A Tertiary Sulfonium Compound, Dimethylsulfoniopropionate in Green Sea Algae, Completely Suppresses Crucial Ehrlich Ascites Carcinoma in Mice

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Abstract. *Background/Aim: Dimethylsulfoniopropionate (DMSP) has been intensively studied in bacteria, plankton, algae and salt-resistant plants to investigate its metabolism and fate in the atmosphere. However, its effects on diseased terrestrial animals have not yet been examined. We have found that DMSP exerts a great healing effect for a variety of inflammatory disorders in rodents. In the present study, effects of single and high concentration of DMSP on terrible Ehrlich ascites carcinoma (EAC) bearing-mice with unavoidable-rapid death were examined. Results: We found that high concentration of DMSP completely suppresses acute EAC, which has never been eradicated, and accumulates large amounts of activated macrophages with no inflammation on various viscera in the peritoneal cavity of normal mice. Conclusion: These results show that DMSP is a new and potent anticancer compound with no side-effects, most likely playing a vital role for cancer immune therapy.*

Cancer has remained a major cause of death and the number of individuals with various cancers has continued to expand over a long period. Since several decades, a number of anti-cancer drugs have been developed (1, 2) but they have been proven clinically ineffective due to serious side-effects to patients at present (1, 3). Furthermore, there has never been reported successful cancer eradication even by many therapies under various strategies for cancer chemoprevention without any side effects.

Dimethylsulfoniopropionate (DMSP) has been found to exert a healing effect for a variety of inflammatory disorders in rodents while many studies on the metabolisms of DMSP and its derivatives have been reported in bacteria, plankton, algae, and halophytic plants in estuarine, coastal and oceanic water (4-6). However, there is no report on the physiological roles of DMSP in diseased terrestrial animals, except for the function as an osmoregulant and a cryoprotectant (5,6). In this study, we report that initial intraperitoneal (*i.p.*) supplementation of a highly purified DMSP solution (70 mM, 0.5 ml) completely suppresses powerful proliferation of crucial Ehrlich ascites carcinoma (EAC) cells without any toxicity, accompanied likely by accumulation of large amounts of activated macrophages in peritoneal cavity of EAC-bearing mice.

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Materials and Methods

Reagents and cells. Dimethylsulfoniopropionate (DMSP) was synthesized by refluxing dimethylsulfide (DMS) and 3-bromopropionic acid, washing with ethyl ether and crystallization in methanol to a purification level of 99.8 % (by element analysis) (7). 3-Bromopropionate was purchased from Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan and other chemicals were purchased from Wako Pure Chemicals Co. Ltd., Osaka, Japan. Three-week-old ICR/Jcl male mice were purchased from CLEA Japan Inc., Osaka, Japan. Unless otherwise stated, the mice had free access to distilled water and solid diets ("MF," CLEA Japan Inc.) and were reared under a dark/light cycle (12/12 h) at around 60% relative humidity and at 24±2˚C, accompanied by preliminary acclimatization for one week. EAC cells were kindly donated by the Cancer Cell Repository, Research Institute for Tuberculosis and Cancer, Tohoku University, Japan. The cells were maintained *in vivo* in mice by weekly *i.p.* transplantation of an appropriate volume (1 ml) of ascites fluid to 4-week-old normal mice.

Preparation of test mouse for determination of body weight. The mice (34.6±0.18 g, n=8) were divided into seven groups (eight mice in each group). Subsequently, 0.5 ml aliquots of saline solution (two groups of mice) or DMSP solutions at 10, 20, 30, 50 and 70 mM concentrations (1.1-7.7 mg/mouse) were *i.p.*-administered to all the seven groups of mice. In contrast, an appropriate volume of ascites

fluid containing EAC cells was withdrawn from EAC-bearing mice, centrifuged and then suspended in Harm's 12F medium (Wako Pure Chemicals, Co. Ltd., Osaka, Japan), followed by further centrifugation (8). EAC cells obtained were suspended in Harm's medium containing 8% fetal bovine serum and a suspension (0.5 ml) of these cells (2×10^6 cells) was *i.p.*-administered immediately into one control (Carcinoma-control) and five groups of mice treated with DMSP solutions as mentioned above.

