RADIOPROTECTION OF BONE MARROW STEM CELLS BY SUPEROXIDE DISMUTASE

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SUMMARY In mouse bone marrow cells the radioactivity from intravenously injected ^{125}I-superoxide dismutase reached its maximum level 1 h later. The 1 h interval was used while examining the effect of the unlabelled enzyme on the proliferative capacity of X-irradiated hematopoietic stem cells, as measured by their ability to form colonies in host spleens. When administered to donor mice in the amount of 35 µg/g body weight, the enzyme protected the cells, exposed to 350 rads, by a factor of 2.16 ±0.18. At doses of 15, 70, and 100 µg/g, this factor was reduced to 1.44 ± 0.18, 1.15 ± 0.15, and 0.7 ± 0.1,respectively. The value of 2.16 at 35 µg/g was increased to 2.5 by giving an additional therapeutic dose of the same size 1 h after the X-irradiation.

INTRODUCTION Recently, it was shown that an intravenous dose of bovine superoxide dismutase protected Swiss white mice from the lethal effects of ionizing radiation (1). This protection was elicited within a radiation dose range where depression of hematopoiesis is the principal defect governing animal survival. It suggested the present study in which the radioprotective effect of the enzyme on the blood forming organ in mice is examined both in terms of its association with bone marrow stem cells and in its ability to protect the proliferative capacity of these cells, as measured by the spleen colony assay technique (2).

MATERIALS AND METHODS C3H/HeB inbred male mice weighing 16-22 grams each, were obtained from BioBreeding Laboratories of Canada Ltd., Ottawa, and from Canadian Breeding Farms and Laboratory Ltd., St. Constant, Quebec. Each shipment of mice, upon arrival, was divided randomly into donor and host groups and given food and water ad libitum. The host mice were housed in groups of 3 per cage.

Lyophilized superoxide dismutase, obtained from Truett Laboratories, Dallas, Texas was dissolved in sterile normal saline (0.9%) for intravenous administration. In the solution, the enzyme concentration was 0.60, 1.4, 2.8, or 4.0 mg/ml depending on whether a 20 gram donor mouse was to receive 15, 35, 70, or 100 µg of enzyme per gram body weight, respectively, in an injected (via tail vein) volume of 0.5 ml. Enzyme-treated donor mice of lesser or greater weight received proportionately smaller and larger volumes, respectively. Control donor mice were injected either 1 h before or

1 h after X-irradiation or at both times in tandem.

Donor mice were exposed to 350 rads, at a rate of 100 rad/min, with 250 KV, 15 mA X-rays filtered by 0.5 mm Cu and 1 mm Al. Host mice received no prior treatment and were supralethally irradiated to 800 rads with X-rays of identical quality during the evening preceding the day in which they were intravenously injected with donor bone marrow stem cells.

One hour after completion of the irradiation or post-exposure treatment, the donor mice were sacrificed and the femora removed. The bone marrow stem cells from each femur were eluted as described previously (2) with 0.5 ml of Hank's balanced salts (HBS) solution. In any particular experiment, samples from individual mice in each treatment category were pooled and stored on ice prior to use. The number of nucleated cells in the pooled samples was counted in a hemocytometer. Each sample was divided into two parts with adjustment in the cell number in one part to 18 x 10^6 and in the other part to 4.5 x 10^6 cells/ml, by dilution with HBS solution. The diluted cell suspensions were injected by tail vein into the host mice, with each mouse receiving 0.5 ml. Each dilution was injected into a minimum of 27 mice.

The host mice were observed for 10 days during which some of them died. The surviving fraction was generally larger in groups that had received donor stem cells treated with superoxide dismutase rather than 0.9% saline. The survivors were sacrificed, the spleens removed and fixed in Bouin's fixative. The number of colonies on the surface of each spleen was counted with the aid of a low power (20X) stereomicroscope. In instances where the colonies were too numerous and confluent, the spleen weight was taken as an indicator of stem cell proliferative activity.

