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20. ABSTRACT (continued)

lung superoxide dismutase specific activity increased with increasing endotoxin dose. Reduction of superoxide dismutase specific activity in mouse lung following endotoxin challenge may be necessitated by a requirement for an elevated  $O_2^-$  concentration. Superoxide anion is a required substrate for at least one endotoxin-inducible enzyme found in mouse lung.

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## Effects of Superoxide Dismutase on Lipopolysaccharide-Stressed Mice and Alteration of Lung Enzyme Levels by Endotoxin<sup>1</sup>

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Effects of Superoxide Dismutase on Lipopolysaccharide-Stressed Mice and Alteration of Lung Enzyme Levels by Endotoxin. GRAY, B. (1981). *Toxicol. Appl. Pharmacol.* 60, 479-484. Superoxide dismutase (SOD) injected into mice prior to lipopolysaccharide (endotoxin) challenge enhanced mouse survival. However, mice administered prophylactic SOD at levels sufficiently above or below the optimum concentration were not protected. The levels of glutathione peroxidase and catalase in extracts from lungs of mice challenged with endotoxin did not change appreciably. SOD enzyme specific activity in lung extracts was reduced about 50% within 1 hr of endotoxin challenge. The reduction in lung superoxide dismutase specific activity increased with increasing endotoxin dose. Reduction of superoxide dismutase specific activity in mouse lung following endotoxin challenge may be necessitated by a requirement for an elevated  $O_2^-$  concentration. Superoxide anion is a required substrate for at least one endotoxin-inducible enzyme found in mouse lung.

One effect of endotoxin administration in mammals is rapid sequestration of platelets and polymorphonuclear leukocytes into pulmonary capillaries and other vascularized organs (Stetson, 1951). Microscopic lung damage is evident minutes after endotoxin injection, and death may follow within days at sufficiently high doses (Coalson *et al.*, 1970). Active oxygen, particularly  $O_2^-$  and  $H_2O_2$ , is an important mediator of cell damage produced by polymorphonuclear leukocytes that could lead to observed lesions in lungs (Sacks *et al.*, 1978).

Administration of endotoxin or other stresses alters profiles for those enzymes in lungs that utilize  $O_2^-$  or  $H_2O_2$  as substrates. A 50-fold increase in indoleamine 2,3-diox-

xygenase levels in mouse lung was observed 24 hr after endotoxin administration (Hayashi and Yoshida, 1979). Hyperbaric stress plus endotoxin increased levels of superoxide dismutase (SOD), glutathione peroxidase, and catalase in rats (Frank *et al.*, 1978). Modest increases in SOD levels were observed in mouse lungs subjected to hyperbaric oxygen stress (Crapo and Tierney, 1974). These and other studies support the hypothesis of a common mechanism for lung damage caused by hyperbaric oxygen, ozone, radiation, or endotoxin stress (Menzel, 1970).

SOD has been used therapeutically to treat arthritis, cataracts, and radiation cystitis (Ahlengard *et al.*, 1978; Marberger *et al.*, 1974; Cobble and Lynd, 1977). Also the enzyme has been used as a radioprotectant in mice and in tissue culture studies (Petkau *et al.*, 1975; Petkau, 1978). The free enzyme or derivatives of SOD affects model inflammatory systems, and the importance of  $O_2^-$

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levels in inflammation has been documented (McCord, 1974; Salin and McCord, 1975; McCord and Wong, 1979; Petrone *et al.*, 1980). On the other hand, SOD was not effective in modifying pulmonary oxygen toxicity or in reducing central nervous system toxicity due to hyperbaric oxygen (Crapo *et al.*, 1977; Hilton *et al.*, 1980). The following studies were done to determine if exogenous SOD acts to alter lethality caused by endotoxin. In addition, since the lung is an organ suffering early trauma after endotoxin administration, the enzymatic activities of SOD, catalase, and glutathione peroxidase were assayed in mouse lungs to determine if their levels changed. No other tissues were examined in this study.

