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 survival 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?

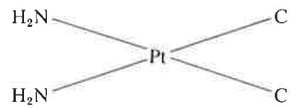
McGINNESS: No, we monitor food intake and weights of the rats. Sprague–Dawley rats tolerate these doses. Animals that show the highest blood urea nitrogen levels die first. Furthermore, the time scale for toxicity correlates with the final pathology reports. We find dramatic differences among species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

LARRY W. OBERLEY, SUSAN W. H. C. LEUTHAUSER, GARRY R. BUETTNER, JOHN R. J. SORENSON, TERRY D. OBERLEY, AND ISABEL B. BIZE

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

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Escherichia coli (6) and *Streptococcus mutans* (7). The fourth type of superoxide dismutase contains iron (FeSOD) and has been found in the periplasmic space of *E. coli* (8,9). The purpose of this chapter is to review the role of superoxide dismutase in the treatment of cancer and to discuss how knowledge of the levels of this enzyme in tumor cells may lead to potentially better treatment.

LEVELS OF SUPEROXIDE DISMUTASE AND SUPEROXIDE RADICAL IN CANCER CELLS

Many comparisons have been made between superoxide dismutase activities in normal and in malignant cells. A typical pattern has emerged from these studies. Cancer cells have in general smaller amounts of both CuZnSOD and MnSOD than do their normal cell counterparts. Exceptions to this pattern have been found in the case of CuZnSOD but not in the case of MnSOD. The activity of MnSOD is greatly reduced in *in vivo, in vitro,* spontaneous, transplanted, virally induced, and chemically induced tumors (unpublished observations). This work has been the subject of a recent review (10). A typical example is the mouse liver system (11). Normal mouse liver was found to contain 122 ± 12 units of CuZnSOD activity per milligram protein and 35 ± 5 units of MnSOD activity per milligram protein. Regenerating liver, a rapidly dividing normal cell system, also contained both CuZnSOD and MnSOD. However, the superoxide dismutase activity varied with time after partial hepatectomy. The amount of CuZnSOD was greatly diminished 4 days after surgery, a time at which a nearly synchronous wave of cell division occurs, whereas MnSOD was present at all times after surgery and did not decrease. In contrast, H6 hepatoma tumor cells contain a greatly diminished amount of CuZnSOD and no detectable MnSOD. Isolated mitochondria from normal liver cells contained a large amount of superoxide dismutase activity, but isolated mitochondria from H6 hepatoma mitochondria contained no measureable superoxide dismutase activity. Thus, in this system, a loss of MnSOD was representative of tumor tissue and not of any form of normal tissue.

We have questioned the significance of these enzyme changes because the cancer cell exhibits numerous enzyme alterations. Why is superoxide dismutase any more important than any other enzyme? The answer may be that superoxide dismutase is fundamentally different from most other enzymes in that it is a *protective* enzyme. Indeed, Fridovich has provided substantial evidence that this enzyme is necessary for life in all oxygen-

metabolizing cells (3). We believe that the loss of the enzymatic activity of superoxide dismutase in cancer cells leads to changes in key subcellular structures because of the presence of oxygen-derived radicals.

However, superoxide dismutase is a protective enzyme only if its substrate, O_2^-, is present in the cancer cell. If O_2^- is not produced in the cancer cell, then a loss of MnSOD should not have any harmful effects. In this case, research should focus on the loss of superoxide-producing capacity and not on the loss of MnSOD. From these considerations, it can be seen that, in order to establish that the loss of MnSOD is important in malignancy, it is also necessary to show the production of superoxide in tumor cell mitochondria. Dionisi *et al.* (12) have shown that two tumor types have the capacity to produce superoxide. Using adrenochrome formation as an indicator of O_2^- production, we have been able to show that mouse H6 hepatoma tumor mitochondria also produce superoxide. The data are shown in Table I, which indicates the amount of adrenochrome formed each minute per milligram of submitochondrial particles isolated from H6 hepatoma. In all cases studied, superoxide dismutase inhibited adrenochrome formation, showing that O_2^- was responsible for the reaction. Antimycin A (an inhibitor of electron transport) alone caused superoxide production in a reaction system that contained tissue, epinephrine, and diethylenetriaminepentaacetic acid. Succinate enhanced the amount of O_2^- produced by a factor of 3. Antimycin A caused more O_2^- production

