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**SUMMARY**

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**UNCLASSIFIED**
Trehalose Dimycolate Enhances Survival of Fission Neutron-Irradiated Mice and Klebsiella pneumoniae-Challenged Irradiated Mice¹,²

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INTRODUCTION

The effects of radiation on mammalian hematopoietic, myelopoietic, and gastrointestinal systems are diverse and depend on both the exposure dose and the quality of radiation. Several studies have focused on the effects of X rays (1-4) and ⁶⁰Co γ rays (5, 6) on these systems in mice. Other studies have examined the effect various specific and non-specific immunomodulators have on survival in mice given ⁶⁰Co γ rays (7, 8). However, there are a limited number of reports (5, 9, 10) on the effects of fission neutron radiation on these systems and the effect immunomodulators have on these animals' survival.

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Research was conducted according to the principles enunciated in the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council.

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Nuclear weapons detonations or nuclear criticality accidents can produce mixed radiation fields of various proportions of photon and neutron radiations. Therefore, it is important to determine the response of animals to mixed-field radiation in order to evaluate realistically the effect cytokines and immunomodulators of nonspecific resistance to infection might have on the animals' survival. Several different formulations of the immunomodulator trehalose dimycolate (TDM) have been shown to be effective in increasing survival of mice exposed to ⁶⁰Co γ rays (7). Therefore it is of interest to evaluate TDM formulations for their ability to increase survival in animals irradiated with mixed-field fission neutrons.

Exposure to fission neutron radiation is more effective in causing severe leukocytopenia in mice within 4 days than exposure to ⁶⁰Co γ radiation (11, 12). Hemopoietic recovery occurs in animals exposed to 7.0 Gy ⁶⁰Co radiation by Day 14 after exposure (13, 14). Mice exposed to 3.5 Gy fission neutrons recover in a similar time period. Exposure to higher levels of radiation produces leukocytopenia and irreversible gastrointestinal damage. Death normally occurs in these mice in less than 14 days due to denudation of the intestinal mucosa, fluid and electrolyte imbalance, and bacteremia (15). Infection is a major cause of death in animals exposed to radiation doses sufficient to depress the immune system severely but not to produce irreversible gastrointestinal damage. For example, 5.75 Gy fission neutrons or 10.5 Gy ⁶⁰Co. The source of the infection can be translocation of normal intestinal flora or an external source. In these circumstances, bacteria of the family Enterobacteriaceae often become opportunistic pathogens. One member of this family, Klebsiella pneumoniae, is associated with a high incidence of mortality in immunocompromised patients (16, 17).

In this paper, we report on the effects of TDM on the survival of fission neutron-irradiated mice when TDM is used as a protectant (before exposure) and as a therapeutic agent (after exposure) in irradiated mice. We also report the effects of TDM as a therapeutic agent for irradiated mice challenged with K. pneumoniae.

MATERIALS AND METHODS

Mice — JAX B6D2F1 female mice, 12-15 weeks of age (20-25 g), were quarantined on arrival and screened for evidence of disease before being
reduced for experimental use. They were maintained in an AAALAC-accredited facility in plastic Micro-Isolator cages containing autoclaved hardwood chip, contact bedding. Mice were provided commercial rodent chow and acidified tap water (pH 2.5 with concentrated HCl) ad libitum. Animal rooms were maintained at 70 ± 2°F with 50 ± 10% relative humidity using at least 10 air changes per hour of 100% conditioned fresh air. The mice were on a 12-h light/dark full spectrum lighting cycle with no twilight. All research was conducted in accordance with NIH and our Institutional Animal Care and Use Committee guidelines for the care and use of laboratory animals.