## METHODS

B6CBF1 mice (Cumberland Farms) were housed in a colony and provided with a standard laboratory diet and chlorinated water *ad libitum*. Mice were maintained until they were at least 10 weeks old and weighed about 25 g before being used experimentally. Mice were given 0.25-ml iv injections of solutions of SOD (Sigma, bovine blood type I, 2900 units/mg) prepared by dissolving the enzyme in pyrogen-free sterile saline (Cutter Laboratories). *Salmonella typhosa* lipopolysaccharide W 0901 (Endotoxin, lot No. 355643 Difco Laboratories), dissolved in pyrogen-free sterile saline, was injected ip in 0.25-ml volumes within 1 hr of the prophylactic SOD injection. Each repetition to assess SOD prophylaxis was done over the range from 0 to 320 mg/kg SOD with the same age animals (11–17 weeks), on the same day, using the same endotoxin preparation. Mice were housed in similar cages in the same room before and after treatment. These precautions were necessary due to the heterogeneity of all endotoxin preparations examined plus the variable biological responses of mice based on age or environmental factors (Nowotny, 1969). After the original endotoxin was expended, a second lot, No. 678325, was used to determine the 1-hr dose response of endogenous lung SOD in 26-week-old, female mice.

Mouse lung extracts were prepared following endotoxin or sterile saline injection. Mice were dispatched by cervical dislocation, the descending aorta was severed, and lungs were flushed by injecting 10 ml of sterile saline into the right ventricle. Lungs from four or five animals were excised, rinsed in ice-cold 0.05 M sodium phosphate buffer (pH 7.8), minced, rinsed again, and homogenized in buffer at a 1:10 (w/v) ratio (Polytron,

Brinkman Instruments). Homogenates were centrifuged at 5000g<sub>max</sub> for 15 min at 0–4°C. Supernatant fluids were divided into two aliquots, and one sample was dialyzed for about 14 hr against buffer at 5–10°C.

Supernatant fluids were assayed for enzymes and protein with the aid of a Varian Model 219 spectrophotometer. Glutathione peroxidase was assayed in 3-ml reaction mixtures at 25°C with continuous stirring (Paglia and Valentine, 1967). Supernatant fluids were assayed for catalase, based on the method of Beer and Sizer (1952). The time required for the OD<sub>240 nm</sub> to decrease from 0.45 to 0.40 was noted. Since this corresponds to decomposition of 3.45 μmoles of H<sub>2</sub>O<sub>2</sub> in a 3-ml reaction mixture, the enzyme activity in the supernatant fluid could be calculated. Supernatant fluids that had been dialyzed were assayed for SOD by modification of the method of Sun and Zigman (1978). The assay mixture was maintained at pH 10.0 and 25°C with continuous stirring. The rate of increase in the OD<sub>320 nm</sub> was calculated at 5 to 6 min after addition of epinephrine, and data from several assays using varying concentrations of supernatant fluids were used to calculate the number of units of SOD present. One unit of enzyme activity was defined as that quantity that reduced the change in OD<sub>320 nm</sub> by 50%. Protein was assayed by the Bradford method (1976) with bovine plasma gamma globulin as a standard (Bio-Rad Laboratories).

## RESULTS

Mice administered prophylactic injections of SOD at about 40 mg/kg had survival rates twice as high as those injected with sterile saline when challenged with doses of endotoxin that killed 75–95% of the control animals. The endotoxin used killed 75 to 95% of the mice at doses ranging between 20 and 28 mg/kg. Endotoxin was administered by ip injection, and the mice died 2 to 7 days after treatment. Thirty-day observations were used to determine that the mice were in fact surviving and not simply dying after a 7-day delay. Figure 1 illustrates survival rates for mice challenged with endotoxin following prophylactic iv injections of SOD and ip injections of endotoxin. Mice administered up to 320 mg/kg SOD followed by 0.25 ml of sterile saline remained active, eating and drinking with no apparent signs of illness until they were sacrificed more than 30 days after treatment. Further,

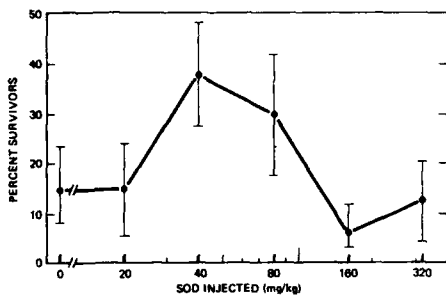


FIG. 1. Percentage 30-day survivors as function of SOD administered before endotoxin challenge. Bars represent 95% confidence limits. Numbers of mice treated were controls, 89; 20 mg/kg, 53; 40 mg/kg, 82; 80 mg/kg, 54; 160 mg/kg, 68; and 320 mg/kg, 65.

mice given prophylactic injections from an SOD solution placed in a boiling water bath for 30 min had survival levels similar to those of control mice.