Determination of body weight and ascitic fluid. The mice were housed for 10 days and body weights were measured in all the groups at the specified times (Figure 1). Ascites fluids in the peritoneal cavity of the test mice were collected carefully into plastic tubes first from a hole in the abdomen by a syringe and then from the cut end of the abdominal cavity with scissors under deep ether anesthesia and the volume of ascites fluid was measured at 10 days (Figure 2).

Isolation of macrophages. To collect highly purified macrophages, 1 ml of 40 mM DMSP solution was *i.p.*- injected into five normal 4-week-old mice (n=5), followed by collection of peritoneal cells into plastic Petri dishes by washing the peritoneal cavity with cold phosphate-buffered saline (hand-made PBS) three days after the injection. The cells were then allowed to adhere for 2 h at 37˚C; non-adhering cells were removed by aspiration and washing. The adherent cells (macrophages) were then washed twice and carefully collected by a rubber scrapper after the cells had been dislodged by chilled PBS.

Incubation of EAC cells and macrophages with DMSP in vitro. To examine effects of DMSP, EAC cells and macrophages on each other, incubation of 30, 50, or 70 mM DMSP solutions (final) with freshly-prepared EAC cells or macrophages (5×10^4 cells/ml each) (Table I), and incubation of EAC cells and macrophages (equal amounts of 5×10^4 cells/ml) (Figure 3) were performed for 5 h in Harm's medium (8 ml) with 8% fetal bovine serum in a humidified atmosphere of 5 % CO_2 in air at 37˚C, respectively. Then, 1 ml aliquots were withdrawn from the Harm's medium at the indicated times. The number of EAC cells and macrophages was counted in a Neubauer haemocytometer using the trypan blue dye exclusion method.

Effects of DMSP and thioglycollic acid (TG) on various viscera in peritoneal cavity. One-ml aliquots of 20 and 40 mM DMSP, and 40 mM TG (3.7 mg/mouse) were *i.p.*-injected into normal 4-week-old mice (n=5) and the accumulated macrophages were collected three days after the injection and counted in the same way, as described above (Figure 4).

In contrast, to determine the efficacy of DMSP and TG on inflammation and cell death in viscera of the peritoneal cavity, 1 ml of DMSP or TG (40 mM each) was *i.p.*-administered to normal 4-week-old mice (n=3). Three days later, the whole body of test mice was sterilized, the abdominal cavity was wide opened carefully and a trypan blue dye solution was sprayed lightly and uniformly on exposed viscera. The blue-dyed viscera were photographed (Figure 5) and their areas were calculated carefully using Image software (Table II).

Calculation of area and depiction of structures. The area which dyed blue were calculated by Image software (1.45 m, Scion Image, Set Scale, Scion Co., Ltd., Bethesda, Maryland, USA.) and two dimensional structures of molecules were depicted using the software of CSC ChemDraw (Cambridge Scientific Computing, Inc., ver. 3.1).

Table I. *Effect of high concentrations of DMSP on proliferation of EAC cells (A) and macrophages (B) grown on Harm's medium containing fetal bovine serum.*

DMSP (mM)	Incubation (h)		
A	1	3	5
	EAC cells ($\times10^5$)		
0	14.8±1.13	18.9±1.31	19.3±2.06
30	15.9±0.94	19.1±1.50	20.4±1.69
50	18.8±1.31[a]	20.3±0.94	17.1±1.50
70	18.0±1.13	16.7±1.69	11.6±1.73[abc]
B			
	Macrophages ($\times10^5$)		
0	5.3±0.37	6.1±0.37	6.3±0.56
30	6.0±0.31	7.5±0.50[a]	7.2±0.62
50	5.9±0.25	6.8±0.43	6.2±0.50
70	5.0±0.37[bc]	5.1±0.50[bc]	3.6±0.68[abc]

Values: mean±SD (n=5). [a, b, c] $p<0.05$ (*vs.* 0, 30 and 50 mM DMSP groups at the indicated times).

