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Trehalose Dimycolate Enhances Resistance to Infection in Neutropenic Animals

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Bacterial infections are lethal complications of neutropenia, and antibiotics alone are inadequate therapy for these infections. Irradiated mice become severely neutropenic and remain susceptible to infection for 2 to 3 weeks, depending on the dose and quality of radiation. Some bacterial cell wall derivatives stimulate nonspecific host defense mechanisms against a variety of microbes which might cause postirradiation infection. In this study we determined if the cell wall glycolipid trehalose dimycolate (TDM), derived from *Mycobacterium phlei*, or a synthetic preparation of TDM was able to (i) enhance survival in mice when given before or after lethal doses of ^{60}Co radiation and (ii) increase nonspecific resistance to postirradiation infection. Treatment with TDM oil-in-water emulsions and with synthetic TDM significantly enhanced survival before and after lethal doses of ^{60}Co irradiation. This result correlated with the ability of TDM to reduce the translocation of intestinal bacteria and to stimulate hematopoiesis. With respect to nonspecific resistance to infection, TDM injected 1 h after sublethal irradiation increased resistance to a lethal *Klebsiella pneumoniae* challenge (10 50% lethal doses of *K. pneumoniae* in 30 days [$LD_{50/30}$]) 4 or 14 days later. Increasing the dose of *K. pneumoniae* to 5,000 $LD_{50/30}$ on day 4 overwhelmed the ability of TDM-treated mice to overcome infection. However, TDM treatment 1 h postirradiation combined with ceftriaxone antibiotic therapy (days 5 through 14) enhanced survival, even when the higher dose of bacteria (5,000 $LD_{50/30}$) was used. These results indicate that in irradiated mice, TDM can be used to enhance survival and, as a potent stimulant of nonspecific resistance to infection in neutropenic mice, can act synergistically with antibiotic therapy to reduce sepsis and mortality.

Bacterial infections in immunocompromised persons continue to be a major medical problem, occurring in at least several hundred thousand cases annually in the United States alone (5, 40). Prolonged neutropenia is associated with increased susceptibility to endogenous infections, often of enteric origin. Current therapeutic regimens do not assure survival. However, present antimicrobial and supportive therapies may soon be supplemented by new approaches which enhance the restoration of host immunocompetence. Some of these new approaches include the use of new immunomodulators to augment the production of mature cells in the bone marrow and stimulate existing nonspecific host defenses.

Ribi and colleagues (23, 28) developed immunomodulators that retain the beneficial properties of the bacterial cell walls from which they were obtained but are devoid of toxic cell wall contaminants. One of these, trehalose dimycolate (TDM), is a cell wall glycolipid produced by mycobacteria, nocardia, and corynebacteria (reviewed in references 10 and 12). It consists of 6,6'-diesters of α,α-D-trehalose and is an active component of Freund complete adjuvant (2). Another immunomodulator, monophosphoryl lipid A (MPL), is a chemical modification of toxic lipid A from heptoseless Re mutants of *Salmonella* species (23, 26, 27, 32, 33). It consists of a monophosphorylated diglucosamine unit substituted with ester- and amide-linked long-chain fatty acids. TDM and MPL have been shown to possess a variety of beneficial properties, including enhancement of resistance to bacterial and parasitic infections (8, 17, 19, 29, 38) and activation of macrophage activity (8, 25, 39), with the production of important mediators such as interleukin-1 (34), colony-stimulating factor, and interferon (14). Injection of MPL-TDM combined in an oil-in-water emulsion has been shown to increase synergistically the resistance of BALB/c mice to *Salmonella enteritidis* infection (29) and confer long-term protection in mice against lethal aerogenic challenge with influenza virus (15). Recently, we found TDM in oil-in-water emulsion to be more effective than MPL as a means of enhancing resistance to *Klebsiella pneumoniae* infection in irradiated and unirradiated B6D2F1 mice (13).

In this report, we evaluated the effects of TDM in mice made neutropenic by irradiation. High sublethal and low lethal doses of ionizing radiation cause a hematopoietic syndrome in which rapidly multiplying cells in the bone marrow are killed (4). If this damage is extensive enough, experimental animals will succumb to infection by endogenous flora (7). Bacteria generally appear in host organs about 9 to 10 days after lethal radiation exposure, and death begins within 1 to 2 days. All animals die within 2 weeks after exposure (37). In this report lethal- and sublethal-radiation models were used. In the latter, *K. pneumoniae* was given as a subcutaneous (s.c.) challenge 4 or 14 days after exposure to sublethal radiation and immunomodulator treatment. Therapy with the antibiotic ceftriaxone combined with TDM treatment postirradiation was also studied. The data showed the TDM increased survival and enhanced nonspecific resistance to infection in mice made severely neutropenic by irradiation.