TABLE I

Adrenochrome Formation in Submitochondrial Particles[a]

Reactants	−SOD	+SOD
Succinate	1.44	Not done
Antimycin A	0.96	0.00
Succinate + antimycin A	2.88	0.19
Succinate + rotenone	1.44	Not done
NADH + antimycin A	1.44	0.34
NADH + rotenone	1.15	Not done

[a] Membrane fragments (0.5 mg protein per milliliter) from mouse H6 hepatoma tissue were in all cases suspended in 0.25 *M* sucrose, 50 m*M* HEPES, pH 7.5, 0.5 m*M* diethylenetriamine pentaacetic acid, and 1 m*M* epinephrine. In some of the tubes, 3 m*M* succinate, 2.5 μg/ml antimycin A, 13 μ*M* NADH, or 2 μM rotenone was also added. Measurements were performed in a Cary 15 spectrophotometer at both 480 and 575 nm. Values are given in nanomoles adrenochrome formed per minute per milligram of submitochondrial particles.

than rotenone, and succinate was a more effective substrate than NADH. These results, as well as those of Dionisi, show that cancer cells have the capacity to produce O_2^-.

A diminished amount of MnSOD coupled with superoxide production appears to be a general characteristic of the tumor cells studied. This undoubtedly leads to changes in subcellular structures because of the presence of oxygen-derived chemical species (such as hydroxyl radical, singlet oxygen, and hydroperoxides). These changes are in turn probably responsible for at least part of the phenotypic properties of the cancer cell.

TREATMENT OF CANCER

We have described a general characteristic of the tumor cell: a diminished amount of MnSOD coupled with superoxide production. What use can we make of this in the treatment of cancer? Three different techniques are being used at present: (a) production of an excess flux of O_2^- or other superoxide-derived radicals in tumor cells, (b) inhibition of CuZnSOD, and (c) addition of superoxide dismutase activity to tumor cells.

The first technique is the oldest and was actually developed long before the association of cytotoxicity with O_2^- was known. Many antitumor agents produce or cause the production of O_2^- or superoxide-derived radicals such as $HO^\cdot$. If an equal amount of O_2^- or superoxide-derived radicals can be delivered to both cancer cells and normal cells, the cancer cells should be preferentially killed because they have lower MnSOD activity. An example of this type of mechanism is the action of the anticancer agent bleomycin, a glycopeptide antibiotic thought to be cytotoxic to tumor cells because of DNA chain breakage. *In vitro* DNA chain breakage by bleomycin was shown to be enhanced by the addition of xanthine–xanthine oxidase (13). The effect of the xanthine oxidase system disappeared completely when superoxide dismutase was added. From these results, it was concluded that superoxide radical is one of the mediators for the enhancement of the DNA chain breakage action of bleomycin. Sausville *et al.* have shown that DNA degradation by bleomycin requires oxygen and Fe(II) (14). Reducing agents such as ascorbate and H_2O_2, as well as O_2^-, greatly increase the DNA degradation. These observations have led Sausville *et al.* to propose the following model for the action of bleomycin (14). Bleomycin can bind to DNA in the absence of metal ion or reducing agent. Iron(II) can then attach to the bleomycin and thus form a ternary complex. The ternary complex can produce a species that degrades DNA.