Table II. *Blue-dyed area, which were dyed by spraying trypan blue solution onto viscera in peritoneal cavity.*

Compound	n	Blue-dyed areas (cm^2)	Total areas (cm^2)
Control	1	0.739	
	2	0.646	1.519
	3	0.134	
DMSP	1	0.763	
	2	0.416	2.011
	3	0.832	
TG[a,b]	1	1.992	
	2	1.936	5.885
	3	1.957	

[a, b] $p<0.01$ (*vs.* Control (saline) and DMSP groups).

Statistical analyses. Statistical analyses were performed using ANOVA and Tukey–Kramer tests in Figures 1, 2, 3 and Table I and ANOVA and Fisher's Protected Least Significant Difference (PLSD) tests (StatView-SAS Institute, Inc., ver. 5) in Table II.

Treatment of animals. Care and treatment of the experimental animals were performed in accordance with the National Institute of Health Guide (NIH Publication No. 80-23, 1996) and also with the Koshien University Guide for the Care and Use of Laboratory Animals.

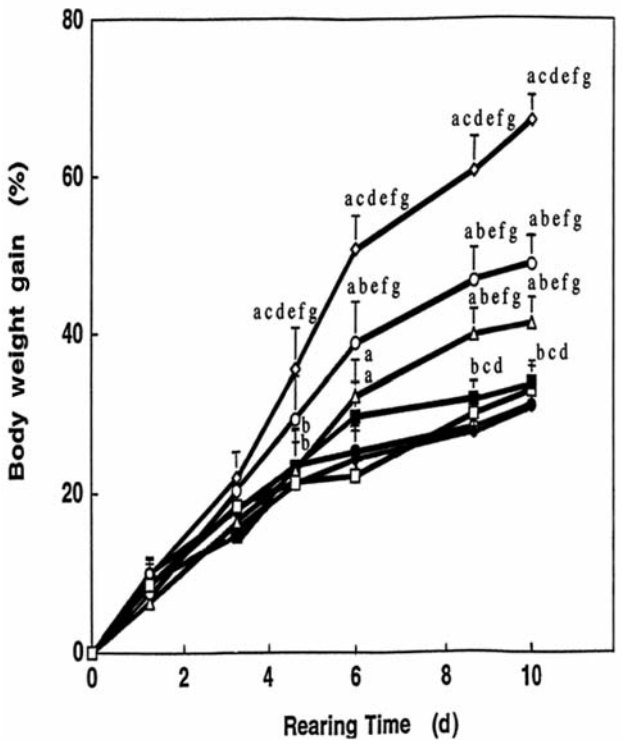

Figure 1. *Effects of various concentrations of DMSP on body weight of EAC-bearing mice. Values: mean±SD.* □, ◇, ○, △, ■, ◆, ●*; control, carcinoma-control, 10, 20, 30, 50 and 70 mM DMSP groups, respectively.* [a, b, c, d ,e, f, g] *p<0.05 (vs. control (saline), carcinoma-control, carcinoma 10, 20, 30, 50 and 70 mM DMSP groups at the indicated times).*