The percentage of 30-day survivors as a function of the quantity of prophylactic SOD given mice is shown in Fig. 1. Statistical analysis yields an optimal protection dose around 40 mg/kg SOD prior to endotoxin challenge. However, higher SOD concentrations combined with the same endotoxin dose resulted in lethality at the same levels as the controls. Both the 40 and 80 mg/kg SOD treatments differ from controls with a significance at the  $p = 0.001$  and  $0.047$  levels, respectively. The 160 mg/kg SOD treatment differed from controls at the  $p \approx 0.055$  level.

The activities of SOD, glutathione peroxidase, and catalase were measured in cell-free extracts prepared from lungs removed from mice previously subjected to a 10 mg/kg endotoxin challenge. The variation in percentage specific activity for each enzyme at different times following endotoxin challenge is shown in Fig. 2. There was a significant drop in SOD specific activity in endotoxin-treated mouse lung compared to controls. Recovery of SOD specific activity to the same relative activity as glutathione peroxidase and catalase required 24 to 48 hr in endotoxin-challenged mice. There was

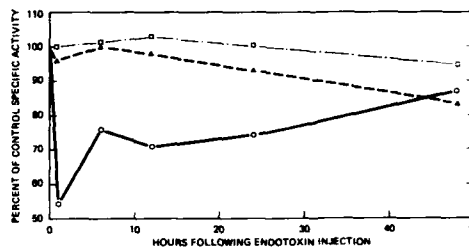


FIG. 2. Percentage of control specific activity vs time after ip injection of 10 mg/kg endotoxin. Assays were done on lung extracts for SOD,  $\circ$ — $\circ$ ; catalase,  $\triangle$ — $\triangle$ ; and glutathione peroxidase,  $\square$ — $\square$ . The 95% confidence limits for controls were SOD (80–120%), glutathione peroxidase (92–113%), and catalase (87–113%).

a slight decline in glutathione peroxidase and catalase specific activities at about 48 hr after endotoxin challenge.

A group of 6-month-old female mice were assayed for endotoxin lethality. An ip injection of 10 mg/kg endotoxin was not lethal in these mice. The animals became visibly ill, but all mice 10 weeks to 6 months old tested with this concentration of endotoxin survived 30 days. The lung SOD specific activity as a function of endotoxin dose for lungs removed from mice 1 hr after challenge is shown in Fig. 3. Therefore, even mice challenged with less than the  $LD_{50,30}$  endotoxin dose responded by reducing lung SOD activity by approximately 50%.

Assays for SOD were done with various levels of endotoxin added to reaction mix-

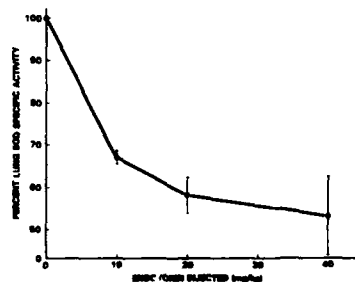


FIG. 3. Percentage lung SOD specific activity as a function of endotoxin dose injected ip 1 hr before sacrifice of mice.

tures to determine the effect of endotoxin on enzyme activity. Reactions were done with purified bovine superoxide dismutase and endotoxin as described above. The percentage inhibition of purified SOD as a function of endotoxin concentration in the reaction mixture is shown in Fig. 4.

### DISCUSSION

Generation of  $O_2^-$  is an important factor contributing to mortality in mice challenged with endotoxin. The only known function for SOD is catalysis of  $O_2^-$  dismutation (McCord and Fridovich, 1969). This enzyme enhances survival at optimum doses in endotoxin-challenged mice. Also, denatured enzyme resulted in no protection from endotoxin challenge. These observations support the conclusion that endotoxin challenge results in  $O_2^-$  generation in mice at levels that may ultimately result in death.