Reducing agents, including O_2^-, enhance the breakage by regenerating Fe(II) from Fe(III) and thus continuing the reaction. The nature of the toxic species is not identified in this model. Since the mechanism is similar to that observed by us (15) and others (16–18) for the production of hydroxyl radical from xanthine–xanthine oxidase, the authors thought that this radical might also be responsible for the degradation of DNA by bleomycin. Using the technique of spin trapping, we have observed that bleomycin and Fe(II) produce $HO^\cdot$ (19). Because of the high reactivity of $HO^\cdot$, it is likely that this radical is responsible for the toxicity caused by bleomycin. Since bleomycin binds preferentially to DNA, the net result is a site-specific free radical. As mentioned earlier, reducing agents such as H_2O_2 and O_2^- are necessary for bleomycin to degrade DNA effectively. What is the source of reducing agent in the tumor cell? Since tumor cells apparently have a lower level of MnSOD and many have a diminished amount of CuZnSOD, tumor cells should have a greatly increased level of O_2^-. Moreover, O_2^- has been shown to be produced in tumor cell nuclei (20). The increased level of O_2^- in tumor cells as compared to normal cells may explain the differential toxicity exhibited between normal and malignant cells upon treatment with bleomycin.

There are many other examples of antitumor agents that produce superoxide or superoxide-derived radicals. The antitumor antibiotic streptonigrin causes DNA strand breaks *in vivo* (21). This antibiotic has been shown to generate the superoxide anion upon reduction and autoxidation *in vitro* (22,23), and the superoxide anion has been shown to cause strand breaks in closed circular double-stranded DNA (24,25). These observations have led to the formulation of a mechanism in which antibiotic generates superoxide during a reduction–oxidation cycle, and this radical brings about single-strand breaks (22,25). It was also shown that superoxide radicals are formed by the redox cycling of the antitumor anthracycline antibiotics daunomycin and adriamycin (26). It was found that NADPH and purified cytochrome *P*-450 reductase caused oxygen consumption from these drugs in excess of the amount of drug present. A reduction–autoxidation of sulfite may be initiated. The latter reaction was inhibited by superoxide dismutase, suggesting that O_2^- was formed. Hydrogen peroxide was also generated, presumably by nonenzymatic dismutation of superoxide. Rat liver microsomes also catalyzed this redox cycling, which was accompanied by the peroxidation of lipids. These experiments suggested that the formation of oxygen radicals followed by lipid peroxidation may be the basis for the cardiotoxic effects of these drugs.

Thayer has shown that adriamycin stimulates superoxide formation in submitochondrial particles (27). At a concentration of 400 μM, adriamy-

cin stimulated the rate of O_2^- formation sixfold to 25 nmol/min mg. Measurements of the relative catalase activity of blood-free tissues of rabbits and rats indicated that heart contained 2–4% of the catalase activity of liver or kidney. The author concluded that an enhanced production of O_2^- and H_2O_2 and the relatively low catalase content of heart tissue may be factors in the cardiotoxicity induced by adriamycin chemotherapy.

Bachur *et al.* (28) extended these measurements and proposed a unifying theory for their mechanisms of action. They found that the highly active quinone-containing anticancer drugs, adriamycin, daunorubicin, carminomycin, rubidazone, nogalamycin, aclacinomycin A, and steffimycin (benzathraquinones), mitomycin C and streptonigrin (*N*-heterocyclic quinones), and lapachol (naphthaquinone) interacted with mammalian microsomes and functioned as free-radical carriers. These quinone drugs augmented the flow of electrons from reduced nicotinamide adenine dinucleotide phosphate to molecular oxygen. This reaction was catalyzed by microsomal protein and produced a free-radical intermediate form of the drugs as determined by ESR spectroscopy. Several nonquinone anticancer agents were tested and found to be inactive in this system. Since quinone anticancer drugs are associated with chromosomal damage that appears to be dependent on the metabolic activation of these drugs, the authors proposed that intracellular activation of these drugs to a free-radical state is primary to their cytotoxic activity. Because of their high affinity and selective binding to nucleic acids, these drugs, as free radicals, have the potential to be "site-specific free radicals" that bind to DNA or RNA and either react directly or generate oxygen-dependent free radicals such as O_2^- or $HO^\cdot$ to cause the damage associated with their cytotoxic actions.