MATERIALS AND METHODS

Mice. In one series of experiments, JAX:B6D2F1 female mice, 12 to 15 weeks old and weighing 20 to 25 g, were obtained from Jackson Laboratory (Bar Harbor, Maine). In a second series of experiments, C3H/HeN female mice, 10 to

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12 weeks old and weighing 20 to 25 g, were obtained from the National Cancer Institute Animal Breeding Facility (Frederick, Md.). All mice were quarantined upon arrival and screened for evidence of disease before being released from quarantine. They were maintained in plastic Micro-Isolator cages (Lab Products, Inc., Maywood, N.J.) on hardwood chip contact bedding and provided commercial rodent chow and acidified tap water ad libitum. Animal-holding rooms were maintained at 70 ± 2°F (ca. 21.1°C) and 50% ± 10% relative humidity, with at least 10 air changes per h with 100% conditioned fresh air. The mice were on a 12-h light-dark full-spectrum lighting cycle with no twilight. Research was conducted in an American Association for Accreditation of Laboratory Animal Care-accredited facility and reviewed by an Institution Animal Care and Use Committee.

Bacteria. A clinical isolate of *K. pneumoniae*, capsule type 5 (strain AFRRI [Armed Forces Radiobiology Research Institute] 7), that was kept frozen at −70°C in skim milk was grown overnight at 35°C on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.). Five typical colonies were inoculated onto 8 ml of brain heart infusion (BBL) and incubated overnight at 35°C. One milliliter of this overnight suspension was inoculated onto 9 ml of brain heart infusion. The culture was incubated at 35°C with shaking for approximately 2.5 h. The optical density of bacterial growth was monitored with a spectrophotometer at a wavelength of 550 nm. Late-log-phase cells were washed and suspended in cold 0.9% NaCl solution (saline) to yield 10^9 viable bacteria per ml. Dilutions were made in cold saline solution, inoculated onto Trypticase soy agar to determine CFU, and injected s.c. into mice.

Bacterial sepsis caused by the spread of endogenous microorganisms following lethal irradiation (10.25 Gy of ^{60}Co) was detected by the following procedure. Five mice were selected at random from treated and control groups on days 1, 3, 5, 7, 9, and 11 postirradiation. Mice were euthanized by cervical dislocation. The entire liver of each mouse was excised aseptically, added to 1 ml of sterile water, and homogenized in a sterile ground-glass homogenizer. The homogenate was diluted 1:2, and dilutions were made in sterile 0.9% NaCl solution. Appropriate suspensions (0.1 ml) were inoculated onto two plates of 5% defibrinated sheep blood in soybean casein digest agar and one plate of MacConkey agar. One soybean casein digest agar plate and the MacConkey agar plate were incubated in air and 5% carbon dioxide, and the other soybean casein digest agar plate was incubated in an anaerobic jar (GasPak; BBL) at 35°C. The numbers of CFU of the isolated bacteria per liver were calculated from the number of colonies that grew on each plate.

Irradiation. Mice were placed in aerated Plexiglas containers and bilaterally irradiated with a ^{60}Co gamma source administered at 0.4 Gy/min. The midline absorbed doses were 7.0 or 10.25 Gy for B6D2F1 mice and 8.0 and 8.5 Gy for C3H/HeN mice. The 50% lethal dose of radiation in 30 days ($LD_{50/30}$) for B6D2F1 female mice at AFRRI is 9.6 Gy, and that for C3H/HeN female mice is 7.9 Gy. The techniques of irradiation and dosimetric determination were previously described (29).

Immunomodulators and antimicrobial agent. (i) Saline suspensions. Natural TDM (derived from *Mycobacterium phlei*) and synthetic TDM (RIBI ImmunoChem Research, Inc., Hamilton, Mont.) were prepared as aqueous suspensions (TDM/s and S-TDM, respectively) by a modification of the procedure of Vosika and Gray (36) as described previously (13). Briefly, 2 mg of TDM or S-TDM per vial was solubilized in 0.2 ml of chloroform-methanol (9:1), placed in a 5-ml Potter-Elvehjem tissue homogenizer, and dried under nitrogen. TDM was homogenized for 4 min at 1,000 rpm in 5 ml of 0.2% Tween 80–saline solution. S-TDM was prepared essentially by the method of Numata et al. (16) by condensation of potassium mycolate containing 32 carbon atoms with 2,3,4,2′,3′,4′-hexa-*O*-trimethylsilyl-α,α-trehalose activated at the 6 and 6′ positions. Potassium mycolate was obtained by Claisen condensation of methyl palmitate, followed by sodium borohydride reduction and saponification, as described by Polonsky and Lederer (22). Potassium mycolate obtained by this method was a mixture of erythro- and threodiastereomers and was used without fractionation.

