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High-dose Antibiotic Therapy Is Superior to a 3-Drug Combination of Prostanoids and Lipid A Derivative in Protecting Irradiated Canines

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Antibiotics / Radioprotection / Canines / Prostanoids / Lipid A

There is an urgent need to develop non-toxic radioprotectors. We tested the efficacy of a 3-drug combination (3-DC) of iloprost, misoprostol, and 3D-MPL (3-deacylated monophosphoryl lipid A) and the effects of postirradiation clinical support with high doses of antibiotics and blood transfusion. Canines were given 3-DC or the vehicle and exposed to 3.4 Gy or 4.1 Gy of ^{60}Co radiation. Canines irradiated at 4.1 Gy were also given clinical support, which consisted of blood transfusion and antibiotics (gentamicin, and cefoxitin or cephalexin). Peripheral blood cell profile and 60-day survival were used as indices of protection. At 3.4 Gy, 3-DC- or vehicle-treated canines without postirradiation clinical support survived only for 10 to 12 days. Fifty percent of the canines treated with 3-DC or vehicle and provided postirradiation clinical support survived 4.1-Gy irradiation. Survival of canines treated with vehicle before irradiation significantly correlated with postirradiation antibiotic treatments, but not with blood transfusion. The recovery profile of peripheral blood cells in 4.1 Gy-irradiated canines treated with vehicle and antibiotics was better than drug-treated canines. These results indicate that therapy with high doses of intramuscular aminoglycoside antibiotic (gentamicin) and an oral cephalosporin (cephalexin) enhanced survival of irradiated canines. Although blood transfusion correlated with survival of 3-DC treated canines, there were no additional survivors with 3-DC treated canines than the controls.

INTRODUCTION

Exposure to ionizing radiation can occur under a variety of scenarios. Recent incidences of terrorism, deterrence by nations with nuclear weapons capability, transport of radioactive waste materials, aging nuclear power plants, and prolonged space travel are some of the avenues, where exposure to ionizing radiation can occur accidentally or intentionally. The criticality accident in Tokai-mura, Japan[1)], is the latest example of one of the many ways exposure can accidentally occur in a nuclear fuel-operated power generating facility. The victims of this accident could not be saved, despite an aggressive application of currently available treatments, whose origins span some 50 years of research in developing treatments for radiation injuries (prophylactic and therapeutic). From standard off-the-shelf prophylactic chemicals like cysteine[2)] and recombinant products like IL-1[3)] (interleukin-1), most have undesirable toxic side effects that impeded their

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Abbreviations: 3-DC, 3-drug combination; 3D-MPL, 3-deacylated monophosphoryl lipid A; AFRRI, Armed Forces Radiobiology Research Institute

development beyond preclinical large-animal models, as radioprotectors for human use. As toxicity became a limiting factor in the development of radioprotective drugs, the search for non-toxic and effective drugs was redirected on the basis of mechanistic factors. Since radiation is known to generate free radicals in cellular aqueous milieu, a large number of previous studies[4–7] used free radical scavengers, alone or in combination, as radioprotectors. Amifostine, the ëgold standardí for radioprotection, is an example of a radioprotector that protects by free radical scavenging as well as by ensuring the fidelity of DNA repair[8] and other mechanisms.

We reported that 3D-MPL provided radiation protection in mice over a wide range of doses and did not exhibit the toxicity that characterized the parent lipid A at the same doses[9]. We also reported that two prostaglandins – iloprost and misoprostol – are radioprotective and that in non-toxic combination provided synergistic protection in mice[10]. When the combination of iloprost, misoprostol, and 3D-MPL was used in mice there was additive protection (unpublished results). Based on this finding, the drug combination was transitioned to a larger, long-lived animal model (canines) with and without clinical support. Since previous studies have shown that clinical support enhanced radioprotection efficacy of cytokines in canines, we have tested the effect of clinical support on 3-DC. The results of these studies are presented here.

MATERIALS AND METHODS

Animals

Animal holding, care, handling, and experimental procedures were approved by the Armed Forces Radiobiology Research Institute (AFRRI) Animal Care and Use Committee. Research was conducted according to the *Guide for the Care and Use of Laboratory Animals*, prepared by the Institute of Laboratory Animal Resources, National Research Council, US National Academy of Sciences. Purpose-bred beagles weighing 9 to 10 kg were housed in environmentally controlled rooms of the AFRRI animal facility, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care-International. The canines were used for the experiments after a 2-week quarantine, physical examination, and serological test for infection. All canine holding rooms were maintained at 21 ± 2°C with 10 cycles of change with fresh air daily and a relative humidity of 50 ± 10%.

