KUEHL: It is not possible to obtain an observable spectrum at room temperature because the reaction is too rapid. Ascorbic acid does inhibit the signal.

ŌYANAGUI: The Haber–Weiss reaction is active in alkaline reactions. Areas of tissue inflammation are said to be acidic. How is it possible, then, that the Haber–Weiss reaction contributes significantly to the generation of HO· in inflamed areas *in vivo?*

KUEHL: I don't feel qualified to assess the role of the Haber–Weiss reaction. Our data suggest the possibility of an enzymatic alternative to the Haber–Weiss reaction as a source of HO· in inflammation.

DEMOPOULOS: We have found that corticosteroids are antioxidants and prevent the oxidation of polyunsaturated fatty acids while they are still attached to phospholipids. Do you think this may be relevant to the effects of steroids on phospholipid synthesis? Can radicals breach the ester bond, and can we do without phospholipase?

KUEHL: I do not know if radicals can breach the ester bond.

MICHELSON: Ultraviolet light can breach phosphate–ester covalent bonds.

ŌYANAGUI: The activation of arachidonic acid by active oxygen radicals is apparently required to obtain maximum prostaglandin synthesis. Have you some comments on this point?

KUEHL: Dr. William Lands (University of Michigan) has provided evidence that a hydroperoxide is necessary to initiate the cyclooxygenase reaction. Consistant with this concept, we have found that high levels of phenol compounds (which act as scavengers of oxygen radicals) inhibit cyclooxygenase at low substrate levels. To rule out the possibility that cyclooxygenase is inhibiting cyclooxygenase rather than arachidonic acid release from macrophages, we employed fully inhibitory doses of indomethacin, which permitted a direct readout of arachidonic acid release.

An *in Vivo* Enzymatic Probe for Superoxide and Peroxide Production by Chemotherapeutic Agents

JOHN E. MCGINNESS, PETER H. PROCTOR, HARRY B. DEMOPOULOS, JAMES A. HOKANSON, AND NGUYEN T. VAN

INTRODUCTION

Oxygen is no longer considered to be without risk to aerobic living systems which require oxygen to sustain life. The biochemical nature of toxic active oxygen species and its medical implications have opened a new area of medical investigation. The topic of this chapter, however, is restricted to superoxide (O_2^-) and hydrogen peroxide (H_2O_2), two important species of active oxygen, and the enzymes superoxide dismutase and catalase, which have evolved to protect aerobic cells from these two toxic species.

A number of chemotherapeutic agents have been shown to stimulate the production of O_2^- [1,2]. The data presented here relate only to the anticancer drugs *cis*-platinum and adriamycin. In addition to having antitumor activity, *cis*-platinum and adriamycin are toxic to the host. At present there is only limited information as to the precise relationship between toxicity directed toward the tumor and toxicity toward the host. The possibility that the cardiac toxicity of adriamycin might be suppressed with-

out interfering with the antitumor activity was explored using a pretreatment with reduced vitamin E (3). The use of vitamin E, however, has not completely solved the clinical problem, and more basic information is required.

The enzymes superoxide dismutase and catalase can provide answers to a number of important questions. Injections of superoxide dismutase and catalase can be expected to increase the extracellular levels of these enzymes. The specificity of the enzymes makes it possible to carry out an independent evaluation of the role of O_2^- and H_2O_2 in the toxicity of anticancer drugs. Our final goal is to develop a set of extracellular and intracellular antioxidants that can be used to evaluate the specific role of each type of active oxygen species. One important step in this direction is understanding the extracellular contribution of O_2^- and H_2O_2 to the toxicity of adriamycin and *cis*-platinum (3,5). In this chapter, we present results obtained by increasing the extracellular levels of either superoxide dismutase or catalase (or both) and then challenging the animals with *cis*-platinum and adriamycin. The response was found to depend on both dose and time.

CHEMOTHERAPEUTIC AGENTS

cis-Diaminodichloroplatinum(II)

The molecular events that occur in *cis*-platinum-induced nephrotoxicity are not known. Both superoxide and hydrogen peroxide appear to be generated. The nephrotoxicity associated with acute doses of *cis*-platinum (up to 10 mg/kg) is ameliorated by superoxide dismutase. Previously reported results are briefly summarized as follows (6,7). Increased survival was correlated more closely with the capacity of superoxide dismutase to minimize *cis*-platinum-induced diarrhea than with the measured kidney toxicity. Examination of the pathological kidney tissue, however, did show that less damage was present in the rats receiving *cis*-platinum and superoxide dismutase than in the rats receiving *cis*-platinum alone.

