PROTECTIVE EFFECT OF SUPEROXIDE DISMUTASE ON ERYTHROCYTES OF X-IRRADIATED MICE

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SUMMARY Following X-ray doses of 550 and 675 rad, the erythrocyte count and percentage reticulocytes in mice first decline and then gradually recover. Bovine superoxide dismutase, injected intravenously at 35 μg/g body weight 1 h before and after the X-irradiation, did not significantly affect the initial phase but hastened the recovery such that at 22 days post-exposure, the erythrocyte count and percentage reticulocytes were both significantly different from the control values. It is estimated that the circulating red cell gains approximately 10 molecules of enzyme per injection. Almost all of it is in the cytoplasm.

INTRODUCTION Recently, it was shown that bovine superoxide dismutase protected the bone marrow stem cells of X-irradiated mice (1). In this study the biological endpoint was the proliferative capacity of the cells as assayed by the spleen colony technique (2). It is known that the spleen colonies observed by this method are of mixed composition and may consist of erythroid, megakaryocytic, granulocytic, and lymphocytic cells (3, 4). Since all of these are ultimately derived from pleuripotent stem cells, it is of interest to know whether the protection afforded by superoxide dismutase affects all of the cell types or only some. In this communication the evidence for radioprotection of the erythroid series is presented.

MATERIALS AND METHODS Six weeks old, white, female Swiss mice were obtained from BioBreeding Laboratories of Canada Ltd., Ottawa. Their irradiation to graded doses with 250 KV, 15mA X-rays at 100 rad/min and their treatment with bovine superoxide dismutase at 35 μg/g has previously been described (5). The lyophilized enzyme was obtained from Truett Laboratories, Dallas, Texas and, after dissolution in 0.1 N saline to a concentration of 1.4 mg/ml, injected intravenously into the mice. Control groups of animals received equivalent quantities of 0.1 N saline by the same route.

On selected days over a 4 week period following the radiation exposure, 5 mice from each enzyme-, and saline-, treated group were separately bled into fresh tubes containing anticoagulant. The blood

samples were examined by standard techniques (6) for a variety of parameters including the erythrocyte number and the reticulocyte count.

The extent to which an intravenous dose of bovine superoxide dismutase enters mature, circulating erythrocytes was examined using an enzyme preparation labelled with ^{125}I as previously described (1). Within 1 - 6 h after an intravenous injection of the labelled enzyme, blood from the mice was collected as before. After counting the ^{125}I activity in the erythrocyte packed volume, these cells were washed in 0.1 N saline and counted both for cell number and ^{125}I activity. Following lysis in distilled water, the cells were centrifuged at 100,000 x g for 30 min to precipitate the cellular particulates. The particulate fraction was separated from the supernatant containing the soluble cytoplasmic constituents. Both fractions were counted for ^{125}I. In some instances, the soluble fraction was run on a Bio-gel P-100 column to determine the fraction of ^{125}I bound to superoxide dismutase after its entry into the cells.

RESULTS In X-irradiated mice, the initial decline in erythrocyte number is followed by a gradual return towards a normal level. Fig. 1(a) and (b) illustrate this biphasic response for 675 and 550 rad, respectively. At either dose, the initial phase of a declining cell number lasted for 18 days in the enzyme-treated animals and for 22 days in animals injected with 0.1 N saline. The earlier recovery in the former group is accompanied by a rise in the reticulocytes (Fig. 1), whose reappearance in the blood is a hallmark for active proliferation of the erythroid series. This earlier response of the hematopoietic system toward cellular replacement is reflected in a higher erythrocyte count 22 days after the enzyme treatment. The difference is statistically significant (Table 1). Protection by superoxide dismutase is also apparent after 400 rad, although the effect fell short of being significant statistically (Table 1). At 50 - 300 rad (data not shown), the depression of the erythrocytes and the subsequent rise in reticulocytes were minor. The enzyme by itself has no effect on the erythrocyte count (Table 1 data compared with baseline values, Fig. 1(a)) or on the reticulocytes (Table 2 data compared with the normal level, Fig. 1(b)).