Drugs and chemicals

Antibiotics were obtained from the pharmacy of the Uniformed Services University of the Health Sciences, Bethesda, MD, USA through the Veterinary Sciences Department (VSD) and were dispensed under guidance from VSD. Iloprost and misoprostol were obtained from Schering AG, Germany, and Searle, Skokie, USA, respectively. 3D-MPL (3-deacylated monophosphoryl lipid A) was purchased from Ribi Immunochem, Hamilton, MT, USA.

3D-MPL is a purified product from *Salmonella minnesota Re* 595 lipopolysaccharide (LPS) obtained by acid hydrolysis. It is an immunostimulant, which has only 100 to 10,000-fold less acute lethal toxicity as compared to parent LPS. Misoprostol (Cytotec) is an esterified prostaglandin E derivative. It undergoes hydrolysis to its free acid, which is the active component. Iloprost is chemically stable analogue of prostacyclin, which is a vasodilator, platelet suppressant, fibrinolytic, and cytoprotective agent. Cefoxitin and cephalexin are cephalosporin antibiotic active against a variety of gram-positive and gram-negative bacteria such as *Streptococci* and *Escherichia coli*. Gentamicin is an aminoglycoside antibiotic active against a variety of microorganisms such as *Escherichia coli* and *Pseudomonas aeruginosa*.

All other chemicals and drugs were purchased from general vendors.

Irradiation

Irradiation was done in the AFRRI ^{60}Co facility. The AFRRI Dosimetry Division conducted the dosimetry using a canine phantom. Canines were placed in well-ventilated Plexiglas boxes. The canines were par-

tially restrained, with their head and neck unrestrained. Canines were irradiated, two at a time, bilaterally at a dose rate of 40 cGy/min for a total radiation dose of either 3.4 Gy or 4.1 Gy, as measured at midline tissue depths. The radiation doses, 3.4 Gy and 4.1 Gy were selected to reflect, respectively, about 30% and 60% increase in the $LD_{50/30}$ radiation dose after various treatments compared with that of the untreated control. The $LD_{50/30}$ radiation dose for untreated control canine was estimated to be 2.6 Gy[11,12].

Preirradiation and postirradiation treatment

Two experiments were conducted on two canine groups (drug-treated and control). In experiment 1, each of four canines in the drug-treated group was given a preirradiation, intravenous injection of a 3-ml mixture containing the following drugs: 0.003 mg/kg of iloprost, dissolved in a solution containing 0.9% NaCl and 10 mM Tris (pH 8), 0.003 mg/kg of misoprostol, dissolved in a vehicle containing 0.9% NaCl and 0.005% alcohol, and 0.5 mg/kg of 3D-MPL, dissolved in 0.5% triethanolamine. Each of four canines of the control group received only 3 ml of vehicle. Two of the drug-treated canines had some transient discomfort and one had mild diarrhea 10 minutes after administration of the drug combination. Vehicle-treated canines remained clinically normal and did not exhibit any signs of toxicity. Twenty minutes after the drug administration, all canines were normal; and 30 minutes after the drug/vehicle administration, all canines were irradiated at 3.4 Gy. Each irradiation was conducted with one canine from the drug-treated group and one from the control group. No clinical support was provided after irradiation in experiment 1.

In experiment 2, the radiation dose was 4.1 Gy and postirradiation clinical support was provided to 10 canines in both drug treated and control groups.

Clinical support consisted of antibiotics and blood transfusion. When the white cell (WBC) count dropped below 1,000/μl of blood or the body temperature exceeded 39.4°C, cefoxitin (90 mg/kg) and gentamicin (6 mg/kg) were given intramuscularly (IM) twice a day (BID) for the first day and once a day thereafter until the white cell count returned to 5,000/μl of blood. Since the injection site appeared irritated, after three days of cefoxitin treatment, it was replaced by oral administration of cephalexin (40 mg/kg, BID) so that only gentamicin needed to be given IM. Blood obtained from matched donors and irradiated at 15 Gy was given when the platelet count dropped below 40,000/μl of blood and the hemoglobin level dropped to 6.6 g/dL or packed cell volume (PCV) was less than 20%. Table 1 summarizes preirradiation and postirradiation treatments.