Although O_2^- production appears to be stimulated by *cis*-platinum, it alone cannot account for the nephrotoxicity. Studies of the effects of chronic administration of *cis*-platinum (5 mg/kg) revealed a synergistic toxicity when the *cis*-platinum was combined with superoxide dismutase. Since superoxide dismutase can convert O_2^- to H_2O_2 and O_2, a study of the combined effects of *cis*-platinum, superoxide dismutase, and catalase was undertaken (Fig. 1). The synergistic toxicity observed with chronic
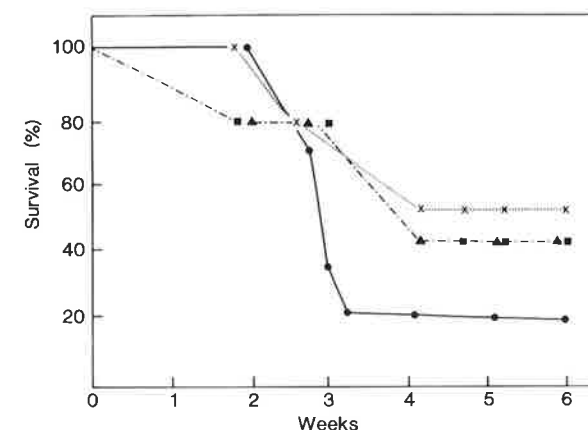
Fig. 1. Percent survival of Sprague–Dawley rats receiving three-weekly ip injections of 5 mg/kg *cis*-diaminodichloroplatinum(II) (CDDP). The mortality was greatest for rats receiving superoxide dismutase (SOD) and *cis*-platinum. Key: ▲, CDDP only; ●, CDDP + 1.0 mg/kg SOD; X, CDDP + 1.0 mg/kg catalase; ■, CDDP + 1.0 mg/kg SOD + 1.0 mg/kg catalase.

application of *cis*-platinum and superoxide dismutase was reversed by catalase. Catalase appeared to offer some protection at these doses but was far less effective than might be expected if H_2O_2 alone were responsible for the chronic toxicity.

Since the greatest toxic effects observed were obtained with superoxide dismutase and *cis*-platinum, a tumor experiment was undertaken with these two drugs. A transplantable leiomyosarcoma (accession no. 14072, experimental no. LX 7330-M09-TB-MU) from Syrian hamster ductus deferens tissues (8,9) was injected into the hamsters subcutaneously in the hind area. Superoxide dismutase did not interfere with the antitumor action of *cis*-platinum at these doses (Fig. 2).

Adriamycin

During our initial work with *cis*-platinum, the observation that O_2^- is produced *in vitro* by adriamycin appeared in print (5). We began experiments with adriamycin *in vivo* in combination with superoxide dismutase and *cis*-platinum. Our first observation was that the cardiac toxicity induced by three pulse doses of 5 mg/kg adriamycin was ameliorated by daily im injections of 1 mg/kg superoxide dismutase and 1 mg/kg catalase (Table I). Representative photomicrographs were prepared from sections cut across the entire heart, which was fixed in formalin while still beating.
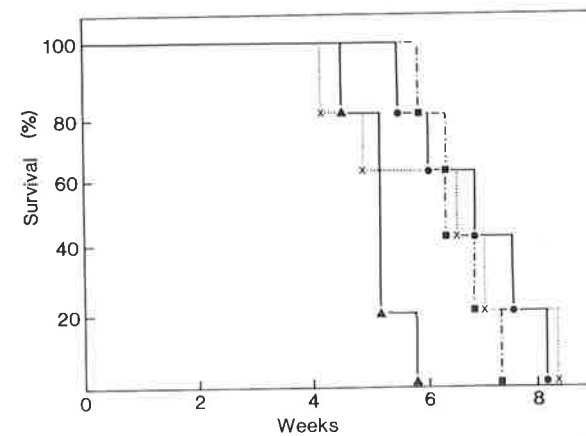
Fig. 2. Percent survival of tumor-bearing Syrian hamsters receiving 5 mg/kg *cis*-diamino-dichloroplatinum(II) in three-weekly injections. Rate of survival increased with both *cis*-platinum and the enzyme superoxide dismutase. The antitumor activity of the *cis*-platinum was not affected by these agents. Key: ■, CDDP + 10⁶ tumor cells; X, 1 mg/kg SOD + 10⁶ tumor cells; ●, CDDP + 1 mg/kg SOD + 10⁶ tumor cells; ▲, 10⁶ tumor cells.