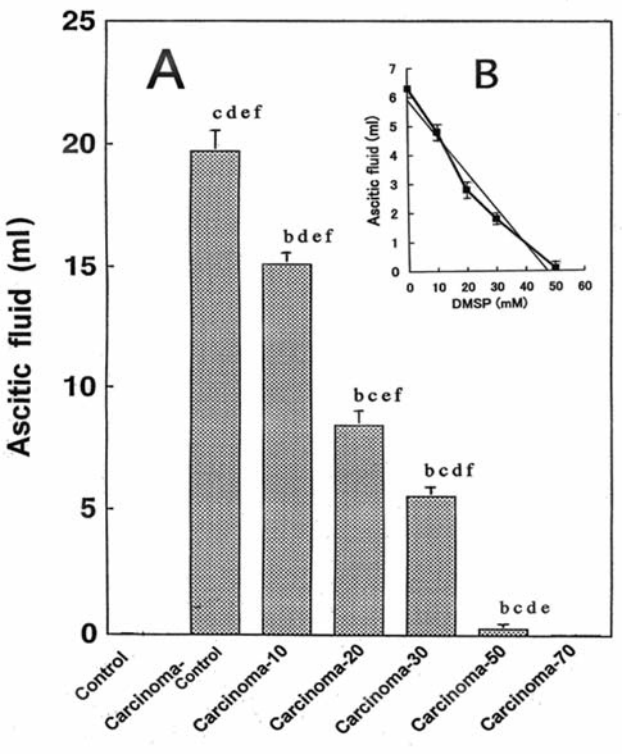

Figure 2. *Effects of various concentrations of DMSP on accumulation of ascitic fluid in EAC-bearing mice. (A) Values: mean±SD.* [a, b, c, d, e, f] *p<0.05 (vs. control (saline), carcinoma-control, 10, 20, 30 and 50 mM DMSP groups).* [a]*control; not detectable). (B) Fitted curve between various concentrations of DMSP and ascitic fluid volumes.*

Results

Determination of body weight and ascitic fluid. The effects of various concentrations of DMSP on the body weight of control mice or EAC-bearing mice were examined for up to 10 days. Results are given in Figure 1. Initial *i.p.* supplementation of 10 and 20 mM DMSP solutions significantly reduced the increase of body weight in the form of ascites fluid, whereas administration of 30, 50 and 70 mM DMSP solutions maintained normal body weight with the increasing rearing times for up to 10 days. Effects of various concentrations of DMSP on accumulation of ascites fluid were examined on the 10th day. Results are given in Figure 2A. Administration of 10-70 mM DMSP solutions almost linearly restricted accumulation of ascites fluid in a dose-dependent manner, showing the linear-fitted curve ($y=-0.125x+5.904$, $R^2=0.964$; Figure 2B).

Incubation of EAC cells or macrophages and of EAC cells with macrophages with DMSP. The effects of 30, 50 and 70 mM (final) DMSP solutions on EAC cells or macrophages (5×10^4 cells /ml, each) were examined *in vitro* for up to 5 h. Results are given in Table I. EAC cells tended to multiply with increasing incubation times and concentrations of DMSP, whereas macrophages almost remained unchangeable under both conditions. However, proliferation of macrophages and EAC cells was restricted at the same levels only at 5 h at 70 mM solution. Furthermore, proliferation of EAC cells and macrophages were examined singly or in combination for up to 5 h. Results are given in Figure 3. EAC cells and macrophages proliferated rapidly with increasing incubation times, although proliferation rate of macrophages was fairly low compared to that in EAC cells for up to 5 h. In contrast, EAC-cells in the mixture containing macrophages were highest at 1 h and then continued to decrease up to 5 h, showing very low values. Macrophages in the mixture containing EAC cells increased up to 1 h, thereafter reached maximal values at 3 h and then slightly decreased up to 5 h, showing smaller numbers than those in macrophage cells-alone.

Effect of DMSP and TG on accumulation of macrophages and viscera in peritoneal cavity. The effects of initial ip