Table 1. Summary of preirradiation and postirradiation treatment protocols of canines

Exp #	# of Canines	Rad. Dose (Gy)	Treatment 30 minutes preirradiation	Postirradiation treatment	
				Indication	Treatment
1	4	3.4	Iloprost and misoprostol (0.003 mg/kg each), 3D-MPL (0.5 mg/kg), all in 3-ml	No treatment	
	4	3.4	10 mM Tris, pH 8.0, 0.005% ethanol, and 0.9% NaCl, all in 3-ml		
2	10	4.1	Iloprost and misoprostol (0.003 mg/kg each), 3D-MPL (0.5 mg/kg), all in 3-ml	Platelet count < 40,000/μl	Blood transfusion
	10	4.1	10 mM Tris, pH 8.0, 0.005% ethanol, and 0.9% NaCl, all in 3-ml	WBC count < 1,000/μl or body temp >39.4°C	Gentamicin, 6 mg/kg, and Cefoxitin, 90 mg/kg, or cephalexin, 40 mg/kg

Iloprost was dissolved in a solution of 10 mM Tris, pH 8.0, in 0.9% NaCl. Misoprostol was dissolved in 0.005% alcohol in 0.9% NaCl. 3D-MPL was dissolved in 0.5% triethanolamine in 0.9% NaCl.

Parameters of radiation protection

The canines were monitored in the morning and evening every day for temperature, pulse, and respiratory rate for 60 days after irradiation. On days 1, 3, 7, 10, 14, 17, 21, 24, 28, 30, 40, 50, and 60 after irradiation, blood was collected and standard hematological and clinical chemistry parameters were determined. These included total and differential cell counts and estimation of various serum analytes and enzymes. In those canines surviving after 60 days, blood samples were collected periodically till the cell counts returned to the normal range. Survival at the end of 60 days and the return to normal blood profile were used as parameters of radioprotective efficacy.

Statistical analysis

Statistical analysis of data on the relationship between blood transfusion and antibiotics treatments and survival was conducted with the Jonckheere-Terpstra test for trend, using StatXact-4 software, Cytel Software Corporation, Cambridge, MA. The p-values were exact and two-sided.

RESULTS

Radioprotective efficacy of the drug combination
Experiment 1 at 3.4 Gy

There was no survival protection by the three-drug combination (Fig. 1A).

Although the experiment was planned for 10 canines in each group (drug-treated and control), the experiment was terminated after 4 canines in each group showed no difference. All canines died within 10 to12 days after irradiation. There was no difference in the recovery profile of blood cells (erythrocytes, platelets, neutrophils, and lymphocytes) in drug- or vehicle-treated canines. In fact, the blood cells levels never recovered in either of the groups.

Experiment 2 at 4.1 Gy

Since the canines in experiment 1 might have died due to lack of clinical support, the second experiment provided clinical support described under 'Materials and methods'. It is known from previous studies[13] from this Institute that $LD_{50/30}$ radiation dose for canines with clinical support alone is 3.4 Gy, com-