(ii) Oil-in-water emulsions. TDM emulsion (TDM/o) was reconstituted in accordance with manufacturer instructions (RIBI). TDM emulsions consisted of either 1 or 2% squalene in 0.2% Tween 80–saline solution. Control squalene emulsions (1 or 2%) were prepared by adding squalene (Sigma Chemical Co., St. Louis, Mo.) and 0.2% Tween 80 in saline to a tissue homogenizer and homogenizing the ingredients for 4 min at 1,000 rpm. All glassware used for the preparation of immunomodulators was depyrogenated by heating in air at 175°C overnight.

(iii) Drug. Ceftriaxone sodium (Hoffmann-La Roche Inc., Nutley, N.J.) was reconstituted with sterile distilled water to 90 mg/ml and diluted further to 18.75 mg/ml. A 0.1-ml amount was injected intramuscularly (i.m.) into the gluteus medius of each restrained mouse once per day for 10 days beginning 1 day after s.c. inoculation of bacteria. The daily dose was 75 mg/kg i.m. for a 25-g mouse. A 0.1-ml amount of pyrogen-free water was injected daily i.m. into each control mouse.

Ceftriaxone was assayed in sera by an adaptation of high-pressure liquid chromatography assay HL009 (Hoffmann-La Roche). This assay was performed on mouse sera collected 1.3 and 25.9 h after injection on day 5 of antimicrobial therapy from groups of five mice that received 10.0 Gy. Ceftriaxone therapy was begun 4 days after irradiation. The sera contained an average of 142.6 μg (±2.2 μg) of ceftriaxone per ml 1.3 h after injection and 2.3 μg (±0.6 μg) per ml 25.9 h after injection ($n = 5$). *K. pneumoniae* AFRRI 7 was found susceptible to an MIC of <8 μg of ceftriaxone per ml by an AutoMicrobic System (Vitek Systems, Inc., Hazelwood, Mo.).

E-CFU assay. Endogenous spleen CFU (E-CFU) were evaluated by the method of Till and McCulloch (35). C3H/HeN mice were given a total-body dose of 6.5 Gy to cause partial ablation of endogenous hematopoietic stem cells. Twelve days after irradiation, the mice were euthanized by cervical dislocation; their spleens were removed, weighed, and fixed in Bouin fixative; and the number of grossly visible spleen colonies was counted. Student's *t* test was used to evaluate E-CFU data statistically.

Survival measurements and statistical evaluation. Data for survival of mice in experimental groups during 30-day periods after irradiation were compared by the generalized Savage (Mantel-Cox) procedure (program 1L; BMD Statistical Software, Inc., Los Angeles, Calif.). Fifty percent lethal doses of radiation or *K. pneumoniae* were calculated by probit analysis or by the method of Reed and Muench (24).

RESULTS

Enhancement of survival by immunomodulators given before and after radiation exposure. Previous studies showed

TABLE 1. Percent 30-day survival of mice following lethal irradiation and treatment with TDM preparations[a]

Expt	Immunomodulator prepn (μg)	% 30-day survival with immunomodulator administered on:		
		−1 day	+1 h	+1 day
1 (n = 16)	S-TDM (100)	100[b]	56[c]	63[c]
	TDM/o (50) (1%)	94[b]	56[c]	56[c]
	Oil (1%)	81[c]	25[d]	13[d]
	RC (no injection)	12	12	12
2	TDM/s (100) (n = 20)	75[b]	45[d]	15[c]
	RC (0.2% Tween 80–saline) (n = 10)	20	40	0
3 (n = 20)	S-TDM (100)	NT[e]	20[c]	NT
	S-TDM (200)	NT	30[c]	NT
	S-TDM (400)	NT	30[c]	NT
	S-TDM (800)	NT	60[b]	NT
	RC (0.2% Tween 80–saline)		0	

[a] Three separate experiments were performed. Radiation controls (RC) were included in each experiment. Groups of 16 or 20 B6D2F1 mice received immunomodulator i.p. before (−1 day) or after (+1 h or +1 day) receiving 10.25 Gy of ^{60}Co. Data represent the percentage of mice that survived for 30 days in one of two replicate experiments. Levels of significance represent comparisons to 30-day survival data for untreated (radiation control) mice for each treatment time.