All hearts were fixed and stained at the same time. Micrographs of the tissue, stained with hematoxylin and eosin, were taken from the muscle tissue of the wall of the left ventricle to show longitudinal muscle bundles and cross-sectional muscle bundles (Fig. 3). The damaged hearts show decreased eosinophilic staining and no infiltration by leukocytes (Fig. 3A, C).

Chronic administration of adriamycin consisted of three doses of 4 mg/kg given at 1-week intervals. The survival data indicate that superoxide dismutase alone increased the toxicity of adriamycin on a chronic schedule at this dose (Fig. 4). Catalase enhanced survival. Treatment with superoxide dismutase and catalase appeared to support a slightly higher

TABLE I

Effects of Superoxide Dismutase plus Catalase on Adriamycin Cardiotoxicity[a]

Number of rats	Treatment	% affected	Extent[b]
6	Adriamycin alone	83 (5/6)	20 ± 10
6	Adriamycin with superoxide dismutase plus catalase	16 (1/6)	15

[a] Masked pathology analysis of heart sections of rats receiving 1 mg/kg superoxide dismutase and 1 mg/kg catalase in addition to 5 mg/kg adriamycin in three-weekly doses.
[b] Extent refers to the approximate percentage of myocardial fibers affected.
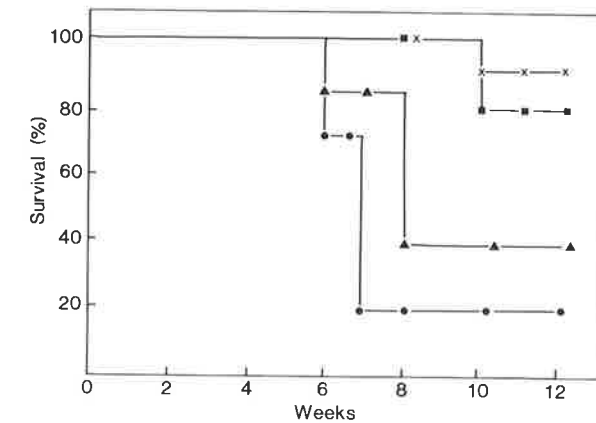
Fig. 4. Percent survival of 12 rats per group receiving 4 mg/kg adriamycin in three-weekly doses. Mortality was greatest for rats receiving 1 mg/kg superoxide dismutase in addition to adriamycin. The combination of 1 mg/kg catalase and adriamycin significantly reduced the mortality ($p < .01$). The greatest protection was seen with superoxide dismutase and catalase together. Experiment was terminated at 12 weeks for necropsy. Key: ▲, adriamycin only; ●, adriamycin + 1 mg/kg SOD; ■, adriamycin + 1 mg/kg catalase; X, adriamycin + 1 mg/kg SOD + 1 mg/kg catalase.