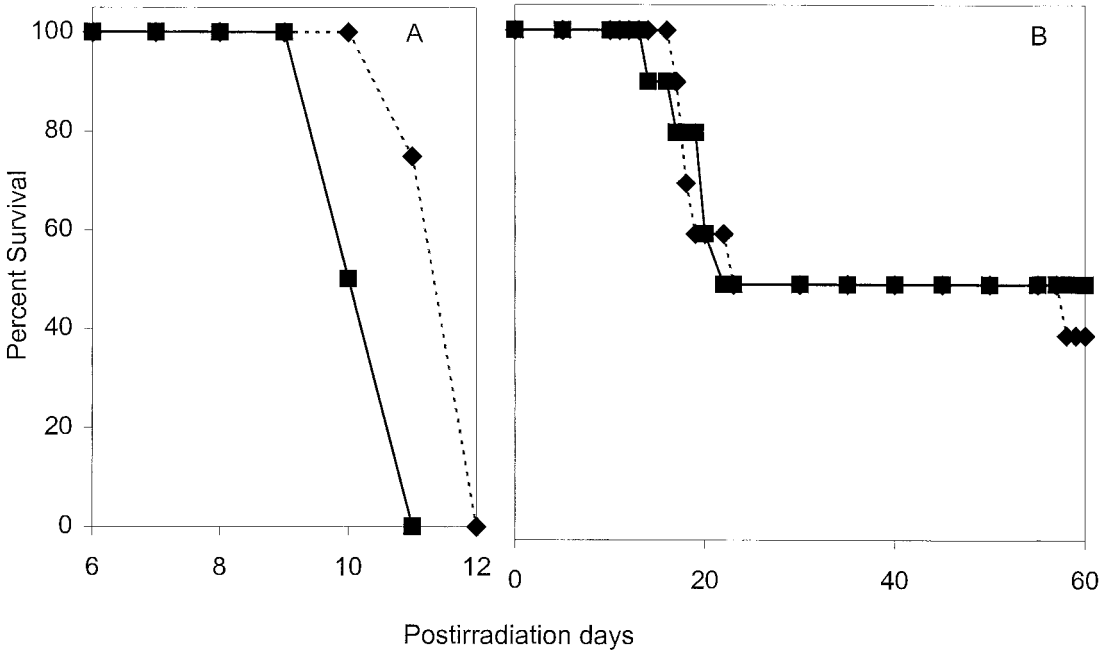

Fig. 1. Survival patterns of canines treated intravenously with vehicle (■) or a drug combination (◆) of 0.003 mg/kg of iloprost and 0.003 mg/kg of misoprostol and 0.5 mg/kg of 3D-MPL 30 minutes before irradiation at 3.4 Gy (A, without clinical support) and 4.1 Gy (B, with clinical support).

pared with the $LD_{50/30}$ radiation dose of 2.6 Gy without clinical support. Hence we used a radiation dose of 4.1 Gy, expecting at least a 20% increase in radiation protection when radioprotective drugs were given before providing clinical support postirradiation. However, there was no significant difference (Fig. 1B) in the survival of the animals in the group provided clinical support alone and the group given radioprotective drugs and clinical support. In both groups, 50% of the animals survived at least 57 days after irradiation (n = 10 each) indicating that the approximate $LD_{50/30}$ radiation dose for these groups was 4.1 Gy and that the three-drug combination had no effect on the survival of irradiated canines.

Response to clinical support

There were two important components in the clinical support regimen – blood transfusion and antibiotics. Fig. 2 compares survival for the treated and control canines in terms of the number of blood transfusions (2A) or the total volume of blood transfused (2B). Fig. 3 compares survival in terms of the number of antibiotics treatments.

Statistical analysis of data on the relationship between blood transfusion and antibiotics treatments and survival using the Jonckheere-Terpstra test for trend, and StatXact-4 software, indicated that, with the three-drug combination, both the number of transfusions (Fig. 2A) and the volume of transfusions (Fig. 2B) were positively associated with survival (p = 0.01 and 0.01 respectively). The data shown later (see Figs. 4A–D) that the drug treated animals could not sustain higher cell numbers in blood as in controls, suggest that endogenous production of blood cells was suppressed. Therefore, the positive correlation observed