[b] $P < 0.001$.

[c] $P < 0.01$.

[d] Not significant ($P > 0.1$).

[e] NT, Not tested.

that injection of mice with endotoxin (30) increased hematopoietic recovery and survival when administered either 1 day before or, to a lesser extent, 1 h after exposure to lethal radiation. To test whether TDM increased postirradiation survival, we injected immunomodulators in saline suspension or oil emulsion intraperitoneally (i.p.) into groups of 16 or 20 B6D2F1 mice. Preparations were administered 24 h before mice received 10.25 Gy of ^{60}Co or 1 or 24 h after exposure.

All of the immunomodulator preparations and the oil control preparation enhanced survival to 70 to 100% ($P < 0.01$) when administered 1 day before irradiation (Table 1). Injection of mice 1 h after irradiation with either S-TDM or TDM/o enhanced survival to 56%. Increasing the dose of TDM/o to 100 or 200 μg per mouse 1 h postirradiation did not significantly increase survival more than that induced by 50-μg doses (data not shown).

Because of the possible side effects of oil emulsions, we determined the effect of increasing the dose of one immunomodulator, S-TDM, not in oil emulsion. S-TDM (100, 200, 400, and 800 μg per mouse) was injected i.p. into groups of 20 mice 1 h after exposure to 10.25 Gy of ^{60}Co. Survival increased in a dose-dependent fashion (Table 1).

Reduction of postirradiation endogenous infection by TDM. To determine whether the survival-enhancing effect of TDM could be attributed to a reduced spread of bacterial organisms, we gave groups of 20 mice 10.25 Gy of ^{60}Co and either 100 μg of TDM/o or 0.5 ml of saline solution (control) 1 h postirradiation. At 1, 3, 5, 7, 9, and 11 days after irradiation, the mice were euthanized by cervical dislocation and their livers were removed, homogenized, and cultured for bacteria. At 1, 3, and 5 days no bacteria were isolated from livers of either TDM/o- or saline-treated irradiated mice (Fig. 1). However, on days 7, 9, and 11 20 to 40 bacteria were isolated from livers of TDM/o-treated mice, as compared with 50 to 3,000 bacteria isolated from livers of saline-treated mice.

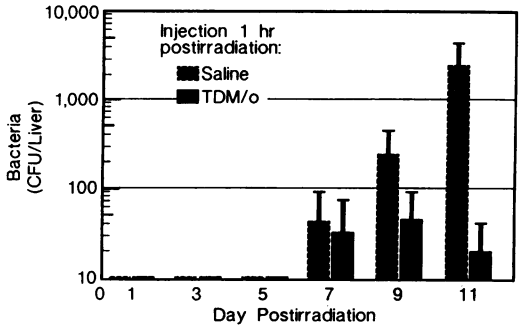

FIG. 1. Numbers of bacteria in livers of irradiated mice treated with TDM. Either saline or TDM/o (100 μg in 2% squalene oil emulsion per mouse) was injected i.p. into 20 B6D2F1 female mice 1 h after radiation exposure. Mice received 10.25 Gy on day 0. On the indicated days mice were euthanized and homogenized livers were cultured for numbers of bacteria. Each point represents the mean plus standard deviation of data obtained from five irradiated mice ($P < 0.5$ on day 11).

Stimulation of hematopoiesis by TDM. It was found in a separate experiment that the increased survival of lethally irradiated mice treated with TDM/o or S-TDM was overcome by increasing the dose of radiation to 11.00 or 11.75 Gy of gamma radiation (data not shown). However, all of the treated mice in these groups lived longer than control (untreated) mice. The diminished effects of TDM at higher doses of radiation could be due to a progressive destruction of hematopoietic stem cells. Thus, the E-CFU assay was used to determine the effects of TDM preparations on hematopoiesis. Various preparations and doses of TDM were injected i.p. into groups of 15 C3H/HeN mice either 1 day before or 1 h after irradiation with 6.5 Gy. Both TDM/o and S-TDM increased the number of E-CFU per spleen ($P < 0.01$) and spleen weight in a dose-dependent fashion when given either before or after irradiation (Fig. 2). The number of E-CFU per spleen increased two to six times over control levels, and spleen weights increased 1.8 to 3.0 times over control weights. Injection of 2% oil emulsion 1 h after irradiation increased the E-CFU number ($P < 0.05$). As a control, survival induced by TDM was also evaluated in C3H/HeN mice. Groups of C3H/HeN mice injected with TDM/o or S-TDM before receiving a high radiation dose exhibited 80 to 100% survival as compared with 0% for controls ($P < 0.001$). Increased survival was also seen when TDM/o or S-TDM was administered 1 h postirradiation ($P < 0.001$). Oil emulsions increased survival when given after ($P < 0.001$) but not before irradiation.