**Radiation** The techniques and dosimetry of exposing mice to mixed radiation fields produced by the AfirRI TRIGA reactor were previously described (18). All radiation doses reported in this paper are the midline tissue dose as measured using ionization chambers (19). In the present study, a neutron to γ kerma ratio of 1:1 (NeG = 1) at midline in the animals was achieved by using a 15.2-cm lead shield in front of the reactor wall. The neutron to γ-radiation kerma was chosen as representative of conditions which might prevail during a nuclear weapons detonation or nuclear criticality incident. Mice challenged with *K. pneumoniae* were given a nonlethal total (neutron plus γ-ray) dose of 3.5 Gy midline tissue dose at 0.4 Gy/min. Mice not challenged with *K. pneumoniae* received 5.75 Gy; this is the radiation dose that kills 80% of the mice of this strain receiving no supportive therapy within 30 days (LD<sub>100</sub>). Mice were exposed individually in well-ventilated aluminum restraining tubes that rotated at 1.5 rpm.

**Dose reduction factor** The dose reduction factor was determined for irradiated mice receiving intraperitoneal (ip) injections of the various TDM formulations or control formulations either 1 day before or 1 h after irradiation with fission neutrons. Groups of 10 mice were exposed to increasing doses of radiation and their 30-day survival was monitored. Probit analysis of the survival data was used to determine the best fit, and thus the LD<sub>100</sub> to LD<sub>10</sub> values, and to determine the dose reduction factor (DRF).

**Immunomodulators** Synthetic trehalose dimycolate (S-TDM), a product containing corynycholate acid and trehalose, and native TDM in a mixture of 2% squalene and 0.2%; Tween 80 were produced by Ribi ImmunoChem Research Inc. (Hamilton, MT). S-TDM was prepared as previously described (20, 21). The native TDM was suspended in saline to give a native TDM: squalene: Tween 80: saline emulsion (TDM-O). The concentration of both TDM formulations was 200 μg TDM/ml. Controls for these preparations were 0.2%; Tween 80:saline (TS) and 2% squalene in 0.2% TS (squalene emulsion). Mice received intraperitoneal injections of 0.5 ml of the appropriate TDM formulation or control formulations.

**Bacteria** A clinical isolate of *K. pneumoniae*, serotype 5, was prepared as previously described (7). The pellet was washed twice with cold saline and suspended to an optical density at 650 nm, known to yield 1 × 10<sup>8</sup> viable bacteria/ml. The actual number of viable bacteria was determined by plate counts on Trypticase Soy Agar (BBL, Cockeysville, MD). Dilutions for injection into mice and plate counts were made in sterile saline.

**Bacterial LD<sub>100</sub>** The dose of *K. pneumoniae* lethal to 50% of mice within 30 days (LD<sub>100</sub>) was determined for control and irradiated mice by subcutaneously injecting 10-fold dilutions of *K. pneumoniae* (10<sup>-1</sup>-10<sup>0</sup>) bacteria/0.1 ml and monitoring survival for 30 days. Mice were injected with bacteria on Days 1, 4, 7, 10, and 14 postirradiation. To assure precise delivery of bacteria and prevent injury to the animal, mice were anesthetized by inhalation of methoxyflurane prior to injection of bacteria. Eight mice were used for each treatment group and a total of six bacteria challenge concentrations were used to determine each LD<sub>100</sub> endpoint for irradiated mice; four challenge concentrations were used for unirradiated control mice. Groups of irradiated mice challenged on Days 1, 4, and 7 received 10<sup>7</sup>, 10<sup>8</sup>, 10<sup>9</sup>, 10<sup>10</sup>, or 10<sup>11</sup> CFU/mouse. Irradiated mice challenged on Days 10 and 14 received 10<sup>7</sup>, 10<sup>8</sup>, 10<sup>9</sup>, 10<sup>10</sup>, or 10<sup>11</sup> CFU/mouse. Groups of unirradiated control mice were injected with 10<sup>7</sup>, 10<sup>8</sup>, 10<sup>9</sup>, or 10<sup>10</sup> CFU/mouse.

**Hematology** Mice received either 3.5 or 5.75 Gy of mixed-field radiation. Experimental groups received either 100 μg/50 ml TDM-O or 0.5 ml of Tween–saline, squalene emulsion. TDM-O, or S-TDM. The above formulations were injected ip into groups of 10 mice either 1 day before (filled) or 1 h after (hatched) radiation exposure.