survival than treatment with catalase alone. One rat from each group was necropsied. The rat receiving adriamycin alone was found to have extreme congestion of all myocardial and subequicardial vessels. At this lower level of adriamycin (12 mg/kg total) the hearts did not show the muscle damage seen in the experiment with three-weekly injections of 5 mg/kg. The rats receiving catalase and superoxide dismutase plus catalase were free of heart congestion. Peritonitis was observed with adriamycin and superoxide dismutase or adriamycin and catalase. The combination of superoxide dismutase and catalase seemed to prevent the adriamycin-associated peritonitis under these experimental conditions. A single pulse dose of adriamycin (10 mg/kg) produced significant mortality within 6 weeks and was chosen as the test dose to study the effects of superoxide dismutase and catalase treatment. The dose–response data with adriamycin and superoxide dismutase are presented in Fig. 5. Animals treated with superoxide dismutase were spared during the first few weeks. These animals showed no diarrhea or other signs of illness until the fourth week. Superoxide dismutase was administered every day. Since these data were obtained, we have found that posttreatment should be discontinued after 2 weeks. The dose–response data with catalase and adriamycin are presented in Fig. 6. Even with large doses of catalase little protection was evident. The activity of catalase was 64,000 units/mg, and that of superox-
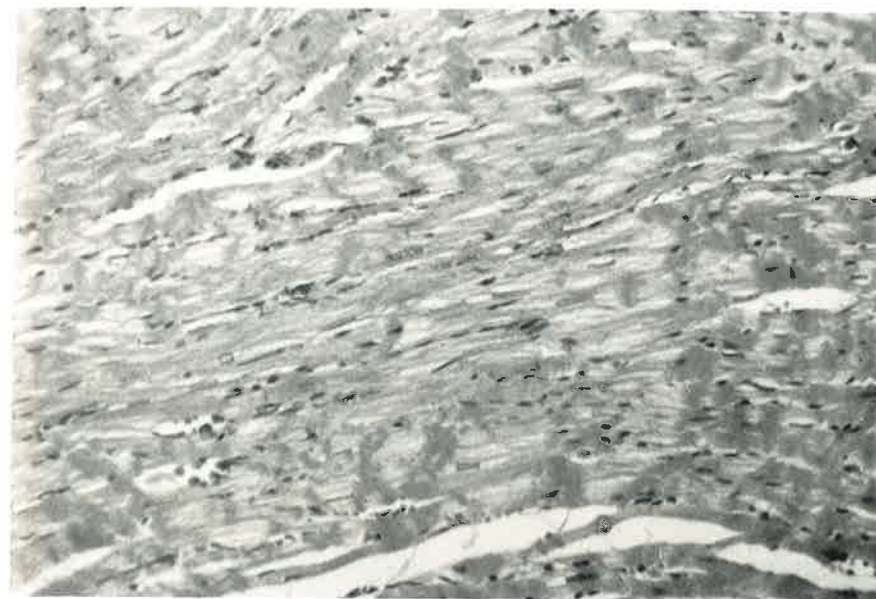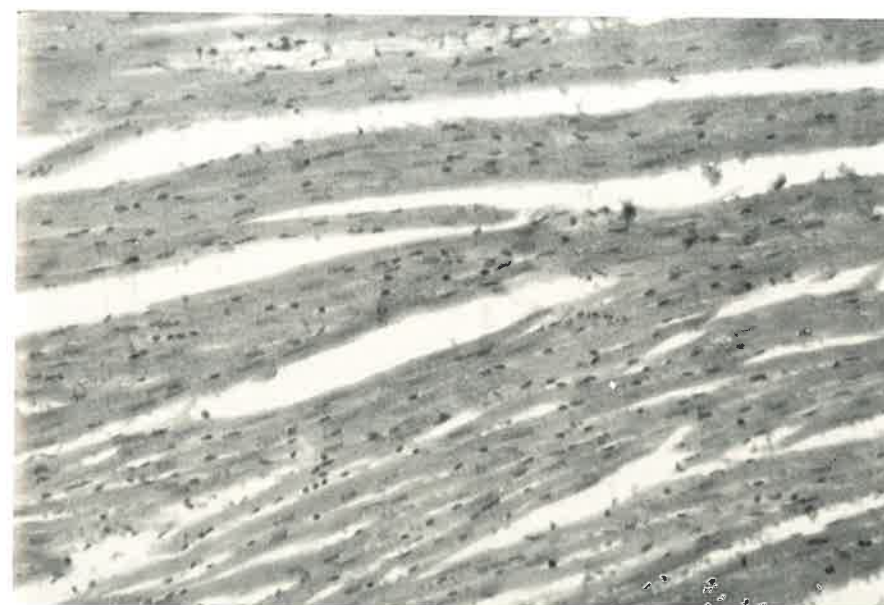
a