TDM-enhanced resistance to challenge with *K. pneumoniae*. To determine the range of susceptibility of mice following radiation exposure, we challenged groups of 16 mice s.c. with 10-fold dilutions of *K. pneumoniae* 1, 4, 7, 10, and 14 days after sublethal irradiation (7.0 Gy). The $LD_{50/30}$ of *K. pneumoniae* for unirradiated mice was 6.5×10^6 CFU. Susceptibility to infection increased by nearly 10,000-fold 4 days after irradiation. The $LD_{50/30}$ decreased to as low as 2.5×10^2 CFU on day 4 and remained in the range of $10^{2.3}$ to $10^{4.0}$ CFU in irradiated mice through day 10. Increased susceptibility correlated with the known reduction in the number of circulating leukocytes at this dose of radiation in B6D2F1 mice (T. B. Elliott and I. Brook, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, B 123, p. 44). Resistance to infection increased by day 14 postirradiation, when the $LD_{50/30}$ was 4.6×10^4 CFU.

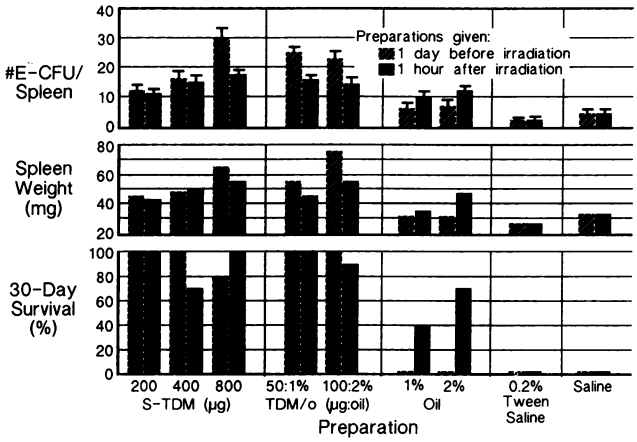

FIG. 2. E-CFU, spleen weight, and percent 30-day survival of lethally irradiated C3H/HeN female mice injected with TDM/o or S-TDM before or after radiation. TDM/o (50 or 100 μg in 1 or 2% squalene oil emulsion, respectively), S-TDM (200, 400, or 800 μg per mouse), squalene oil emulsion (1 or 2%; 0.5 ml per mouse) or 0.2% Tween 80–saline (0.5 ml per mouse) was injected i.p. either 1 day before or 1 h after radiation exposure. Controls received saline. Mice received 6.5 Gy for measurement of E-CFU and spleen weight as described in Materials and Methods. Survival was determined in mice injected either 1 day before receiving 8.0 Gy or 1 h after receiving 8.5 Gy. Each datum point for E-CFU represents the mean plus or minus standard error of data obtained from five mice. Each datum point for spleen weight represents the average value calculated from total weights of all spleens weighed at once ($n = 5$).

We then tested TDM/o and TDM/s preparations to determine which would increase the resistance of irradiated mice to *K. pneumoniae*. Groups of 16 mice received 7.0 Gy. TDM/o, TDM/s, oil, or saline was injected i.p. 1 h later. On day 4, mice were injected s.c. with approximately 10 $LD_{50/30}$s of *K. pneumoniae* (10^4 CFU per mouse). All preparations increased survival ($P < 0.01$) as compared with saline (control); however, TDM/o optimally increased survival to 80% ($P < 0.01$) after lethal challenge with *K. pneumoniae* (Fig. 3A).