**RESULTS**

In a series of protection experiments, mice were injected ip with the TDM preparations 1 day prior to exposure to a 5.75-Gy MLT dose of fission neutron radiation. TDM-O provided greater protection (90%) than S-TDM (60%), while 10% of mice receiving Tween–saline survived and 20% of those receiving the 2% squalene emulsion survived (Fig. 1). The TDM-O (*P* < 0.01) and S-TDM (*P* < 0.05) were significantly better than TS at increasing 30-day survival of the mice exposed to radiation. The TDM-O was significantly better (*P* < 0.01) than the 2% squalene emulsion.

When the TDM preparations were given therapeutically 1 h after exposure to 5.75 Gy fission neutron radiation, TDM-O provided the most benefit (88%) (Fig. 1). The TDM-O formulation was significantly better than the squalene emulsion alone (*P* < 0.001) when given 1 h after radiation. No mice survived in the groups receiving S-TDM, TS, or 2% squalene emulsion. Incremental increases in the amount of S-TDM given up to 800 μg/mouse did not significantly increase the survival of mice when given 1 h after mixed-field radiation over that observed with 100 μg/mouse (data not shown).

Dose reduction factors were determined for irradiated mice which received S-TDM, TDM-O, Tween–saline (con-
FIG. 2. Bacterial LD$_{50/30}$ of mice challenged with *K. pneumoniae* following radiation exposure. B6D2F1 mice were given 3.5 Gy (N:G - 1) radiation. *K. pneumoniae* was injected into groups of eight mice on Days 1, 4, and 7 (10$^3$, 10$^4$, 10$^5$, 10$^6$, 10$^7$, 10$^8$ CFU/mouse), and on Days 10 and 14 (10$^5$, 10$^6$, 10$^7$, 10$^8$, 10$^9$, 10$^{10}$ CFU/mouse). Groups of eight unirradiated control mice were injected with 10$^3$, 10$^4$, 10$^5$, or 10$^6$ CFU/mouse. The LD$_{50/30}$ was determined by probit analysis and plotted for each time of injection after radiation. Vertical bars represent the upper and lower 95% confidence limits for each LD$_{50/30}$.

The TDM formulations given 1 h after exposure to 3.5 Gy fission neutron radiation had an effect on the 30-day survival of mice challenged with 10, 100, 1000, or 5000 times the LD$_{50/30}$ dose of *K. pneumoniae* on Day 4 after exposure to radiation (Fig. 3). At the relatively low challenge dose of 10 times the LD$_{50/30}$, all of the TDM formulations increased 30-day survival, as did the squalene emulsion. When the challenge dose of *K. pneumoniae* was increased to 100 times the LD$_{50/30}$, only the TDM-O, S-TDM, and squalene emulsion increased the number of mice surviving over that of the control. The therapeutic effect provided by the squalene emulsion was not significantly ($P > 0.25$) different than control. At 1000 and 5000 times the LD$_{50/30}$ dose of *K. pneumoniae*, only TDM-O and S-TDM

![Graph 1](https://via.placeholder.com/150)

**TABLE I**

Probit Analysis of Mortality Data for Mixed-Field Irradiated Mice Receiving Formulations Pre- or Postirradiation

<table>
<thead>
<tr>
<th>Time of treatment</th>
<th>Vehicle</th>
<th>Treatment</th>
<th>LD$_{50/30}$ (Gy)</th>
<th>DRF$^{a}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Day</td>
<td>Tween-saline</td>
<td>Tween-saline</td>
<td>530</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Tween-saline</td>
<td>S-TDM</td>
<td>569</td>
<td>1.07</td>
</tr>
<tr>
<td></td>
<td>Squalene emulsion</td>
<td>Squalene emulsion</td>
<td>555</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Squalene emulsion</td>
<td>TDM-O</td>
<td>596</td>
<td>1.07</td>
</tr>
<tr>
<td></td>
<td>Tween-saline</td>
<td>None</td>
<td>542</td>
<td>0.98</td>
</tr>
<tr>
<td>1 h</td>
<td>Tween-saline</td>
<td>Tween-saline</td>
<td>514</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Tween-saline</td>
<td>S-TDM</td>
<td>548</td>
<td>1.07</td>
</tr>
<tr>
<td></td>
<td>Squalene emulsion</td>
<td>Squalene emulsion</td>
<td>540</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Squalene emulsion</td>
<td>TDM-O</td>
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<td>1.08</td>
</tr>
<tr>
<td></td>
<td>Tween-saline</td>
<td>None</td>
<td>542</td>
<td>0.95</td>
</tr>
</tbody>
</table>

$^{a}$ DRF = LD$_{50/30}$ vehicle.

