Synthetic Trehalose Dicorynomycolate (S-TDCM):
Behavioral Effects and Radioprotection

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This study evaluated synthetic trehalose dicorynomycolate (S-TDCM), an immunomodulator, for its survival enhancing capacity and behavioral toxicity in B6D2F1 female mice. In survival experiments, mice were administered S-TDCM (25-400 µg/mouse i.p.) 20-24 hr before 5.6 Gy mixed-field fission-neutron irradiation (n) and γ-photon irradiation. The 30-day survival rates for mice treated with 100-400 µg/mouse S-TDCM were significantly enhanced compared to controls. Toxicity of S-TDCM was measured in nonirradiated mice by locomotor activity, food intake, water consumption, and alterations in body weight. A dose-dependent decrease was noted in all behavioral measures in mice treated with S-TDCM. Doses of 100 and 200 µg/mouse S-TDCM significantly reduced motor activity beginning 12 hr postinjection with recovery by 24 hr. A dose of 400 µg/mouse significantly decreased activity within the first 4 hr after administration and returned to control levels by 32 hr following injection. Food and water intake were significantly depressed at doses of 200 and 400 µg/mouse on the day following drug administration, and were recovered in 24 hr. Body weight was significantly decreased in the 200 µg/mouse group for 2 days and in the 400 µg/mouse group for 4 days following injection. A dose of 100 µg/mouse effectively enhanced survival after fission-neutron irradiation with no adverse effect on food consumption, water intake, or body weight and a minimal, short-term effect on locomotor activity.
INTRODUCTION

Trehalose dimycolate (TDM), a naturally produced substance also known as cord factor, is a glycolipid secreted on the surface of the cell walls of several strains of bacteria including *Mycobacteria*, *Nocardia* and *Corynebacterium*. Bloch observed a rapid weight loss and death in mice injected with TDM; he referred to this substance as a "toxic lipid".

Irradiation compromises nonspecific immune defenses of the hematopoietic and intestinal cell systems, making the host susceptible to bacterial infection. The increasing use of radiotherapy for the treatment of malignant tumors demonstrates the need for effective stimulants of proliferative hematopoietic cells to help reduce aplastic and septic conditions found in irradiated hosts. A number of bacterially and chemically derived agents are able to promote survival after lethal doses of radiation, but most do so only at behaviorally toxic doses. In this study, we examined the use of synthetic trehalose dicorynomycolate (S-TDCM), a biological response modifier known to have beneficial properties in normal and irradiated mice. S-TDCM, for example, enhances survival following pure $\gamma$- or mixed fission-neutron and $\gamma$-photon irradiation, enhances resistance to bacterial infection and activates macrophages that can produce biological response mediators such as interleukin-6, colony stimulating factor, and interferon.

The toxicity of native TDM delivered in oil emulsions is well documented. Mycobacterial TDM produces a toxic effect in mice and leads to anorexia, cachexia, and death. Bloch originally reported that a single injection of TDM (from *Mycobacterium tuberculosis*) administered i.p. or subcutaneously in paraffin oil was not toxic to Swiss-Webster or DBA mice, but both the CF1 and C57 strains exhibited slight weight loss. Three to seven repeated injections resulted in progressive weight loss and death in all mouse strains tested. With highly purified TDM from *M. tuberculosis* prepared in oil, a single injection (3 or 10 $\mu$g) administered to C57BL/6 or C57BL/10 mice resulted in interstitial and hemorrhagic pneumonitis with deaths reported for 5% of the animals at 4 weeks postinjection. Multiple injections increased the number of deaths. In addition, Saito et al. found that a single i.p. injection of 10 $\mu$g TDM in Drakeol resulted in diarrhea and weight loss in C57 mice. Furthermore, repeated administration of TDM suppressed weight gain. Within 3 days after the fifth injection of TDM, 10% of AKR mice and 70% of C57BL mice died. The toxicity may be directly related to the percent of oil used as the vehicle.

Synthetic TDM (S-TDCM) is prepared by the condensation of potassium mycolate containing 32 carbon atoms with 2,3,4,2',3',4'-hexa-O-trimethylsilyl-a,a-trehalose activated at the 6 and 6' positions. S-TDCM has a molecular weight of 1374 daltons, compared with 2860 daltons for naturally occurring TDM isolated from *M. phlei*. The synthetic product retains most of the beneficial effects of the native TDM, is less toxic, and is easier to obtain. S-TDCM can be administered in a nontoxic vehicle, 0.2% Tween 80/saline, instead of the toxic squalene emulsion in which the naturally occurring TDM is often administered.