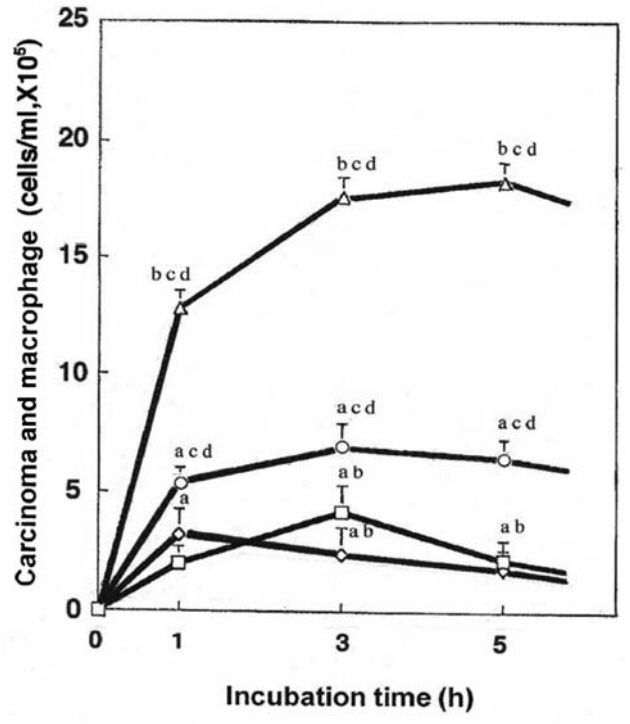

Figure 3. *Effects of macrophages on EAC cells during the incubation period. Values: mean±SD. △, ○, □, ◇; EAC cells, macrophages, and macrophages or EAC cells in the co-culture medium with EAC cells and macrophages, respectively.* [a, b, c, d] *p<0.05 (vs. EAC cells, macrophages and macrophages or EAC cells in the co-culture medium).*

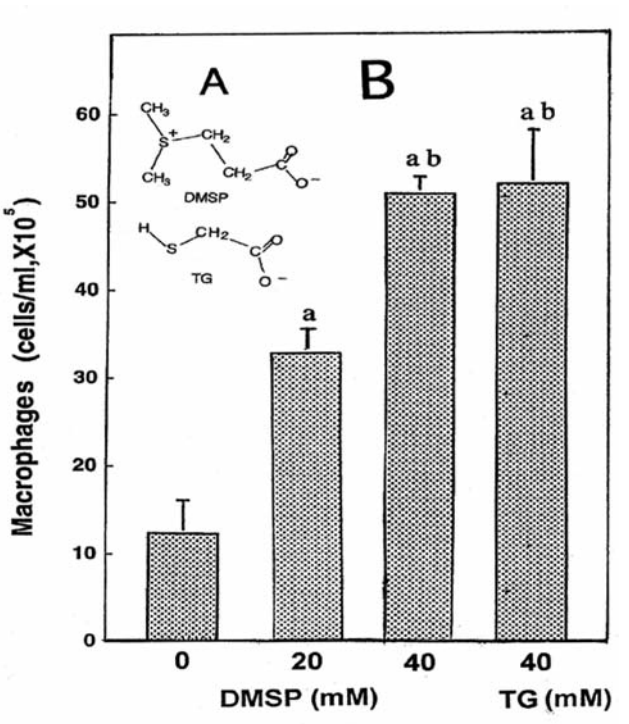

Figure 4. *Effects of DMSP and TG on amounts of macrophages in peritoneal cavity of normal mice. (A) Two-dimensional structures of DMSP and TG. (B) Values: mean±SD.* [a, b] *p<0.01 (vs. Control (saline) and 20 mM DMSP groups).*

administration of 20 and 40 mM DMSP and 40 mM TG solutions on the accumulation of macrophages in the peritoneal cavity of 4-week-old normal mice were examined 3 days after the injection. Results are given in Figure 4. Interestingly, both administrations (40 mM each) accumulated large numbers of macrophages in the peritoneal cavity at the same levels (without significant difference) 3 days after the injection of DMSP or TG. Moreover, to examine the occurrence of inflammation and cell death following administration of DMSP and TG, 1-ml aliquots of 40 mM DMSP and TG solutions were *i.p.*-injected into normal 4-week-old mice (n=3). The abdominal site of the test mice was widely exposed aseptically 3 days after the injection and a trypan blue dye solution was sprayed on the exposed viscera. Pictures of viscera (Figure 5) were immediately taken. The area which dyed blue was carefully calculated. Results are given in Table II, which showed that the control and the DMSP group had the same values (without significant difference), clearly distinguishable from those of the TG group.