During the course of this study the chloramine-T technique (3) was used to label the tyrosine residues of bovine superoxide dismutase with ^{125}I. The chemicals required in the preparation were bovine superoxide dismutase ((SOD), Truett Laboratories) and reagent grade chloramine-T, sodium metabisulfite, potassium iodide, and 0.05 M phosphate buffer, pH 7.5. Sodium (^{125}I) iodide was obtained from New England Nuclear Canada, Dorval, Quebec. It was supplied in aqueous solution, pH 8 - 10, and specified as thiosulfate-free as well as carrier-free, with an isotopic purity > 99%. The reagents were freshly prepared in the 0.05 M phosphate buffer to the following concentrations per ml: SOD 8 mg, chloramine-T 4 mg, $Na_2S_2O_5$ 2.4 mg, and KI 10 mg. To the reaction vial, containing 2 mCi of Na ^{125}I/0.0045 ml, were added 0.025 ml each of 0.05 M phosphate buffer (pH 7.5), SOD, and chloramine-T solutions with brief mixing after each addition. Following the addition of chloramine-T, the reaction was allowed to proceed for 5 min before 0.1 ml of $Na_2S_2O_5$ and 0.2 ml of KI were added. The labelled enzyme was separated from free ^{125}I on a Bio-gel P-60 column, equilibrated beforehand in 0.05 M phosphate buffer and presaturated with bovine serum albumin (25 mg/0.5 ml) to eliminate absorption of the labelled enzyme. During elution, the latter was collected in 0.5 ml fractions which were counted for ^{125}I activity. The labelled enzyme was pooled as indicated (Fig. 1) and further purified by exhaustive dialysis against physiological saline (0.15 M, pH 7.4). The labelled enzyme had an activity of 5 x 10^7 cpm/ml, equivalent to 3.4 x 10^6 cpm/μg SOD. It was administered to mice in uptake studies in which the organ distribution and cellular uptake of the labelled enzyme could be followed. The small quantities (0.1 - 0.3 ml/mouse) of the ^{125}I-SOD were injected intravenously. The mice were sacrificed at various times thereafter to collect serum and bone marrow stem cells for the purpose of counting their ^{125}I activity.

RESULTS The elution profile of the ^{125}I-labelling reaction mixture is given in Fig. 1 which shows that more than 50% of the radionuclide

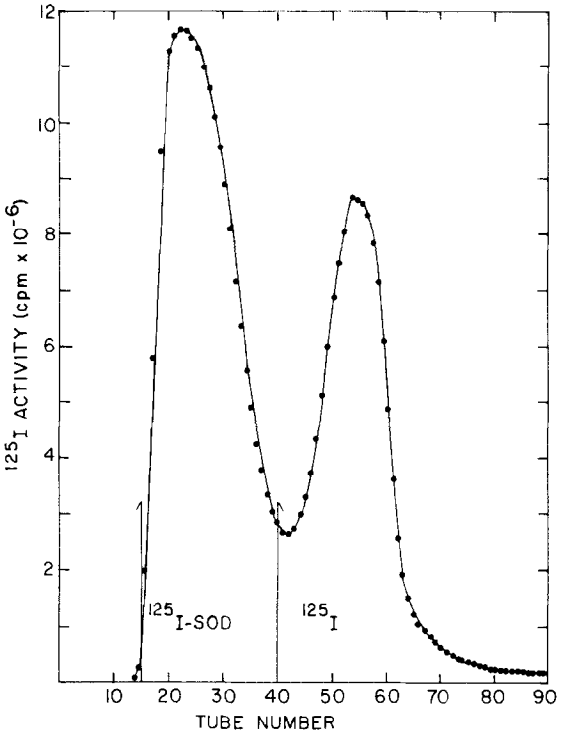

Figure 1. Elution profile on Bio-gel P-60 column, 0.05 M phosphate buffer pH 7.5, of free ^{125}I and ^{125}I-labelled bovine superoxide dismutase (^{125}I-SOD)

was bound to the superoxide dismutase and that the labelled enzyme was also separated from free ^{125}I. Fractions of ^{125}I-SOD within tubes #15-40 were pooled and, after dialysis against physiological saline, used to obtain the in-vivo uptake data of Fig. 2. It shows that in mouse serum the amount of labelled enzyme decreased after half an hour and was ∿ 90% cleared in 6 h. In suspensions of eluted bone marrow cells, the level increased to a maximum at 1 h and thereafter declined in paralled with its drop in the serum. The amount directly associated with the eluted cells also appeared to increase within the first hour and was 10 - 18% of the total in the suspensions during the first four hours. The eluted bone marrow cells contained both erythrocytes and nucleated cells. Removal of the former by differential lysis left 41% of the ^{125}I activity remaining with the nucleated cells. When the latter were lysed and centri-