Administration of S-TDCM increased survival in B6D2F1 mice made neutropenic by exposure to ionizing radiation. In addition, mice given a sublethal $\gamma$ for mixed-field fission-neutron and $\gamma$-photon radiation dose and challenged with *Klebsiella pneumoniae* survived when administered S-TDCM 1 hr postirradiation.

The effectiveness of S-TDCM in increasing survival in irradiated mice has made it an excellent candidate for additional testing. In this study, the survival enhancing properties of S-TDCM administered prior to mixed-field fission-neutron and $\gamma$-photon irradiation were further delineated. In addition, general toxicity, reflected by food intake, water consumption, body weight, and locomotor activity was examined. These parameters are useful in evaluating the toxic effects of radiation or putative radioprotective
MATERIALS AND METHODS

Subjects
Female B6D2F1 mice (15-18 weeks of age; 24-29 g) were obtained from Jackson Laboratories (Bar Harbor, ME). They were quarantined on arrival for 2 weeks, and representative mice were examined by microbiology, serology, and histopathology to assure the absence of specific bacteria, particularly Pseudomonas aeruginosa, and common murine diseases. Mice were housed in plastic Micro-Isolator cages on hardwood chip contact bedding in a facility accredited by the American Association for the Accreditation of Laboratory Animal Care. Rooms were maintained at 21°C ± 1°C with 50% relative humidity on a 12 hr light-dark cycle. Commercial rodent chow (Wayne Rodent Blox) and acidified water (pH = 2.5) to minimize Pseudomonas colonization were freely available. All research was conducted in accordance with National Institutes of Health guidelines and was approved by the AFRRI Institutional Animal Care and Use Committee.

Immunomodulator
S-TDCM was produced by Ribi ImmunoChem Research, Inc. (Hamilton, MT) and was prepared in depyrogenated glassware as an aqueous suspension in a 0.2% Tween 80/saline vehicle.

Radiation
The techniques and dosimetry of exposing mice to radiation fields containing both fission-neutrons and γ-photons produced by the AFRRI Training, Research, Isotope, General Atomic (TRIGA) reactor were previously described. Mice were administered a midline tissue (MLT) dose of 5.6 Gy (1 Gy = 100 rad) at 0.4 Gy/min with a neutron dose to total dose ratio \([n/(n+\gamma)]\) of 0.67 (+/-10%). This ratio was achieved by using a 15.2-cm lead shield in front of the reactor wall. The MLT dose was measured by paired ionization chambers. Chamber calibrations for mixed fields were traceable to standards maintained by the National Institute of Standards and Technology. Mice were individually irradiated in well-ventilated aluminum restraining tubes that rotated at 1.5 revolutions/min.

Survival
To evaluate the protective efficacy of S-TDCM, we selected a radiation dose of 5.6 Gy at which approximately 80% of saline-treated mice of this strain die within 30 days (LD80). Mice were each administered either a single i.p. injection of 0.5 ml of saline vehicle, or 25, 50, 100, 200, or 400 µg/mouse S-TDCM (N = 20/group), 20-24 hr before irradiation.

Locomotor Activity Measurement
A computerized Digiscan Animal Activity Monitor (Omnitech Electronics, Columbus, OH) was used to quantitate locomotor behavior. The apparatus consisted of a 20.3-cm x 20.3-cm acrylic arena with an array of infrared photodetectors spaced 2.5 cm apart to detect locomotor activity, which was expressed as the total distance traveled. Nonirradiated mice each received a single i.p. injection either of the vehicle or 100, 200, or 400 µg/mouse S-TDCM (N = 10-11/group). Immediately after injection the mice were each
placed into the activity monitor where ambulation was recorded at 2-hr intervals for the first 6 hr and again at 24 and 48 hr. All testing began at the onset of the dark portion of the light-dark cycle.