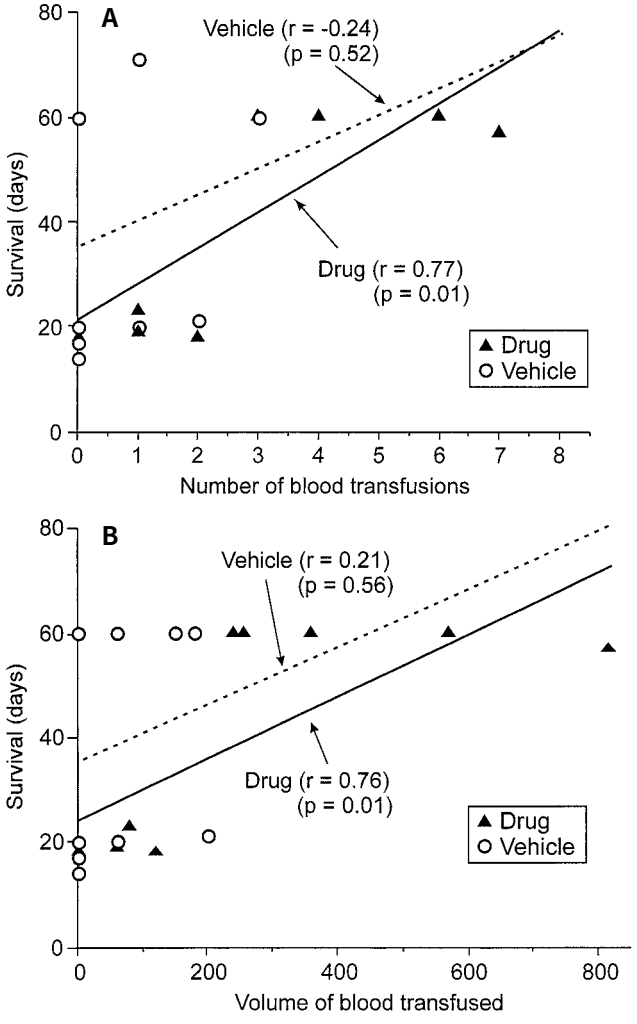

Fig. 2. Relationship between the number of blood transfusions (A) and the volume of blood transfused (B) and survival days in canines treated intravenously with a drug combination (▲) of 0.003 mg/kg of iloprost and 0.003 mg/kg of misoprostol and 0.5 mg/kg of 3D-MPL or vehicle (○) 30 minutes before irradiation at 4.1 Gy and given clinical support after irradiation. r = correlation coefficient.

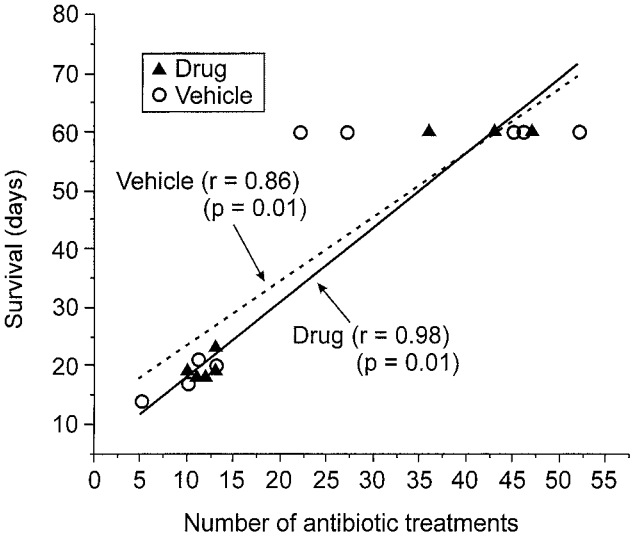

Fig. 3. Relationship between antibiotics treatments and survival in canines treated intravenously with a drug combination (▲) of 0.003 mg/kg of iloprost and 0.003 mg/kg of misoprostol and 0.5 mg/kg of 3D-MPL or vehicle (○) 30 minutes before irradiation at 4.1 Gy and given clinical support after irradiation. r = correlation coefficient.

