell line can be used to induce leukemia in syngenic BALB/c mice for evaluating anti-leukemia effects of agents (24); v) the murine monomyelocytic WEHI-3 leukemia
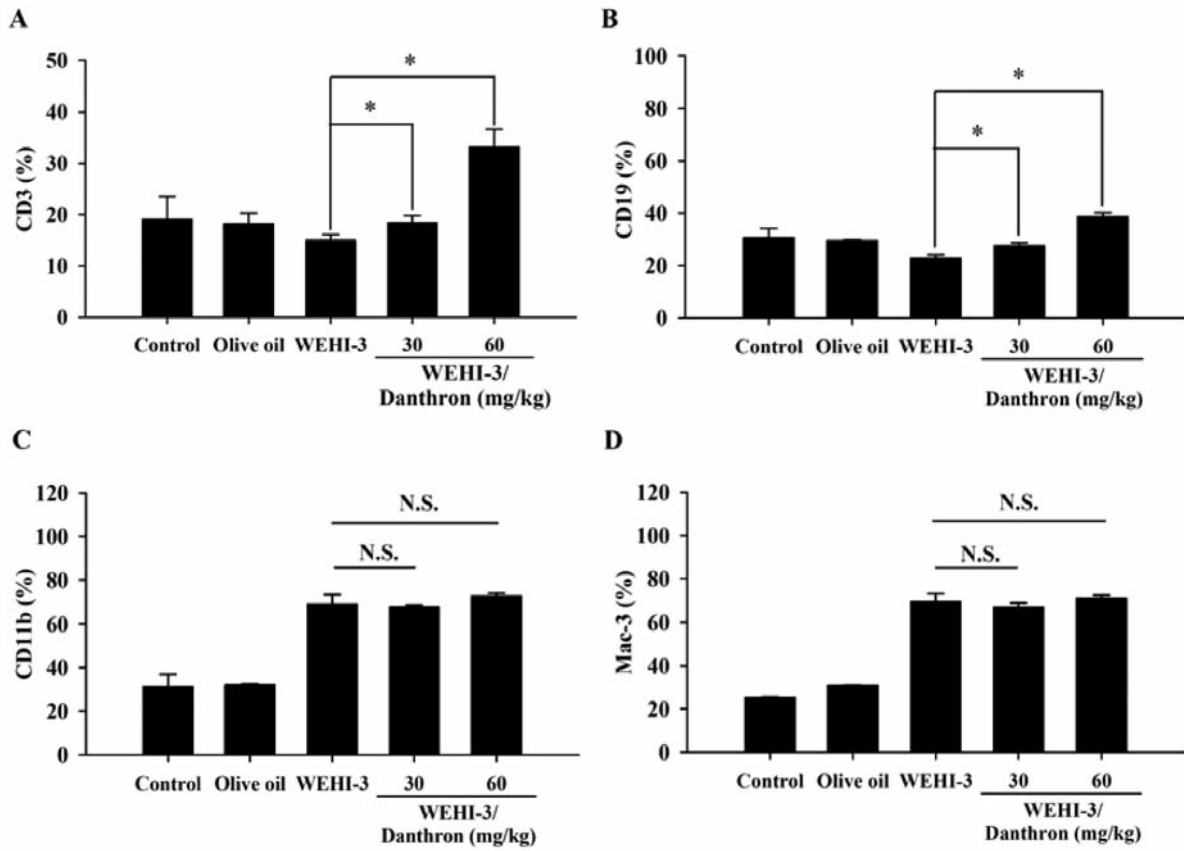
Figure 2. *Danthron affected the cell markers of white blood cells from BALB/c mice. Animals from each group were intraperitoneally injected with WEHI-3 cells (1×10⁵ cells/mice) in RPMI-1640 medium for 2 weeks and treated with or without 30 and 60 mg/kg of danthron for 2 weeks. Blood was collected from individual animals and analyzed for cell markers (A: CD3; B: CD19; C: CD11b and D: Mac-3) by flow cytometry as described in the Materials and Methods. Each point is the mean±S.D. (n=10). *p<0.05 significantly different; N.S., not significant (p>0.05).*
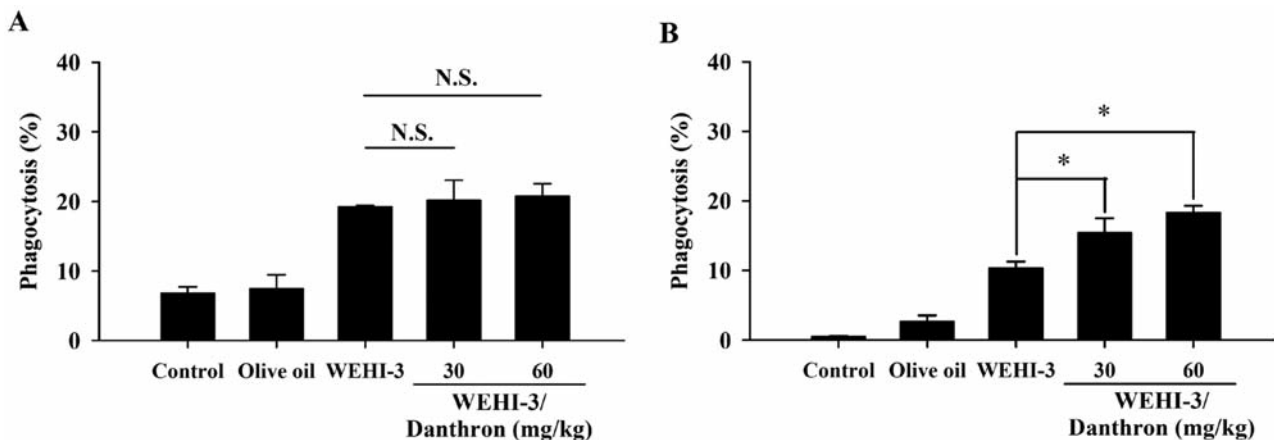