**Food and Water Consumption, Body Weight**

A separate group of nonirradiated mice treated similarly to those in the locomotor study were used to measure food and water intake and body weight. Three days before the drugs were administered, the mice were each weighed and placed into individual cages. Body weight was balanced across groups at the beginning of the experiment so that the mean weights of the groups were within 1 g. One day before the experiment, baseline values for food and water intake and body weight were determined and then measured for 8 consecutive days following injection. The amount consumed was determined by daily weighing of the food hoppers and water bottles. Mice were each injected i.p. (day 0) either with the vehicle (0.2% Tween 80/saline), or with 50, 100, 200, or 400 µg/mouse of S-TDCM. We previously determined that there was no difference in body weight or consummatory behavior following injections of saline or the vehicle.

**Statistical Analysis**

Locomotor activity as well as daily food, water, and body weight data were analyzed by one-way ANOVA. Posthoc comparisons were made with Dunnett’s test. Survival data were analyzed with the Fisher exact test.

**RESULTS**

**Survival**

As illustrated in Fig 1, administration of 25, 50, 100, 200, or 400 µg/mouse S-TDCM 20-24 hr before 5.6-Gy mixed-field neutron irradiation resulted in 30-day survival rates of 35%, 45%, 80%, 80%, and 95%, respectively. The survival rate for saline-treated mice was 15% which was not statistically different from the 30% rate observed for subjects administered the vehicle, Tween-80/saline. Compared to the vehicle control group, significantly (p < .001) greater survival was observed for mice treated with 100, 200, and 400 µg S-TDCM.

**Locomotor Activity**

Fig. 2 illustrates the locomotor activity of mice over a 3-day period after receiving S-TDCM. The mice were on a 12-hr light/dark cycle and testing began at the onset of the dark period (indicated by the solid bar). The activity rhythm, plotted in 4 hr intervals, of these nocturnal rodents is clear. Locomotor activity of control animals was maximal during the first time interval of the dark period and gradually decreased during the remainder of each 24-hr period. Locomotor activity in the 100 and 200 µg/mouse groups was significantly (p< 0.01) depressed beginning 12 hr after injection and recovered by 24 hr. Mice treated with 400 µg/mouse exhibited significantly (p< 0.01) reduced locomotor activity that commenced earlier (4 hr), was sustained longer (up to 28 hr), and was of a greater magnitude than the lower dosed groups.
Fig. 1. Thirty-day survival curves for mice administered synthetic trehalose dicorynomycolate (S-TDCM) 20-24 hr before 5.6-Gy mixed-field fission-neutron and $\gamma$-photon irradiation (N = 20/group).

Fig. 2. Circadian rhythm in locomotor behavior of control and S-TDCM treated mice over a 3-day period. Locomotor activity is expressed as the total distance traveled (meters) in 4-hr intervals. The solid bar along the abscissa represents the dark period of the light-dark cycle. Mice each received a single i.p. injection of vehicle or S-TDCM at the beginning of the dark period on the first day. Vertical lines represent the SEM. (N = 10-11/group) * $p < 0.05$, ** $p < 0.01$ from vehicle control group.
Food and water consumption, body weight

Food (Fig. 3) and water (Fig. 4) consumption was significantly (p< 0.01) decreased within 24 hr (day 1) following administration of doses of 200 and 400 µg/mouse of S-TDCM. A dose of 100 µg/mouse did not significantly decrease either food consumption or water intake. Food and water consumption in all groups returned to control levels by the second day after drug administration.

Body weight (Fig. 5) was not affected by a dose of 100 µg/mouse S-TDCM. Doses of 200 µg/mouse or greater significantly depressed body weights (p< 0.01) beginning 24 hr after injection. Mice administered 200 and 400 µg/mouse S-TDCM had significantly lower weights than control animals for 2 (p< 0.01) and 4 (p< 0.05) days, respectively.

DISCUSSION

In this study, the lowest dose at which S-TDCM exhibited a beneficial radioprotective effect (100 µg), had no significant effect on food and water consumption, or body weight, and had a minimal, reversible effect on locomotor activity. A dose of 200 µg significantly reduced all parameters evaluated, with complete recovery within 24 hr of administration. A dose of 400 µg significantly decreased locomotor activity for 28 hr, consummatory behaviors for 48 hr, and body weight for 96 hr. After these time periods, all parameters returned to control levels. Thus, S-TDCM at the most radioprotective doses, offered short-term, adverse behavioral effects that were completely reversible.
Fig. 4. Water consumption after S-TDCM treatment. Water intake was measured daily for 8 days after a single i.p. injection of S-TDCM. Day 0 is the day of injection. Vertical lines represent the SEM. (N = 12/group) ** p < 0.01 from vehicle control group.