Note: Dose-response survival factors in irradiated mice receiving TDM formulations. B6D2F1 mice received increasing amounts of mixed radiation (N:G - 1). One day before or 1 h after irradiation, groups of 20 mice received 0.5 ml of one of the following formulations (1 treatment) by ip injection: Tween-saline, 2% squalene emulsion, 100 μg TDM-O, or 100 μg S-TDM. Mice used in this experiment received only one ip injection; the formulation in the Vehicle column was not injected unless it also appears in the Treatment column. Survival was followed for 30 days. Probit analysis of the results showed the slopes of the probit lines in the comparisons to be parallel; all reported DRFs are based on the LD$_{50/30}$ radiation dose for ease of comparison.

FIG. 3. Thirty-day survival of B6D2F1 female mice given 3.5 Gy (N:G - 1) radiation and treated with TDM prior to challenge with *K. pneumoniae*. One hour after irradiation, 0.5 ml of one of the following was injected ip into groups of 20 mice: (■) Tween-saline; (□) squalene emulsion; (△) 100 mg TDM-O; (△△) 100 mg S-TDM; (□□) controls. Mice were challenged on Day 4 after irradiation with either 10, 100, 1000, or 5000 times the LD$_{50/30}$ of *K. pneumoniae* serotype 5.
increased survival. TDM-O increased survival to twice that of the S-TDM-treated group. The $L_{D_{50}}$ (i.e., 80% survival) for unirradiated mice is $2.08 \times 10^2$ with a lower confidence limit of $7.95 \times 10^2$, while for the irradiated mice treated with TDM-O the corresponding $L_{D_{50}}$ is $4.38 \times 10^2$. Thus TDM-O is capable of increasing the irradiated mouse's resistance to bacterial challenge to nearly that of a normal mouse.

White blood cell (WBC), red blood cell (RBC), and platelet counts were obtained from mice given 3.5 or 5.75 Gy of mixed-field radiation and treated with either 100 µg S-TDM or 5 ml or an equal volume of the vehicle (0.2% Tween-saline) 1 h postirradiation. White blood cell counts were similar for mice receiving either 3.5 or 5.75 Gy until Day 14 after radiation exposure. At this time the values were significantly lower ($P < 0.05$) for mice exposed to 5.75 Gy (data not shown). This is true whether or not mice received S-TDM 1 h after exposure to radiation.

The effect of the two doses of radiation on the RBC and platelet counts appeared to be similar until Day 14 for mice treated with S-TDM (data not shown). By Day 14, the RBC and platelet counts in mice exposed to 3.5 Gy were recovering, and by Day 28 they were within 20% of unirradiated control values. On Day 14, the surviving mice of the group given 5.75 Gy and S-TDM 1 h after radiation had RBC and platelet levels significantly lower ($P < 0.05$) than those of mice receiving S-TDM 1 h after 3.5 Gy.