Figure 3. *Danthron affected phagocytosis by macrophages in BALB/c mice. Animals were intraperitoneally injected with WEHI-3 cells (1×10⁵ cells/mice) in RPMI 1640 medium for 2 weeks and treated without or with 30 and 60 mg/kg of danthron for 2 weeks. Cells were collected from PBMCs (A) and the peritoneal cavity (B) and were analyzed for macrophage phagocytosis by flow cytometry as described in the Materials and Methods. Each point is the mean±S.D. (n=10). *p<0.05 significantly different; and N.S., not significant.*
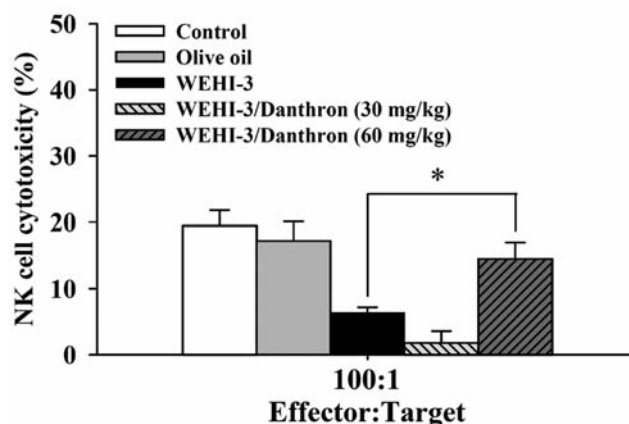
Figure 4. *Danthron affected NK cell cytotoxic activity from BALB/c mice. The mice were intraperitoneally injected with WEHI-3 cells (1×10^5 cells/mice) in PBS for 2 weeks and treated without or with 30 and 60 mg/kg of danthron for 2 weeks. Macrophages were collected from the peritoneal cavity of each animal and analyzed for NK cell cytotoxicity by flow cytometry as described in the Materials and Methods. Each point is the mean±S.D. *p<0.05 (n=10) significantly different.*

cells were originally derived from the BALB/c mouse (25). Moreover, many studies have shown that the WEHI-3 *in-vivo* model is an ideal system for examining the anti leukemia activity of aclacinomycin A, all-*trans* retinoic acid (ATRA), interleukin-6 (IL-6), granulocyte colony-stimulating factor and vitamin D3-induced *in vitro* differentiation of WEHI-3 in monocytic and granulocytic lineages (26-28). Based on our earlier studies, we have shown that danthron induced apoptosis in human stomach cancer SNU-1 cells (14) and brain glioblastoma multiforme (GBM) 8401 cells (12), and it also inhibited the migration and invasion of the latter (8).

In the present study, we investigated the effect of danthron on WEHI-3 leukemia in mice *in vivo*. The results indicated that danthron did not affect the body weight (data not shown), but did increase the splenic weight (Figure 1B and C) when compared with the untreated leukemic mice. However, danthron statistically increased the percentage of CD3 and CD19 (Figure 2A and B)-positive cells, but it did not affect the percentage of CD11b and Mac-3-positive cells (Figure 2C and D) in the blood samples from the leukemic mice. These results also indicated that danthron promoted the proliferation of T- and B-cells from the leukemic BALB/c mice. Based on these results, danthron inhibited splenic tumor growth in this WEHI-3 leukemia BALB/c murine model. It is well documented that agents, whether or not they affect immune responses, should also be examined for effects on phagocytosis by macrophages or monocytes, and NK cell cytotoxic activity (21, 29-30). Our results also show that danthron promoted phagocytosis by macrophages (Figure 3) and NK cell cytotoxic activity (Figure 4) in the WEHI-3 leukemia BALB/c murine model.

In conclusion, danthron inhibited tumor from leukemia and splenic also was associated with the increase of phagocytotic activity from macrophages and NK cell cytotoxicity in leukemic mice *in vivo*.

Acknowledgements

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