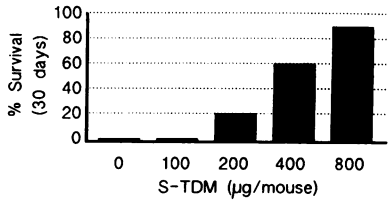

FIG. 4. Percent 30-day survival of sublethally irradiated mice treated with S-TDM and challenged with *K. pneumoniae*. B6D2F1 mice received 7.0 Gy. One hour later, S-TDM (100, 200, 400, or 800 μg per mouse) or saline was injected i.p. into the animals. Mice were challenged s.c. on day 4 with *K. pneumoniae* (10^4 CFU per 0.1 ml per mouse; 10 $LD_{50/30}$s). Results are from one of two experiments performed.

Next, we tested TDM preparations to determine the increase in survival following bacterial challenge 2 weeks after irradiation (Fig. 3B). Mice were injected i.p. with preparations of TDM 1 h after irradiation and challenged s.c. with 10 $LD_{50/30}$s of *K. pneumoniae* (5×10^5 CFU per mouse) 14 days later. Again, TDM/o enhanced survival most effectively.

In addition, as seen in radiation-induced endogenous infection, injection of mice with various doses of S-TDM increased survival in a dose-dependent fashion after *K. pneumoniae* challenge on day 4 (Fig. 4). Survival was increased to 90% with 800 μg per mouse, the highest dose of S-TDM tested. Injection of S-TDM at 100 μg per mouse 1 h postirradiation increased survival to 100% ($P < 0.001$) in mice challenged on day 14 with *K. pneumoniae* (5×10^5 CFU per mouse).

Decrease in the enhancement by TDM of survival from *K. pneumoniae* infection with higher challenge doses of bacteria. Results of two experiments showed that TDM-enhanced resistance to *K. pneumoniae* infection in irradiated mice can be opposed by increasing the challenge dose of bacteria. Groups of 16 mice were irradiated (7.0 Gy) and given

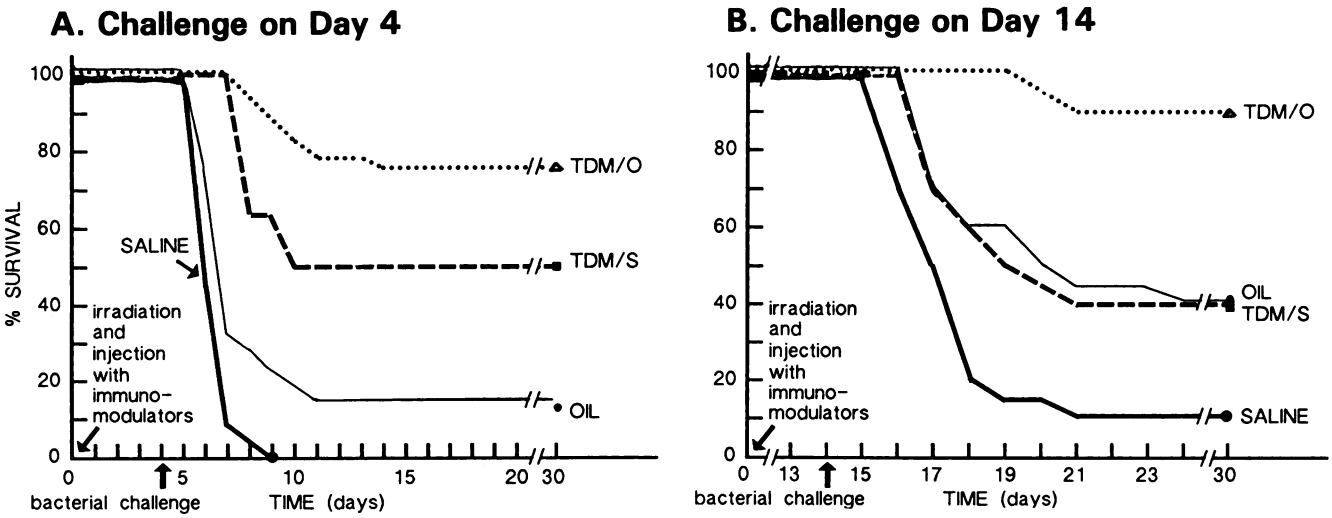

FIG. 3. Percent 30-day survival of sublethally irradiated mice treated with TDM and challenged with *K. pneumoniae*. (A) B6D2F1 female mice received 7.0 Gy. One hour later, 0.5 ml of one of the following preparations was injected i.p. into groups of 20 mice: TDM/o (100 μg in 2% oil per mouse), TDM/s (100 μg per mouse), 2% oil, or saline. Mice were challenged s.c. on day 4 with *K. pneumoniae* (10^4 CFU per mouse; 10 $LD_{50/30}$s). (B) B6D2F1 female mice were irradiated and injections were performed as described for panel A. Mice were challenged s.c. on day 14 with *K. pneumoniae* (5×10^5 CFU per mouse; 10 $LD_{50/30}$s). Results are from a typical experiment.