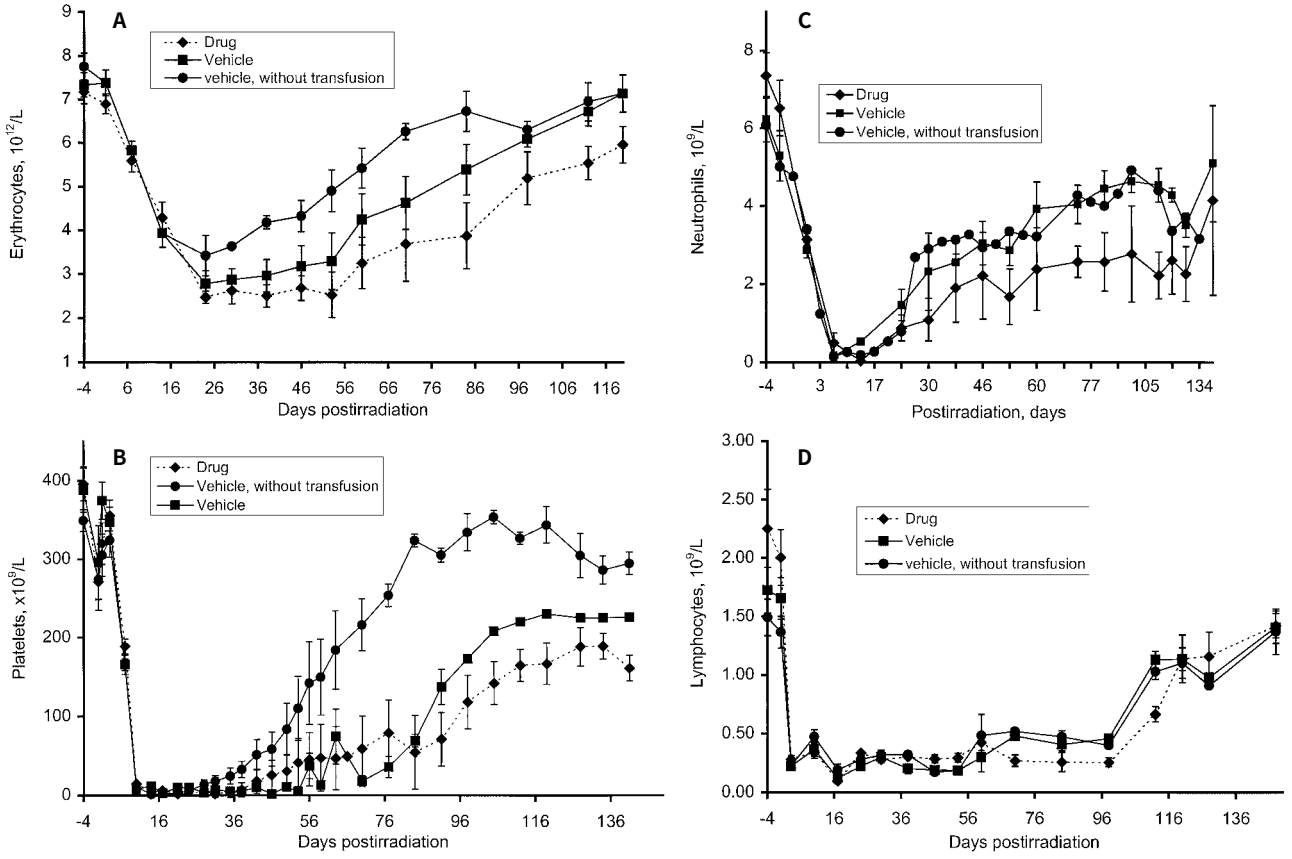

Fig. 4. Recovery profile of erythrocytes (A), platelets (B), neutrophils (C), and lymphocytes (D) in canines treated with a combination of 0.003 mg/kg of iloprost and 0.003 mg/kg of misoprostol and 0.5 mg/kg of 3D-MPL or vehicle 30 minutes before irradiation at 4.1 Gy and given clinical support after irradiation. (◆) drug-treated, (■) vehicle-treated with transfusion, (●) vehicle-treated without transfusion.

between blood transfusion and survival in drug treated canines may be mainly due to the replenishment of cells in blood by transfusion. Although there was statistical correlation between survival and blood transfusion in drug-treated animals, the canines that received the largest number and volume of blood transfusions died three-days before the end of the 60-day postirradiation period. Without the drug treatment, however, the significance between survival time and transfusion was lost ($p = 0.52$ and 0.56 respectively).

The antibiotics treatments showed a positive correlation with survival, both with and without drug treatment (Fig 3. $p < 0.01$ and < 0.01 respectively). Since the three-drug combination had no beneficial effect in the absence of blood transfusion, the protection that is seen in both groups – drug-treated and vehicle-treated- was probably due to the antibiotic treatment given to both groups. There was no positive correlation between blood transfusion and survival in the vehicle-treated canines. Hence, antibiotics treatment appears to be the main factor responsible for the survival of vehicle-treated canines.

Fifty percent of the vehicle-treated canines with antibiotics support survived 60 days although they received fewer transfusions and some of the surviving animals had only antibiotics support and no transfusions.

Recovery profile of peripheral blood cells

The profiles for erythrocytes, platelets, neutrophils, and lymphocytes in canines irradiated at 4.1 Gy and given clinical support indicate that the blood cells recovered faster in the vehicle treated than the drug-treated canines. The data are given in figure 4.