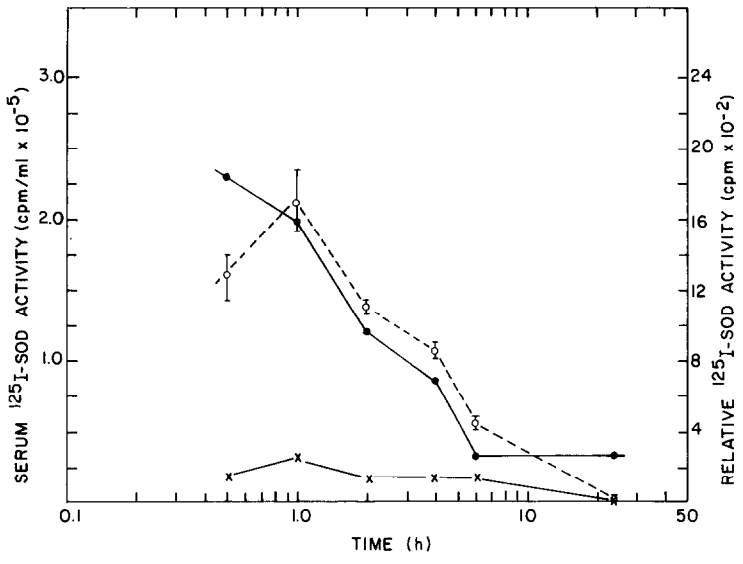

Figure 2. Variation with time of ^{125}I-labelled superoxide dismutase (^{125}I-SOD) in serum (●——●), LHS ordinate), eluted bone marrow (o---o), RHS ordinate), and bone marrow stem cells (X——X), RHS ordinate). Error bars represent standard deviations.

fuged (1 h at 1000,000 x g), the supernatant contained 45% of the residual ^{125}I activity and the particulate material (membranes, mitochondria, ribosomes, nuclei, etc.) accounted for the remaining 55%. While the small volume and low radioactivity of the subcellular fractions has made it difficult to demonstrate that the ^{125}I activity was incorporated into SOD, preliminary results from gel chromatography of various tissue extracts have indicated that 30-70% of the ^{125}I label is associated with the intact enzyme. It suggests that the labelled enzyme may enter stem cells, albeit to a small extent.

The increase during the first hour in the amount of exogenous superoxide dismutase associated with the eluted bone marrow stem cells was also accompanied by an enhancement in the radioprotective effect of the enzyme on the proliferative capacity of the cells. The data, given in Table 1, first two rows, show that this radioprotective effect amounted to a factor of 1.7 and 2.16 times, respectively, for donor stem cells treated with the enzyme one-half and one hour before X-irradiation.

Table 1. Prophylactic and therapeutic effect of an intravenous dose of superoxide dismutase (SOD) on X-irradiated donor bone marrow stem cells with respect to their ability to form spleen colonies (SC) in host mice on day 10. Amount of SOD/injection = 35 μg/g.

Time of treatment of donor mice	No. of spleen colonies (SC) formed				Radioprotective effect
	0.9% saline (NS)		SOD		
	N	Mean ± σ	N	Mean ± σ	SC(SOD)/SC(NS)
½ h before 350 rad	6	6.3 ± 3.6	8	10.5 ± 2.7	1.7
1 h " 350 "	25	9.8 ± 3.3	32	21.3 ± 5.6	2.16
1 h after 350 "	24	10 ± 5.4	23	14 ± 6.6	1.4
1 h before and after 350 rad	21	13.9 ± 8.0	26	35.0 ± 9.8	2.5

The level of enzyme therefore seems to determine the extent of protection it affords.

However, excessive quantities of exogenous superoxide dismutase nullify its radioprotective action as indicated by the data in Fig. 3. At 35 μg/g body weight, the enzyme elicited the largest effect, amounting to a factor of 2.16 ± 0.18, while at 70 and 100 μg/g this factor was reduced to 1.15 ± 0.15 and 0.7 ± 0.1, respectively. The latter value, being less than unity, signifies sensitization and suggests that at 100 μg/g the enzyme is toxic. It appears to be related to the irradiated state, however, since unirradiated stem cells, exposed to the same amount of enzyme for the same (2 h) period, did not show a deleterious effect with respect to their proliferative capacity in the spleens of host mice (Table 2). An account of the apparent toxicity is, in principle, difficult unless the exogenous enzyme is able to act intracellularly by dismutating superoxide anions, generated metabolically for functional purposes.