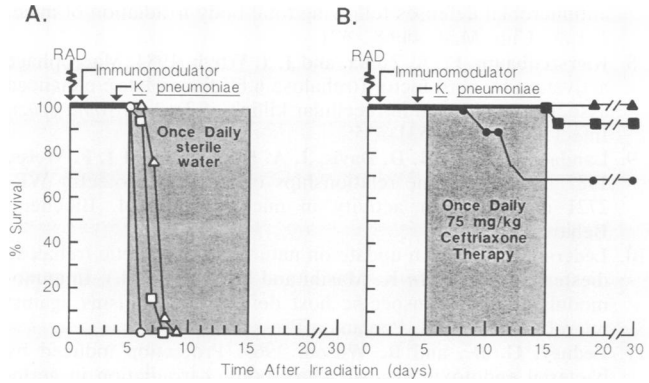

FIG. 5. Percent 30-day survival of mice treated with a combined therapy of TDM and ceftriaxone. Mice received 7.0 Gy of gamma radiation (RAD) followed by TDM/o (△ and ▲; 100 μg in 2% squalene oil emulsion per mouse), TDM/s (□ and ■; 100 μg per mouse), or saline (○ and ●). On day 4, mice were challenged with *K. pneumoniae* (5,000 $LD_{50/30}$s). From days 5 through 14 the mice were injected i.m. with either sterile, pyrogen-free water (A) or ceftriaxone (75 mg/kg) (B). Groups of 16 mice were used, except for saline i.p. and water i.m., for which groups of 10 mice were used. Results are from a typical experiment.

TDM/o, TDM/s, oil, or saline i.p. 1 h postirradiation. Four days later, the mice were challenged with 1.2×10^4, 1.2×10^5, 1.0×10^6, or 5.2×10^6 CFU per mouse. These doses corresponded to 10, 100, 1,000, and 5,000 $LD_{50/30}$s of *K. pneumoniae*, respectively, 4 days postirradiation. TDM/o enhanced the survival of mice given 10, 100, or 1,000 $LD_{50/30}$s of *K. pneumoniae* to 90, 68, and 22%, respectively. TDM/s enhanced the survival of mice given 10 or 100 $LD_{50/30}$s of *K. pneumoniae* to 62 and 18%, respectively. Although no difference was seen in the final numbers of surviving TDM/o-treated mice challenged with 5,000 $LD_{50/30}$s of *K. pneumoniae* or TDM/s-treated mice challenged with 1,000 or 5,000 $LD_{50/30}$s of *K. pneumoniae*, the mean survival times for these groups were longer than that for saline-treated mice ($P < 0.001$). Injection of a 2% emulsion of oil did not significantly enhance the survival of mice challenged with greater than 100 $LD_{50/30}$ of *K. pneumoniae*.

Because of the ability of TDM to enhance the survival of irradiated mice only from lower doses of bacteria challenge, we used a combined therapy of TDM to stimulate nonspecific resistance to infection and an antibiotic to kill a high dose of bacteria. Groups of 16 mice were given 7.0 Gy of gamma radiation and injected 1 h later with TDM/o, TDM/s, or saline. Four days later all mice were challenged s.c. with 5.2×10^6 CFU of *K. pneumoniae* per mouse (5,000 $LD_{50/30}$s). On day 5 postirradiation, a 10-day course of ceftriaxone or pyrogen-free water (control) was begun. The combined therapy of mice with one injection of 100 μg of TDM/o 1 h postirradiation and 10 days of ceftriaxone starting 1 day postchallenge enhanced survival from an otherwise lethal dose of *K. pneumoniae*, as compared with survival of immunomodulator- or antibiotic-treated controls (Fig. 5) (TDM/o-ceftriaxone versus ceftriaxone, $P = 0.0165$; TDM/o-ceftriaxone versus TDM/o, $P < 0.001$; and TDM/s-ceftriaxone versus ceftriaxone, $P < 0.05$). This experiment was repeated with 7.5×10^6 CFU *K. pneumoniae* and groups of 10 mice. The results were similar: for TDM/o-ceftriaxone versus ceftriaxone alone, the P value was 0.0137; for TDM/s-ceftriaxone versus ceftriaxone alone the P value was not significant ($P > 0.05$); but TDM/o-ceftriaxone was more effective than TDM/s-ceftriaxone ($P = 0.0443$).