Discussion

Ehrlich ascites carcinoma cells elicit solid cancers in organs when subcutaneously-(*s.c.*) injected and free cell cancers in the peritoneal cavity when *i.p.*-injected in mice (9). In particular, the latter causes prompt death of all the mice with large volume of ascites fluid for about 2 weeks (9).

However, we found that preliminarily *i.p.* administration (1 ml) of 10 and 20 mM DMSP solutions on alternate days, only two weeks prior to injection of EAC cells, prolonged the lives of the majority (50%-63%) of residual EAC bearing-mice for 300 days. In these experiments, EAC-bearing mice treated with 10 (n=4) and 20 mM (n=3) DMSP solutions died with accumulated volumes (7.7 ml and 4.1ml) of ascitic fluid by 64 and 53 days (8,10). Therefore, residual 4 and 5 mice bearing about 4-8 ml of ascetic fluid per animal are likely in these days to continue to survive for more than 300 days by destroying evolvability of tumor cell populations (11) and killing recruited as well as resided tumor cells in niches and also by regenerating resident stem cells in the microenvironments of various tissues in the peritoneal cavity (12-15).

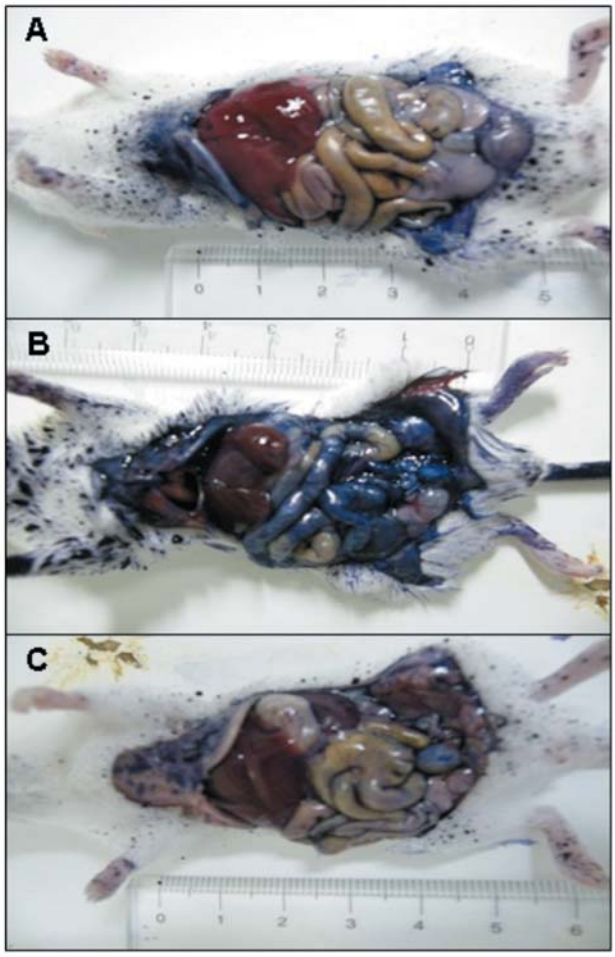

Figure 5. *Representative photograph of the three groups. ((A) Control (saline), (B) TG and (C) DMSP). The saline and the TG and DMSP (40 mM each) solutions were i.p.-administered to normal 4-week-old male mice and the abdominal cavity of mice was opened three days after injection. Then, the photographs were taken immediately after spraying trypan blue dye solution on the viscera.*

In the present work, we found that initial *i.p.* supplementation (0.5 ml) of 10-70 mM DMSP solutions almost linearly restricted accumulation of ascites fluid in a dose-dependent manner. The linear-fitted curve indicates that increasing DMSP doses causes a parallel relation with a decrease of the volume of ascitic fluid and thus with a decline of the death rate of test mice. In fact, the supplementation of 70 mM DMSP solution to EAC bearing-mice completely suppressed the proliferation of EAC cells with no accumulation of ascites fluid in the peritoneal cavity, exhibiting the same body weights as those in control mice.