**DISCUSSION**

For an immunomodulator to be useful as a therapeutic or radioprotective agent in the context of mixed-field radiation, it must be effective over a range of neutron to photon ratios and have a low potential for toxicity. Madonna et al. (7) showed that TDM and a synthetic analog, S-TDM, are capable of increasing survival in mice exposed to an LD$_{50}$ dose of $^{103}$Co radiation. Additionally, they showed that TDM-O and S-TDM increase the survival of mice exposed to a sublethal dose of $^{103}$Co γ radiation and challenged with *K. pneumoniae* serotype 5. In our experiments, we have used the more severe radiation challenge generated by a mixed radiation field of equal doses of fission neutrons and photons. Our results demonstrate that both TDM-O and S-TDM formulations are effective in increasing survival when given 1 day before exposure to an LD$_{50}$ dose of mixed-field radiation. However, only TDM-O is effective at increasing survival at this radiation level when given 1 h after radiation. The inability of S-TDM to increase survival at the LD$_{50}$ radiation level when given 1 h after mixed-field radiation is unclear, especially since S-TDM is effective in increasing survival to an equivalent radiation dose when the radiation is only photons (7). The differences observed are most likely due to the slightly different effect each type of radiation has on cells (21). Specifically, the difference might be related to a difference in the ability of each type of radiation, mixed-field or pure γ radiation, to generate oxygen-derived free radicals and hydrogen peroxide that cause peroxidation of membrane-polyunsaturated fatty acids (22). Additionally, squalene, a long-chain, polyunsaturated hydrocarbon, may act as a quencher to destroy free radicals and hydrogen peroxide before these agents can affect membrane-polyunsaturated fatty acids. This concept is consistent with the increased survival observed (Figs. 1 and 3) when squalene alone is given and might also explain the increased effectiveness of the TDM-O formulation.

Synthetic TDM is an effective therapeutic agent for both endogenous and exogenous infections in irradiated mice. Mixed-field radiation doses of either 3.5 or 5.75 Gy both reduce the number of WBCs, RBCs, and platelets on Days 1 through 7 to similar levels. However, with the sublethal dose, recovery of these cellular fractions begins by Day 14, while with the 5.75-Gy dose they remained depressed. If the mice receiving the sublethal dose of radiation are challenged with *K. pneumoniae* on Day 14 after radiation the number of organisms required for an LD$_{50}$ is equivalent to unirradiated controls. 1.4 $\times 10^7$. However if these mice are challenged on Day 4, the LD$_{50}$ is reduced to 81 organisms. If, however, mice receive S-TDM 1 h after irradiation, 40% of those challenged are able to survive 5000 times the LD$_{50}$ dose of the organisms. An identical experiment using TDM-O had 80% survival at this level of bacterial challenge. From our results, it is clear that S-TDM is an effective therapeutic agent at moderate mixed-field radiation levels and an effective therapeutic agent at both moderate and high levels of $^{103}$Co (7).

Preliminary data suggest that S-TDM increases the amount of tumor necrosis factor (TNF) present in the 24-h supernatants of macrophages isolated from B6D2FI mice given S-TDM either 1 day before or 1 h after exposure to an LD$_{50}$ dose of mixed-field radiation, compared to radiation and S-TDM controls. Measurement of interleukin 1 (IL-1) in the same supernatants showed a decrease in the amount of IL-1 present when S-TDM is given in conjunction with radiation, compared to radiation alone. If these results are confirmed, they suggest that the ratio of TNF to IL-1 may be an important aspect in determining whether mice survive. These results would also be consistent with the work of Neta using γ-irradiated C3H/HEN mice (8) and the work of Cross with unirradiated and bacterial-challenged C3H/HEN mice (23). Furthermore, they would be consistent with the recent finding of Slordal et al. (24), who showed that murine rTNF-α given before sublethal irradiation reduced the decline of neutrophils and total blood counts after irradiation and also accelerated the subsequent normalization of peripheral blood cell counts.

In conclusion, both S-TDM and TDM-O offer substantial protection to endogenous infection when given 1 day before high levels of mixed-field radiation but only TDM-
O is effective at this level when given after irradiation. Additionally, both S-TDM and TDM-O are effective in increasing survival in irradiated and bacteria-challenged mice when given 1 h after mixed-field radiation. The use of TDM-O in future experiments because of its greater effectiveness when compared to S-TDM must be weighed against the reported toxicity of native TDM preparations delivered in oil emulsions and the absence of toxicity of synthetic analogs of TDM at equivalent concentrations (25). We are continuing to explore the interaction of different types of radiation and S-TDM stimulation of TNF and IL-1.

REFERENCES