DISCUSSION

The resistance of mice to infection during neutropenia was clearly augmented by treatment with TDM. As noted previously, TDM was superior to MPL in both the lethal radiation model (data not shown) and the sublethal radiation-challenge model (13). The difference in activities of the MPL and TDM preparations in neutropenic mouse models may relate to the specific physical natures of these substances, i.e., solubility and ability to interact with the membranes of certain mammalian cells, e.g., macrophages. In electron micrographs, MPL appears as lipid bilayers, whereas TDM forms crystal like structures (18). Further, we recently measured the effects of endotoxin, MPL, and TDM on the phase transition of lipid vesicles (E. M. Movius and G. S. Madonna, unpublished observations). In this system both endotoxin and TDM caused a significant perturbation of the membrane lipids, but MPL had virtually no effect on the molecular packing of the membrane lipids.

The effects of TDM were improved when it was emulsified in squalene (oil) rather than suspended in saline. However, S-TDM at increased doses was equivalent to TDM/o when used at various times prior to or after radiation in either of the two models studied.

As in earlier work with endotoxin (11, 30), optimal protection with the immunomodulators occurred when they were administered 1 day prior to radiation. Significant but lesser effects could be obtained with TDM given 1 h after radiation. If immunomodulators stimulate the production of mediators by macrophages at the same time that these cells are activated by radiation (6), it is possible that the combined effects could counter the otherwise beneficial effects of immunomodulators. On the other hand, the reactivity of macrophages with TDM could change at various times relative to the time of radiation exposure. Another factor which probably contributes to the time effect is the extent of radiation-induced depletion of bone marrow cells upon which macrophage-derived mediators may act following stimulation by TDM.

Although the survival of mice given lethal radiation doses would benefit in part from the enhancement of marrow repair, the protective effect of TDM was seen before significant marrow regeneration could occur in mice given sublethal radiation doses and challenged with *K. pneumoniae* (20, 21). Enhanced macrophage antimicrobial activity probably accounts for the short-term beneficial effects of TDM (20, 25). Macrophages from irradiated mice are able to kill *K. pneumoniae* more effectively when stimulated in vitro with TDM than are untreated macrophages from irradiated mice (G. D. Ledney, G. S. Madonna, and S. P. Chock, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, E71, p. 120). Based on the results with *K. pneumoniae*-challenged mice, increased nonspecific resistance lasted at least 2 weeks. Survival at this time after treatment may have been augmented somewhat by hematopoietic activity.

In general, as the concentration of immunomodulator increased, so did survival. Microgram quantities of TDM enhanced survival following radiation and caused no apparent toxic effects (ruffled fur, diarrhea, eye exudate). This conclusion was substantiated in part by a behavioral study in which TDM/s-treated mice maintained normal locomotor activity (M. R. Landauer, personal communication). This

behavioral paradigm has previously been used to assess the toxicity of immunomodulators (M. R. Landauer, M. L. Patchen, and H. D. Davis, Abstr. 4th Int. Conf. Immunopharmacol. 1988, p. 91) and radioprotectors (9).

The finding that squalene provided protection in both the lethal irradiation and sublethal irradiation-bacterial challenge models is noteworthy. Because squalene is a long-chain polyunsaturated fatty acid, it is possible that its multiple bonds can be peroxidized by oxygen-free radicals, which are known to mediate many of the cytotoxic effects of radiation (1). The effect would be a reduction in the number of free radicals available to attack cell membranes, thus reducing cell damage. In support of this theory, some sulfhydryl-containing radioprotectors have been shown to work by reducing oxygen-free radicals (3). Squalene, like any inflammatory agent, could also increase low-level nonspecific resistance to infection by stimulating macrophages. Indeed, present and previous data (13) indicate protection against low but not high doses of bacterial challenge.

In a clinical situation TDM should help protect against opportunistic infections. If overwhelming infections occur, then the immunomodulator would augment the effectiveness of conventional antibiotic therapy. This action was demonstrated in our studies with increasingly larger challenges with *K. pneumoniae* and ceftriaxone treatment. In the future, TDM preparations, particularly S-TDM, may be used to increase nonspecific resistance to infection along with conventional antimicrobial therapy of immunosuppressed individuals. In addition, TDM may be used as an important component of regimens in which cytokines and cell growth factors are used to restore the function or number of cell populations essential in normal host resistance.

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