The  $O_2^-$  generated must transit an extracellular space since it is assumed that exogenous SOD injected into animals remains in those spaces (Crapo, 1977). Since injected SOD affords limited protection in mice against the toxic effects of  $O_2^-$ , extracellular  $O_2^-$  is one factor contributing to mortality. Moreover,  $O_2^-$  at some concentration is a necessary compound formed in response to endotoxin stress. Excess SOD reduced survival in endotoxin-challenged mice to levels

at or below control survival rates. These observations support the contentions that the  $O_2^-$  concentration following endotoxin challenge is important and that its control may ultimately influence survival.

The level of SOD specific activity dropped 1 hr after endotoxin challenge in mice. The loss of SOD specific activity in lungs 1 hr after endotoxin injection could be enhanced by increasing the endotoxin dose. The decreased SOD specific activity was not simply a reflection of endotoxin inhibition in reaction mixtures. About 1000  $\mu$ g of endotoxin would have to be injected into a 25-g mouse to achieve an 8% reduction in activity in SOD assays. This analysis assumes that all injected endotoxin is in the lungs and that it is all recovered in cell extracts. As a result, the mechanism responsible for the drop in SOD specific activity is not known. Also, rapid loss of enzyme following cell lysis does not seem likely since catalase and glutathione peroxidase levels remain fairly constant.

Although the nature of the regulatory mechanisms for SOD is not understood, the necessity for such regulation of this enzyme following endotoxin stress is consistent with previous results. Indolamine 2,3-dioxygenase, an  $O_2^-$ -requiring enzyme, is induced nearly 50-fold in mouse lung 24 to 48 hr after endotoxin stress, even though the  $K_m$  of the enzyme for  $O_2^-$  is  $1.3 \times 10^{-6}$  M (Ohnishi *et al.*, 1977; Yoshida and Hayaishi, 1978). This level of enzyme induction is even more remarkable when considering the deposition of  $O_2^-$ -elaborating granulocytes in lungs minute after endotoxin injection. Reduction of lung SOD activity would be consistent with those mechanisms that increase the  $O_2^-$  concentration. Thus reduction of lung SOD activity could be a critical mechanism for conservation of generated  $O_2^-$  during the early induction period of indolamine 2,3-dioxygenase. Indeed, lung SOD specific activity drops almost immediately after endotoxin challenge and recovers gradually during the induction of indolamine 2,3-dioxygenase. These observations support the idea

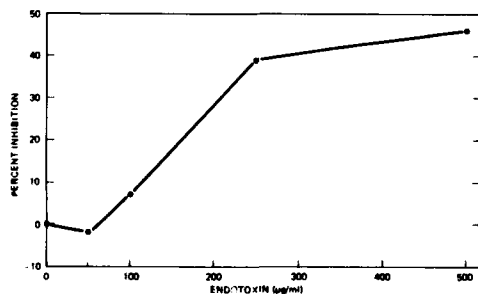


FIG. 4. Percentage inhibition of bovine SOD activity in reaction mixtures vs endotoxin concentration per milliliter.

that the O<sub>2</sub><sup>-</sup> concentration in lungs following endotoxin administration is important for metabolism of 5-hydroxyindoleamines.

Red blood cells were removed from the lungs by the procedure described above before enzymatic assay for SOD. Residual SOD activity in lungs could then be assayed without contamination by SOD in red blood cells. Since exogenous SOD injected into mice remains in extracellular compartments, it was removed and could not be detected in lungs subjected to preflushing to remove red blood cells. Therefore, it was not possible to assay injected SOD in lungs in this study.

The circulating half life of exogenous SOD has been estimated at between 1 and 2 hr in mice (Pyatak *et al.*, 1980). Therefore, it is remarkable that SOD has the protective effect observed in these studies. Fortunately, techniques have been developed to produce protein complexes having greatly extended circulating lives and reduced immunogenicity in mammals (Abuchowski *et al.*, 1977). Further studies using these enzyme complexes are currently under way to determine their effect on endotoxin-treated animals.

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