Moreover, the effects of 30, 50 and 70 mM (final) of DMSP solutions on EAC cells or macrophages were examined *in vitro* for up to 5 h. Proliferation of EAC-cells or macrophages remained almost unchangeable in each of the dose and time determinations up to 5 h, although proliferation of EAC cells and macrophages was reduced at the same levels only at 5 h at 70 mM. In contrast, co-culturing of macrophages with EAC cells indicated that macrophages remarkably reduced proliferation of EAC cells with increasing incubation times up to 5 h *in vitro*.

Only initial *i.p.* administration of 50 and 70 mM DMSP solutions to EAC-bearing mice resulted in same body weights as those in the control group for up to10 days. Of great interest, a single *i.p.* injection of DMSP (180 mM, 1 ml) to juvenile mice, single *i.p.* supplementation of a large amount of DMSP (3.2 M, 1 ml) to rats (8) or oral and sequential administration of a high dose of DMSP (208 mg/day/rat) for up to 33 weeks to young rats (10), caused no toxicity in rodents for a long time. These findings indicate that it is possible to administer *i.p.* and orally higher frequent-doses of DMSP to animals suffering from acute and chronic cancers without side-effects *in vivo*, which strongly suggests that DMSP mitigates and heals different kinds of cancers in different types of animals.

Regarding the healing mechanisms of DMSP in EAC-bearing mice, DMSP has been shown to be a weak antioxidant at low concentrations (5-50 mM) but not at high concentrations (100-500 mM) by assessing radical scavenging activity (10,16).

In contrast, it was found that DMSP activated delayed-type hypersensitive immune reaction in rats (10) and mice (8, 10). Moreover we showed that administration of DMSP to EAC-bearing mice restored the abnormal immune systems to normal levels with decreased ascites fluids at 10 days (10). This re-establishment is likely attributable to increased numbers of macrophages at early rearing time since these cells play a major role in ameliorating inflammation caused by cancer (12-15, 18, 19). In fact, we found that *i.p.* administrations of DMSP into normal 4-week-old mice accumulated large numbers of macrophages in the peritoneal cavity without giving any damage to various viscera of the peritoneal cavity 3 days after the injection, greatly distinguishing from TG causing inflammation and cell death of all viscera in the cavity.

In addition, we demonstrated that free ingestion of 10- and 20-mM DMSP solutions (av. 104 and 208 mg/day/rat) for up to 33 weeks completely healed chronic cancer, 3'-methyl-4-dimethylamino-azobenzene induced-liver cancer in rats with no abnormality in other viscera, showing liver weights and liver weights (/body wt.) with significant difference ($p<0.05$, by Tukey-Kramer test) from controls, cancerous rats fed with 10 mM DMSP and 20 mM DMSP solutions (10,20). In this experiments, the activity of a liver marker enzyme, γ-GTP, in serum of cancerous rats was very high as well (10, 20).

Taken together, our results and other findings suggest it is likely that resident and multi-potent stem cells formed from hematopoietic progenitor stem cells are regenerated by EAC cells leading to a subsequent differentiation of these progenitors to lineage cellular populations in inflamed and/or damaged cells and tissues (13-15, 18), which is accompanied by the up-regulation of the innate immune systems due to high potential of activated macrophages (12, 18-19, 21) *via* the DMSP signaling through complex and yet unspecified ways.

In conclusion, our results demonstrate that 70 mM DMSP completely eradicated crucial Ehrlich ascites carcinoma in mice. This finding is likely attributable to the function of recruited and resided activated macrophages and to the regeneration of tissue specific stem cells in the local microenvironments without a cytokine interference and, in particular, with no side effects.

Conflicts of Interest

The Authors declare that they have no competing interests.

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