Blood transfusion did not seem to help in the recovery of blood cells. The profiles of blood cell

recovery in canines not transfused were better than in canines that were transfused (Figs. 4A–4D). The recovery profile of blood cells in vehicle-treated canines was better than that in drug-treated canines. Thus, neither blood transfusion nor the three-drug combination facilitated the recovery of peripheral blood cells. The only clinical support, other than blood transfusion, that the vehicle-treated canines received was antibiotics. Therefore, antibiotics seemed to have played a major role in the recovery of blood cells, probably by virtue of limiting infection and sepsis and in turn by promoting survival. The number of daily antibiotic treatments in both the control and drug treated groups may be critical in promoting survival (Fig. 3). Fewer than 20 treatments were less effective whereas treatments in excess of 20 provided extended survival and recovery.

DISCUSSION

The three-drug combination did not provide the expected protection for canines irradiated at lethal doses of 3.4 Gy (without clinical support) and 4.1 Gy (with clinical support). As expected at 3.4 Gy, there were no survivors in the control group. But the absence of any survivors in the drug-treated group was unexpected. Considering the protection obtained with mice using 3-DC, the results obtained with canines in the 3-DC treated groups at 3.4 Gy and 4.1 Gy was dismal. However, when clinical support was given, 50% of the animals survived at the higher radiation dose of 4.1Gy even in the absence of treatment with the 3-DC. The 3-drug combination does not seem to have any additional advantage. Blood cell numbers indicate that 3-DC, in fact, suppressed the hematopoiesis. It had been reported that E-type prostaglandins (such as misoprostol in 3-DC) inhibited the proliferation of committed granulocyte-macrophage progenitor cells[14]. The lack of efficacy with 3-DC in canines as compared to mice may be due to the low doses of prostanoids used in canine study. In radioprotection studies with mice, 0.1 mg/kg each of misoprostol and iloprost could be used without any toxic manifestations. But such concentrations were instantaneously lethal to canines. The tolerable doses were only 0.003 mg/kg each. Thus, although we used doses that were not toxic or only marginally toxic in both species, the combination used in canines was not efficacious.

The lack of protection against lethality in canines by 3-DC as against the effectiveness in mice indicate the caution that may be exercised in applying radioprotection data obtained from these species to humans. Several factors may be considered before extrapolating the data to humans. Firstly, in lower animals like mice and rats, the rate of metabolic disposal of the 3-DC drugs may be very high so that a higher dose can be used for radiation protection without any adverse effects. In large animals like canines, if the rate of metabolic disposal of the drugs is low, the toxicity will be more severely manifested limiting the dose that can be used for radiation protection. Secondly, therapeutic window in each species for a specific drug/ drug combination should be considered. With highly potent drugs like prostanoids and endotoxin, the therapeutic ratio will determine the dose range that can be used safely. Finally, it appears that the size of the animals must be an important consideration for radioprotection. Size is important not only in transitioning the drug dose from lower to higher animals to humans, but also in determining the appropriate radiation dose to be used in the studies.

There was no increase in survival with drug treatment and clinical support than with clinical support alone. Although we did not perform tests for renal toxicity, high concentrations of gentamicin are known[15] to cause nephrotoxicity. Since misoprostol is known[16] to aggravate the renal toxicity of gentamicin, the presence of these two drugs (gentamicin and misoprostol) and a renal system already compromised by radiation might have led to the mortality of canines, eliminating any protective advantage that could have been provided by the three-drug combination. It was reported[17] that chronic renal disease was the common cause for mortality when canines were irradiated in juvenile periods of life. But the doses of antibiotics that we used (gentamicin, 6 mg/kg body weight and cephalexin, 90 mg/kg body weight) were the same as in earlier

study by Natanson *et al*.[18], and the dose of gentamicin that we used was lower than the dose known (10 mg/kg body weight) to cause nephrotoxicity.

Radiation induced neutropenia, thrombocytopenia, and associated infectious and hemorrhagic complications all tend to severely compromise the host defense system, and in turn, elevate the risk to radiation lethality[19]. Survival depends upon the radiation dose and dose rate[20]. Even if the acute radiation dose is moderately high and potentially lethal or if the dose is high but the exposure is protracted over time, a critical number of hemopoietic stem cells are generally spared; and if the canines can be kept free from infection during the neutropenic period, they can recover from irradiation exposure, spontaneously or through pharmacologic intervention.