b

c

d
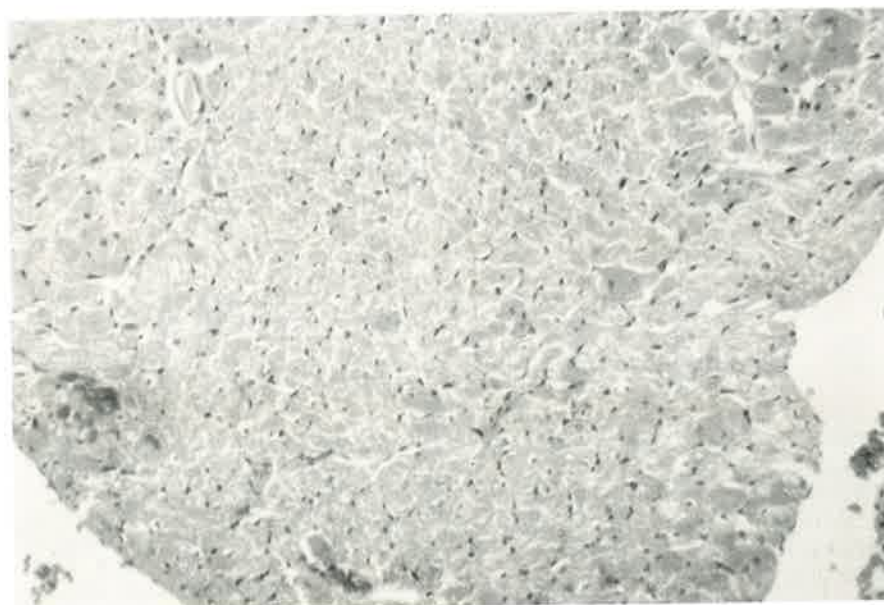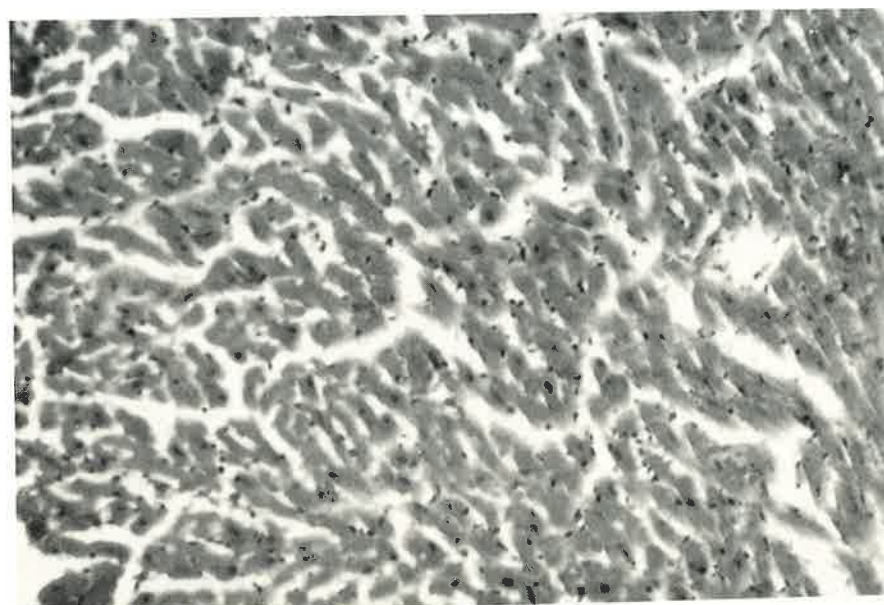
Fig. 3. Micrographs of representative muscle tissue (hematoxylin and eosin stained) taken from the wall of the left ventricle of the rats described in Table 1. The adriamycin produces decreased eosinophilic staining and no polymorphonuclear leukocytic infiltration.
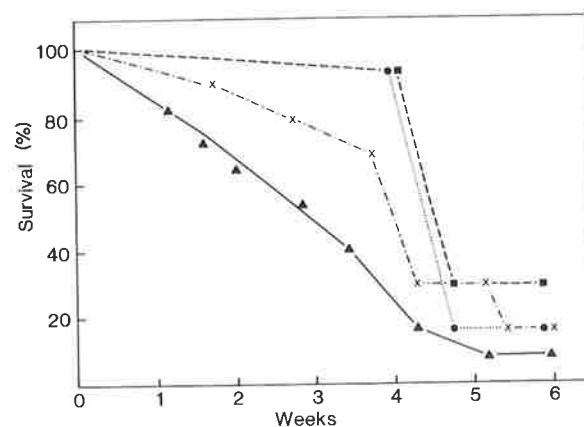
(a) Adriamycin treated (cross section). (b) Treated with adriamycin, superoxide, and catalase (cross section). (c) Adriamycin treated (longitudinal section). (d) Treated with adriamycin, superoxide, and catalase (longitudinal section).

Fig. 5. Percent survival of 36 Lewis inbred rats (300 g weight) receiving a single ip dose of 10 mg/kg adriamycin and daily im injections of superoxide dismutase as indicated. Key: ▲, adriamycin only; X, adriamycin + 0.04 mg/kg SOD; ●, adriamycin + 0.4 mg/kg SOD; ■, adriamycin + 4.0 mg/kg SOD.

ide dismutase was 3300 units/mg. The catalase experiment extended to a higher enzymatic activity.