Fig. 1(b) and data in Table 2 also show that after 550 rad, the reticulocytes in the control mice reappear significantly more slowly than in the enzyme-treated group, despite the fact, that in the former animals,

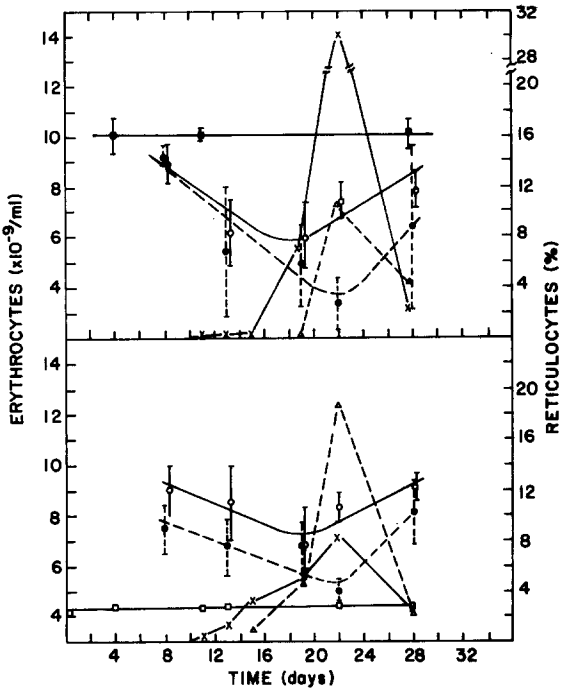

Figure 1. Variation with time in the erythrocyte count and percentage reticulocytes of mice irradiated with X-rays to 675 (panel (a)) and 550 (panel (b)) rad. In both panels the erythrocyte count is represented by o---o (0.1 N saline) and ●——● (superoxide dismutase). Panel (a) also shows the normal erythrocyte count in untreated, unirradiated mice (■——■). Error bars represent standard deviations. The percentage reticulocytes is represented by Δ---Δ (0.1 N saline) and X——X (superoxide dismutase). In panel (b) the percentage reticulocytes in untreated, unirradiated mice is indicated (□——□).

depression of the circulating erythrocytes is more severe, resulting in a greater requirement for cell replacement. It is possible that the hormonal regulatory factors, which control the response of the hematopoietic system (4) to cell loss, may be damaged in some substantive manner when irradiated in the absence of superoxide dismutase in the serum. Nevertheless, the initially slower response is followed by a more rapid rise in reticulocytes so that, by day 22, their percentage in the control group significantly exceeds

Table 1. Effect of superoxide dismutase on erythrocytes of mice X-irradiated 22 days earlier. Amount of enzyme given per injection = 35 μg/g body weight. Injections given 1 h before and after the X-ray dose.

X-ray dose (rad)	Erythrocyte Count (x 10^{-9}/ml) 0.1 N Saline No. $\pm$ σ	Superoxide dismutase No. $\pm$ σ	Significance of difference (p)
400	7.81 $\pm$ 0.67	8.56 $\pm$ 0.19	N. S.
550	4.99 $\pm$ 0.46	8.29 $\pm$ 0.53	<0.005
675	3.34 $\pm$ 1.04	7.39 $\pm$ 0.77	<0.005
0	10.57 $\pm$ 0.20	10.53 $\pm$ 1.26	N. S.

N.S. Difference in erythrocyte counts between groups not significant at $p \leq 0.05$.

Table 2. Effect of superoxide dismutase on reticulocytes of X-irradiated mice. Amount of enzyme given per injection = 35 μg/g body weight. Injection given 1 h before and after the X-ray dose.

X-ray Dose (rad)	Day after X-ray dose	% Reticulocytes 0.1 N Saline % $\pm$ σ	Superoxide dismutase $\pm$ σ	Significance of difference (p)
400	13	1.6 $\pm$ 0.6	2.2 $\pm$ 0.8	N. S.
	15	2.9 $\pm$ 1.2	2.2 $\pm$ 1.4	N. S.
	19	8.1 $\pm$ 1.4	4.9 $\pm$ 1.0	<0.01
	22	3.3 $\pm$ 0.3	3.3 $\pm$ 0.3	N. S.
550	13	0.0	1.3 $\pm$ 0.7	
	15	0.8 $\pm$ 0.8	3.6 $\pm$ 0.7	<0.005
	19	5.2 $\pm$ 1.8	5.0 $\pm$ 2.6	N. S.
	22	18.9 $\pm$ 7.5	8.5 $\pm$ 3.6	<0.005
	28	2.1 $\pm$ 0.3	2.3 $\pm$ 0.5	N. S.
675	19	0.1 $\pm$ 0.1	7.0 $\pm$ 4.2	<0.01
	22	10.6 $\pm$ 0.6	30.2 $\pm$ 16.3	<0.01
	28	4.3 $\pm$ 3.3	2.2 $\pm$ 0.2	N. S.
0	13	1.8 $\pm$ 0.2	2.1 $\pm$ 0.3	N. S.
	15	4.8 $\pm$ 3.5	2.6 $\pm$ 1.0	N. S.
	19	3.3 $\pm$ 1.6	3.7 $\pm$ 0.9	N. S.
	22	1.9 $\pm$ 0.3	2.3 $\pm$ 0.6	N. S.
	28	1.6 $\pm$ 0.8	2.4 $\pm$ 0.5	N. S.