Fig. 5. Body weight after S-TDCM treatment. Body weight was measured daily for 8 days following a single i.p. injection of S-TDCM. Day 0 equals the day of injection. Vertical lines represent the SEM. (N = 12/group) * p < 0.05, ** p < 0.01 from vehicle control group.
The onset of behavioral decrements observed in this study at approximately 4 hr postinjection (400 µg group) corresponded to some of the toxic manifestations previously reported for native TDM in a hexadecane vehicle. Seggev and colleagues\(^{15}\) found that 25% of mice showed mild mononuclear cell infiltrations within 6 hr postinjection and Silva et al.\(^{28}\) reported that mice injected i.p. with TDM in mineral oil exhibited a decrease in body weight and were reluctant to move after a single injection.

The locomotor decrement as well as reductions in food, water, and body weight observed in our study may have resulted directly from the direct action of S-TDCM on the gastrointestinal tract or indirectly through a metabolite, a change in hormone concentration, or the production of cytokines. With reference to cytokines, Silva et al.\(^{28}\) detected tumor necrosis factor (TNF) in the plasma of animals injected with TDM. In other studies, S-TDCM increased the splenic gene expression for interleukin-1 (IL-1) beta, IL-3, IL-6, and granulocyte colony stimulating factor (G-CSF)\(^{15}\). Moreover, decreases in food intake and investigatory or motor behavior were observed in rodents after administration of IL-1 beta, or correlated with a rise in endogenous IL-1 beta\(^{29-31}\), IL-3\(^{32}\), IL-6\(^{29}\), or TNF\(^{29,33}\). In addition, evidence from our laboratory indicated that 400 µg/mouse S-TDCM resulted in a 2 to 4°C rise in mouse basal body temperature within 3 hr of administration. The onset of this temperature increase may also be related to the onset of the locomotor activity decrement observed in the present experiment.

This is the first comprehensive paper evaluating the effects of an acute administration of S-TDCM on consummatory and locomotor behavior. However, the toxic effects of naturally occurring TDM has been well documented especially in instances in which the compound was dissolved in an oil and water emulsion and repeated injections were administered. The toxicity (lung granuloma, weight loss, and death) depended on the percent of oil used in the injection and on the size of the oil droplets\(^{11}\). After i.p. administration of TDM, interstitial and hemorrhagic pneumonitis was likely to occur\(^{17,18}\). Unpublished work from our laboratory indicated that the squalene vehicle used to emulsify TDM was behaviorally toxic as shown by significant decreases in locomotor behavior. Squalene at doses of 0.5%, 1%, and 2% produced locomotor deficits in mice, while a dose of 0.1% did not. However, we found that S-TDCM in a 0.2% Tween-80/saline vehicle did not differ from that in saline-treated animals with respect to locomotor activity\(^{34}\).

TDM in saline is not toxic to mice but also does not offer as much protection from γ rays as does TDM in oil\(^{30}\). In nonirradiated mice injected i.p. with doses of 50-1000 µg TDM/saline, body weight was comparable to noninjected controls and no deaths were reported\(^{35}\). In our laboratory we found that TDM/saline at doses of 100 or 200 µg/mouse did not result in locomotor deficits.

S-TDCM (100 µg) offered protection when given 24 hr before radiation and was comparable to 100 µg TDM/oil\(^{29}\) for γ rays when 30-day survival was used as the end point. S-TDCM (100 µg) was less effective than 100 µg TDM/oil when this preparation was used to protect against mixed-field fission-neutron and γ-photon irradiation (60% versus 90%)\(^{31}\). In general, the toxicity of TDM was shown to be associated with the percent of oil used in the emulsion, the strain of the mouse tested, and the number of injections. Further, there appeared to be a species difference since repeated injections of TDM in Drakeol was more toxic to mice than rats\(^{39}\).

In summary, S-TDCM offers similar levels of radioprotection and therapeutic benefits as naturally occurring TDM. The reduced toxicity of this synthetic analog makes it an ideal compound for continued study.
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REFERENCES