A survival and necropsy experiment was conducted with superoxide dismutase, catalase, and adriamycin (Fig. 7). Superoxide dismutase and catalase were administered for 2 weeks; then catalase alone was administered. Necropsy was performed on two rats from each group at 18 days,
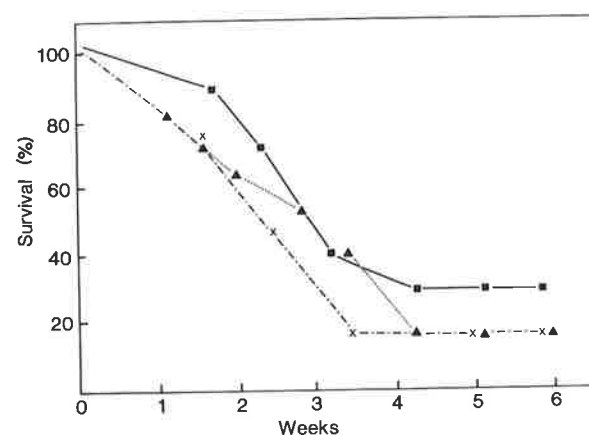
Fig. 6. Percent survival of 36 Lewis inbred rats (300 g weight) receiving a single ip dose of 10 mg/kg adriamycin. Catalase was administered im daily at the doses indicated. Key: ▲, adriamycin only; X, adriamycin + 0.1 mg/kg catalase; ■, adriamycin + 1.0 mg/kg catalase.
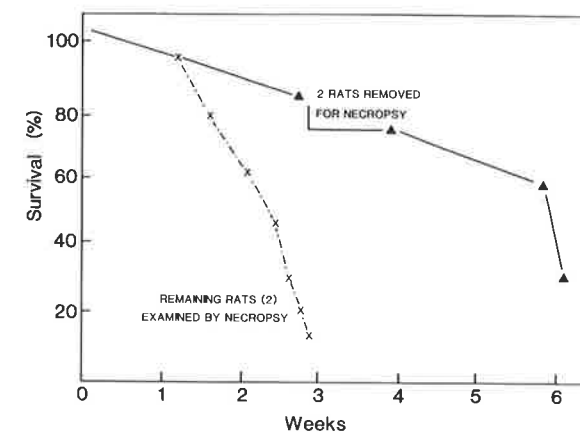
Fig. 7. Percent survival of rats treated with 10 mg/kg adriamycin (high dose) in a single ip injection. The enzymes superoxide dismutase (1 mg/kg) and catalase (1 mg/kg) significantly enhanced survival. Key: X——·—X, adriamycin only; ▲—△, adriamycin plus enzymes.

the point of maximum survival difference. The remaining rats receiving superoxide dismutase, catalase, and adriamycin survived for 6 weeks, which is 2 weeks longer than those given superoxide dismutase alone. Experiments are now in progress to determine whether the increased life span was due to the action of catalase or to the limited exposure to superoxide dismutase or both. The necropsy showed no cardiac damage with the ip injection of 10 mg/kg adriamycin. Death appeared to be due to peritonitis and diarrhea. The animals receiving superoxide dismutase and catalase as described were free of both peritonitis and diarrhea for 5 weeks following injection of 10 mg/kg adriamycin.

Bleomycin

Experimental evidence indicates that superoxide dismutase and catalase can inhibit double-strand DNA breaks by bleomycin (10). In an experiment with 28 mice we administered bleomycin each week for 6 weeks. The mice were split into two groups of 14, each having seven males and seven females. At the end of the 6 weeks all of the females were pregnant in the group receiving bleomycin combined with 1 mg/kg superoxide dismutase and 1 mg/kg catalase. None of the females became pregnant in the group receiving bleomycin alone. These observations support the suggested role of H$_2$O$_2$ and O$_2^-$ in the action of bleomycin (11).

SUMMARY

The data obtained with superoxide dismutase and catalase reveal a complicated time- and dose-dependent relation to *cis*-platinum and adriamycin toxicity. The first reaction to these drugs appears to involve O_2^-. Toxicity that is expressed at later times with high doses appears to involve hydrogen peroxide. There are at least three stages of toxicity. At the first stage, when superoxide dismutase is most protective, catalase does not show much effect. There is a second or intermediate stage in which catalase and superoxide dismutase together produce the best results. This is observed when superoxide dismutase alone increases the toxicity. The third stage involved interrupted administration of superoxide dismutase. Superoxide dismutase can even prevent catalase from being effective if it is continued after the animals begin to die. Since our discovery that superoxide dismutase and catalase can affect the toxicity of *cis*-platinum (6), the capacity of *cis*-platinum to deplete sulfhydryl groups has been reported. The effect of superoxide dismutase and catalase on lipid peroxidation following glutathione (GSH) depletion was measured by the resulting malondialdehyde (MDA) levels (13). Superoxide dismutase and catalase did not inhibit MDA production even when combined. These results show the clear difference between acute *cis*-platinum toxicity, in which superoxide dismutase and catalase are effective, and chronic *cis*-platinum toxicity, in which they have a limited effect.

The schedule of administration as well as the dose of the administered drug is important. Vitamin E is more effective if given in more than one pretreatment (14). The effect of a single vitamin E injection was reported only to delay toxicity (15). A role for GSH in adriamycin toxicity has been reported (16), and an elevation of serum lipid peroxide levels associated with adriamycin treatment can apparently be ameliorated by vitamin E (17). However, when vitamin E application was chronic, this attempt to protect against cardiac toxicity in the rabbit failed (18). Our observation that superoxide dismutase and catalase ameliorated or at least delayed cardiac toxicity suggests that O_2^- and H_2O_2 are more important in chronic adriamycin toxicity than in the toxicity of *cis*-platinum.