N. S. Difference in reticulocyte count between groups not significant at $p \leq 0.05$.

Table 3. Distribution of ^{125}I in erythrocytes of mice injected intravenously with ^{125}I-superoxide dismutase

Time after injection (h)	^{125}I activity in washed erythrocytes			
	Total cell sample		Amount per cell	
	cpm	%+	cpm x 10^7	In cytoplasm(%)	In membrane(%)
1	1431	2.1	2.7	97#	3
2	3995	5.5	6.5	94	3
3	4332	4.5	8.0	98	2
4	841	1.6	2.0	93	3
6	398	1.3	0.8	93	4

+ % of ^{125}I activity in erythrocyte packed volume.
# Only ~ 9% associated with superoxide dismutase.

that in the enzyme-treated animals (Table 2). A similar pattern is observed after 400 rad (Table 2). In contrast, after 675 rad, the reappearance of the reticulocytes in the control group (Fig. 1(a)) occurs later (after day 19) and remains significantly below that in animals treated with superoxide dismutase (Table 2). It seems that at the larger dose of 675 rad, the unprotected erythroid series of stem cells is severely depleted and unable to rapidly respond to the hematological requirements for cellular replacement.

The results in Fig. 1 give little or no evidence that treatment with superoxide dismutase is of any benefit to mature erythrocytes. An explanation for the negative result is available from the data on the extent to which these cells are penetrable by the radioprotective enzyme as measured in-vivo, using ^{125}I-superoxide dismutase. The data are presented in Table 3. They indicate that a small proportion (< 6%) of the ^{125}I, present in the erythrocyte packed volume, gets into the cells. Of this, 93 - 98% is in the cytoplasm. However, only ~ 9% of the ^{125}I activity in the cytoplasm was bound to superoxide dismutase as determined by column chromatography (1). From these values it is estimated that, at a specific activity of 3.4 x 10^6 cpm/μg of enzyme, the amount of

^{125}I-superoxide dismutase in each red cell, on average, was $\sim 0.7 \times 10^{-20}$ µg, equivalent to 0.14 molecules (at a molecular weight of 32,000). The treatment dose of 35 µg/g body weight was ~ 70 times that of the labelled material injected for the uptake studies, suggesting that each erythrocyte gains ~ 10 molecules per treatment. This number is negligibly small compared to the 8×10^4 molecules per erythrocyte of endogenous superoxide dismutase ($\equiv 2.7$ µM for an erythrocyte volume of 50 μ^3 (7)), as determined from direct assays. Moreover, a homogeneous distribution of a micromolar quantity of enzyme is sufficient to catalyze the dismutation of all of the superoxide anions generated by X-ray doses as high as 675 rad. A non-uniform distribution, on the other hand, might conceivably leave some radiation sensitive targets exposed.

DISCUSSION The beneficial effect of bovine superoxide dismutase on erythropoiesis may be humoral in nature. Regulation of erythropoiesis is accomplished by the hormone erythropoietin acting on surface receptors of unipotent stem cells (8), whose commitment to red cell production is already established. By this account, the protective activity of superoxide dismutase may be seen as directed at the hormone, or at the surface receptor and the cell, or both. This view is compatible, moreover, with the presence of the injected enzyme in the serum. On the other hand, the pleuripotent stem cells are thought to respond to the utilization of the committed erythroid series by delivering more committed cells to the pool (9). Protection by superoxide dismutase may then be effected by an alternative mechanism through action of the enzyme on the uncommitted stem cells themselves. In this case, it is expected that protection by superoxide dismutase of the other precursor cells in the megakaryocytic, granulocytic, and lymphocytic series would also be manifest. Some evidence on the latter point will be discussed elsewhere.

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