The rapid turnover of the enzyme in stem cells (Fig. 2), along

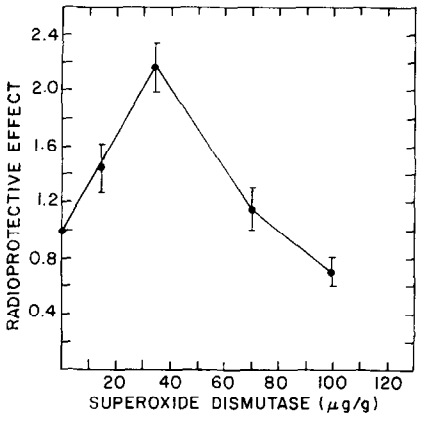

Figure 3. Radioprotective effect on bone marrow stem cells from donor mice, given variable amounts of bovine superoxide dismutase intravenously 1 h before X-irradiation to 350 rad at 100 rad/min. Error bars represent standard deviations.

with its therapeutic potential (4,5) suggested that the enzyme might be more effective if given in multiple small doses over an interval that extended into the post-irradiation period. Accordingly, donor mice were treated with two intravenous injections of SOD at 35 μg/g; the first 1 h before the X-ray dose and the second 1 h after. The stem cells, eluted 1 h later, were found to possess a better proliferative capacity, as determined by the comparative spleen colony assay technique (Table 1, fourth row). The increment of 0.34 from 2.16 to 2.5 in the value of the radioprotective factor is consistent with the therapeutic effect of the enzyme when given 1 h after the X-ray exposure (Table 1, third row). A therapeutic dose of enzyme has also been found to increase the survival of irradiated mice (details to be published elsewhere).

DISCUSSION The capacity of superoxide dismutase to protect against the development of damage in the post-irradiation period is significant in that it occurs well beyond the lifetime of the short-lived primary radicals from water radiolysis. Since the enzyme reacts specifically with O_2^- (6), a requirement is therefore imposed for a secondary supply of the superoxide

Table 2. Effect of bovine superoxide dismutase (SOD) on unirradiated donor marrow stem cells transplanted into supralethally irradiated host mice.

Treatment of donor mice	No. of donor bone marrow stem cells injected per host mouse ($\times 10^{-6}$)	10th day lethality of host mice	Weight of spleens* N	Weight of spleens* Mean ± σ (mg)
Saline (0.9%)	9	1/27	26	105 ± 37
"	2.25	0/30	30	96 ± 23
SOD 100 µg/g	9	0/27	27	116 ± 28
"	2.25	0/30	30	105 ± 31
None	0	63/90	27	78 ± 8
	Weight of normal spleens		6	95 ± 13

* Spleen colonies confluent and too numerous to count reliably. Mean weight of spleens taken as a measure of proliferative capacity of treated stem cells.

anions from which post-irradiation damage may proceed. Potential sources related to the irradiated state and therefore limited in time, are electron transfer reactions between oxygen and radicals of target molecules, of which chain-type oxidations of sulfhydryls and enzyme cofactors (4, 7, 8) are but a few examples. Other sources, of a metabolic and more continuing nature but not specific to the irradiated state, are aspects of electron transfer in respiration and energy production (9). Since the latter are, in principle if not in magnitude, common to both irradiated and unirradiated cells, they might not be expected to effect damage unless, in the irradiated cell, a breakdown in protective mechanisms has occurred or the production of superoxide anions is aberrant and enhanced to the point where they accumulate in excess at critical sites.

The attraction and collection of O_2^- at membrane surfaces has been

noted previously (10) as has the susceptibility of membranes to oxidative damage by the oxygen radical or its derivatives (11, 12). Development of the damage to its full extent in irradiated membranes is slow and can extend for many hours into the post-irradiation period unless inhibited by superoxide dismutase (13). These characteristics of membrane protection by superoxide dismutase provide a basis for the therapeutic effect of the enzyme on stem cells.

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