The pathology studies after adriamycin administration showed mitochondria in the area where tissue has been damaged (19). We have reported an age-dependent retinal degeneration induced by adriamycin with a similar pathological pattern. Vessels are unaltered, phagocytes are not found, and intact mitochondria are present among remnant photoreceptor discs and remnant photoreceptor nuclei (20). The retinal degeneration does appear to be ameliorated by HR (Z12004), which also enhances sur-

vival and maintains a more normal differential white blood cell count. HR (Z12004) was tested further when it was discovered that the administration of this agent after adriamycin administration provided protection. We now know that vitamin E is most effective when given before adriamycin (21), whereas superoxide dismutase and catalase are most effective when given before and after adriamycin. We therefore conclude that the demonstrated dependence on schedule does not allow a complete assessment of a potential ameliorative agent to be made until it has been tested both before and after administration of the anticancer agent. Any compound that cannot be used after adriamycin administration may not be effective in chronic administration. Finally, we have found that such agents as superoxide dismutase, catalase, and HR (Z12004) can be used after adriamycin administration.

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DISCUSSION

BABIOR: Did you measure pharmacokinetics in these studies? Did you look for a nonspecific effect of protein? These types of controls are *crucial* to any interpretation of your data.

McGINNESS: As I pointed out, these experiments were intended to be exploratory. I am presenting the preliminary experimental results. Before we can offer a reasonable interpretation we need more information. We do see opposite effects with superoxide dismutase and catalase, which argues against nonspecific protein effects. Also, I would like to point out that the observable pharmacokinetics of superoxide dismutase are not simply related to its course of action, so perhaps this is not such a "crucial" question, although it is definitely an important one and one that we wish to answer.

PROCTOR: I would like to point out that there is an internal control in this experiment, namely, the experiment with superoxide dismutase and adriamycin. In any case, the total concentration of added protein is so low that it is very unlikely that it would perceptibly increase plasma total protein.

ŌYANAGUI: You said that the animals died of cancer, but what was the real cause of death in your experimental model? Was it thrombosis?

McGINNESS: This study involved the induction of toxicity by anticancer drugs. Rats receiving *cis*-platinum died of kidney toxicity. Rats receiving adriamycin died of cardiac toxicity with chronic doses and extravasation toxicity at high doses given intraperitoneally.

FRIDOVICH: Have you considered using acatalasic mice or animals whose catalase has been depleted with 3-aminotriazole to see whether they are more susceptible than normal animals to the toxicity of adriamycin? This would lend even more validity to the very impressive results you have already achieved.

McGINNESS: I appreciate the suggestion. Yes, we have thought of using acatalasic animals and will try such experiments. If we are looking at extracellular effects, we may not see effects due to differences inside the cells. For example, acatalasic mice do not seem to be more sensitive to high-pressure oxygen.

SCHMIDT: Your dose schedules for adriamycin with respect to both the dose and frequency are quite different (greater) from those used clinically. Can you explain your choice of dose schedules?

McGINNESS: I used values reported in the literature for Sprague–Dawley rats.

McLENNAN: What is known about the tissue distribution of catalase after intramuscular injection? Because it is a large molecule, it does not cross the normal endothelial membrane.

McGINNESS: Very little work has been done *in vivo* with catalase. I do not know the serum clearance time. In the case of toxicity resulting in membrane damage, however, the tissue distribution can be markedly altered.

DEMOPOULOS: Catalase, like any other protein, can be picked up by the lymphatics. It then enters the bloodstream. At sites of damage, such as the heart in adriamycin toxicity, the vessels leak, and there may be a

"beneficial" leakage of plasma that contains free catalase into the injured site.

MICHELSON: You mentioned that injection of superoxide dismutase into solid tumors increased survival but did not decrease tumor size. Some time ago we injected superoxide dismutase into melanomas (in hamsters) and benzopyrene-induced tumors (in mice). Very marked tumor regression accompanied increased survival, *but* very similar results were obtained with denatured superoxide dismutase. Second, we have begun a program using adriamycin and superoxide dismutase packaged together in a liposome so that both agents are conveyed together to the same site. You have convinced me that I should add catalase to these liposomes.

McGINNESS: I would like to see how this works out. It sounds very promising.

PIETTE: What is the evidence that O_2^- is produced by *cis*-platinum, bleomycin, and adriamycin?