MacVittie *et al*.[13] and Schuening *et al*.[21] showed that therapeutic intervention with recombinant human granulocyte-colony stimulating factor (rhG-CSF) could significantly increase the survival of canines exposed to a supra-lethal dose of ^{60}Co radiation. The antibiotic support regimen that we used in our current study (namely, cefoxitin and gentamicin) resulted in 50% survival of animals even at a higher radiation dose of 4.1 Gy. This rather subtle change in antibiotic regimen when compared to earlier antibiotic regimen[12,13] yielded significant survival advantage to the irradiated canines.

It was known for a long time that antibiotics used singly[22] or in combination with whole blood[23] was effective in protecting canines from lethality at the LD_{50} radiation dose. Allen *et al*.[24] showed that blood transfusion was ineffective in reducing the mortality of irradiated canines. Although we used blood transfusion when necessary, many of the canines that were not transfused also survived the radiation dose of 4.1 Gy, indicating that antibiotics may be the most important factor in clinical support.

Our findings indicate the therapeutic benefits of an aminoglycoside antibiotic (gentamicin) and a cepahalosporin, which increased the approximate $LD_{50/30}$ from 3.4 Gy reported earlier[13] to 4.1 Gy representing a significant gain in antibiotic therapy-induced radioresistance. The increase in survival that we obtained may have been due to several reasons. Firstly, although the aminoglycoside antibiotic used by MacVittie *et al*.[13] and by us was same (gentamicin), we used a dosage about four fold more (6 mg/kg as compared to 1.6 mg/kg). We used a second-generation cepahalosporin (cefoxitin) for three days after which we used a first-generation cephalosporin (cephalexin) orally. Secondly, the dose of cephalosporin antibiotic that we used was 18-fold (90 mg/kg vs. 5 mg/kg) more cefoxitin or 8-fold (40 mg/kg vs. 5 mg/kg) more cephalexin than in previous studies. Thirdly, antibiotics administration was continued in our protocol until the white cell count returned to 5,000/μl whereas, in the previous studies, the antibiotics were discontinued when the white cell counts reached 1000/μl. Finally, the different routes of antibiotics administration may also have a beneficial impact on the survival of irradiated canines.

The mechanisms by which an altered regimen of antibiotics worked better to increase survival is not clear, particularly since the class of antibiotics used in both protocols were same. One explanation could be the differential resistance of the cephalosporin antibiotics at high doses versus low doses to β-lactamases produced by bacteria. β-lactamases can hydrolyze the cephalosporin antibiotics[25] and render them inactive against bacteria. The higher dose of cephalosporins used might have overwhelmed the β-lactamases, resulting in the increased efficacy. The bactericidal activity of cephalosporin may generate endotoxin[26], which by itself may cause lethal septic shock. Use of a combination of high doses of aminoglycoside antibiotic and cephalosporin antibiotic reduces the chances of endotoxic shock since aminoglycosides are known to bind endotoxin and render it less toxic[27].

Another mechanism for enhanced protection may have been due to the oral administration of cephalexin after using cefoxitin for three days. The presence of an antibiotic in the gastrointestinal system could disinfect intestinal bacterial flora, which could eliminate any chance of bacterial translocation from intestine to blood. In this respect cefoxitin appears to be similar to OK-432 (a pharmaceutical preparation of low-virulent *Streptococcus pyogenes*), which decreased radiation

induced bacterial translocation in mice[28]. Coupled with the systemic administration of gentamicin, oral administration could have minimized infection and, hence, increased survival.

Irrespective of the mechanisms of protection, an antibiotic regimen consisting of a high dose of intramuscular aminoglycoside and oral cephalosporin started very early after irradiation and continued until the white blood cell counts return to the normal range can increase survival from lethal radiation exposures to an extent not previously recognized. Use of enhanced doses of antibiotics offers another approach to increase rates of survival from potentially lethal radiation exposures. When translated to a radiation exposure scenario, an increase in $LD_{50/30}$ from 3.4 Gy (DRF 1.3 using 2.6 Gy as $LD_{50/30}$ without clinical support) to approximately 4.1 Gy (approximate DRF 1.57), as reported in this paper, can increase the survival from 30% to 57%. Identification and use of an appropriate prophylactic agent coupled with high-dose antibiotic therapy may considerably increase the rates of survival of first responders in radiation rescue scenarios following an accidental or intentional radiation exposure.

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