McGINNESS: The species produced by adriamycin has an electron spin resonance spectrum identical to the known spectrum for O_2^-. Bleomycin-induced DNA degradation is inhibited by superoxide dismutase and catalase. Finally, protection from *cis*-platinum toxicity is provided by superoxide dismutase. In all these cases the final toxic species is not known.

COHEN: In our limited experience with adriamycin, using Swiss–Webster mice and doses similar to yours (experiments of M. Bail), we observed the disappearance of the epididymal fat pad and decreased body weight. It seemed that many of our animals were starving to death. Do you monitor food intake in your experiments? Is starvation a factor in your survival curves?

McGINNESS: No, we monitor food intake and weights of the rats. Sprague–Dawley rats tolerate these doses. Animals that show the highest blood urea nitrogen levels die first. Furthermore, the time scale for toxicity correlates with the final pathology reports. We find dramatic differences among species for tolerance to these drugs.

PROCTOR: I would like to clarify the rationale for using superoxide dismutase and catalase for *cis*-platinum nephrotoxicity. First of all, *cis*-platinum does *not* produce O_2^-. However, Ōyanagui has shown that platinum compounds can stimulate O_2^- production by phagocytes. Likewise,

cis-platinum is ototoxic as well as nephrotoxic; there is some evidence that deafness may be related to a free-radical mechanism.

FRIDOVICH: What is the chemical structure of *cis*-platinum?

McGINNESS:

cis-Diaminodichloroplatinum(II)
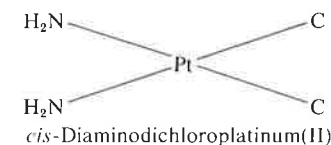
RILEY: With regard to Gerald Cohen's question concerning the nutritional status of the experimental animals, we have been seriously concerned with these experimental parameters. In routine monitoring of food as well as water consumption, we have observed that certain tumors produce a conspicuous anorexia that is reflected in a significant voluntary reduction in food and thus caloric intake. This is known to have effects on tumor behavior. Interestingly, each tumor type has an individual capacity to affect the appetite centers. For example, some tumors have minimal or no capacity to alter food and water consumption, whereas other tumor strains may even stimulate the appetite centers to produce an increase in food consumption as a fraction of increasing tumor mass. Associated stress factors may be stimulated by alterations in food consumption, resulting in an increase in plasma corticosterone levels, destruction of T cells, thymus involution, and an impairment in immunocompetence that may have significant influences on tumor–drug responses.

McGINNESS: This topic is being actively investigated at the University of Texas Cancer Center in connection with patient care. With respect to our rats, we had to use hematological analysis to determine when to sacrifice rats for pathological examination since no behavioral differences were apparent.

JONES: It has been shown that cadmium salts injected into Sprague–Dawley rats affect the immune response in various ways relative to the time of antigen injection. For example, Ca^{2+} injected subcutaneously in very small doses daily for 2 weeks before injection of human γ-globulin (HGG) significantly enhanced both the primary and secondary anti-HGG responses, whereas HGG injected 1 week after or at the same time of Cd^{2+} treatment significantly suppressed the primary response but not the secondary response. Knowing this, one wonders what effect the *cis*-platinum used in your model had on the immune system? Did you measure this effect? Also, do you know if catalase is immunogenic? If it is, there is a

relation between the immune system and the effect of *cis*-platinum; the latter may be associated with the catalase effects that you observed in your model.

McGINNESS: This is an exciting suggestion. The striking difference in the effects of acute and chronic treatment made us think about the immune response. We were unaware of the work you have described. It suggests a number of interesting experiments.

Chapter 13

The Use of Superoxide Dismutase in the Treatment of Cancer

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INTRODUCTION

The enzyme superoxide dismutase (superoxide oxidoreductase, EC 1.15.1.1) is believed to be present in all oxygen-metabolizing cells but lacking in most obligate anaerobes, presumably because its physiological function is to provide a defense against the potentially damaging reactivity of the superoxide radical (O_2^-) generated by aerobic metabolic reactions (1). Superoxide dismutase (SOD) catalyzes the following reaction (2):

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$
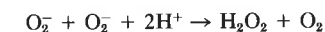
Four different forms of superoxide dismutase have been found to date (4). One of these, which is found in the cytosol and intermembrane space of mitochondria of eukaryotic cells, contains copper and zinc (CuZnSOD) and is entirely unrelated, except in its activity, to the other three. An example of this superoxide dismutase is the erythrocuprein found in bovine and human red blood cells. There are two kinds of superoxide dismutase that contain manganese (MnSOD). One of these is found largely in the matrix of mitochondria (5), and the other in the matrix of bacteria such as