Thus, a large number of anticancer drugs seem to involve O_2^- or O_2^--derived radicals in their mode of action. The differential toxicity of O_2^- to tumor cells as compared to normal cells may be brought about by the lack of MnSOD in tumor cells. This, perhaps coupled with increased O_2^- production in tumor cells, can easily explain their mechanism of action.

All of these anticancer drugs that involve superoxide are also toxic to normal cells and are associated with later induction of cancer. Thus, the capacity to kill tumor cells is less than optimal because the concentrations of drugs that can be used are limited by the damage to normal cells. Since damage to normal cells is also caused by superoxide or superoxide-derived radicals, it is conceivable that normal tissue could be protected by the administration of superoxide dismutase. Of course, in this type of therapy, one must be concerned with whether superoxide dismutase will also affect the antitumor action of the drug. There has been one report that superoxide dismutaste can prevent damage to normal cells. McGinness *et al.* have reported that a subcutaneous dose of CuZnSOD lowers the nephrotoxicity induced by a 5.0 mg/kg dose of the antitumor agent *cis*-platinum (29). The toxicity of *cis*-platinum was studied by comparative measurements of blood urea nitrogen, change in body weight, and renal histology. The results support a role for oxygen radicals in the nephrotoxicity of *cis*-platinum.

A second method that makes use of the inherent differences between normal and tumor cells in superoxide dismutase activity was proposed by Lin and associates (30). They inhibited CuZnSOD with diethyl dithiocarbamate (DDC). Since normal cells still have MnSOD, they were expected to survive this treatment, whereas tumor cells, having only CuZnSOD, were not. The authors carried out preliminary experiments with normal Chinese hamster cells (DON). The cytotoxic effect of DDC on DON cells was dependent on the DDC concentration and exposure time. After 8–10 days of incubation with 10^{-9} *M* DDC, no change in DON cell survival was noted; however, incubation with 10^{-4} *M* DDC showed marked toxicity. When DDC-treated cells were irradiated, they showed a lower percentage survival than cells that were treated with radiation or DDC alone. The combined effects of hyperthermia and DDC were dramatic. Cells treated 8 min at 43°C with 10^{-4} *M* DDC or 10 min at 47°C with 10^{-5} *M* DDC showed a significant decrease in survival. These results suggested that DDC may be a powerful sensitization agent in tumor therapy. However, it still has not been shown whether DDC alone or in combination with other anticancer agents will kill more tumor cells than normal cells. Without such differential toxicity, it is difficult to believe that this therapy will be successful because the damage to normal tissue will be too severe.

The third potential method of treatment involves the addition of superoxide dismutase to tumor cells. The proposed mechanism of action is radically different from those of the two techniques already discussed. The latter two involved using differences in superoxide dismutase activities to kill tumor cells preferentially. The third method does not involve cytotoxicity, but rather addition of superoxide dismutase to tumor cells to try to halt cell division. The rationale of this approach is simply that the level of MnSOD is diminished in tumor cells. This enzyme could be responsible for part of the cancer cell phenotype, and it is possible that the addition of superoxide dismutase to these tumor cells would cause cessation of cell division.

In order to test this method, it is necessary to use a compound that has superoxide dismutase activity and penetrates the cell. The most likely candidate, natural CuZnSOD, would not be expected to be effective because of low penetrability into cells (31). Nonetheless, the enzyme was tested and, surprisingly, was found to have some effect on tumor growth.

Treatment with a single intratumor injection of CuZnSOD 1 hr after intramuscular injection of 4×10^6 sarcoma 180 tumor cells had a small effect on tumor growth (Fig. 1). It also increased animal survival by approximately 20% (Fig. 2). The group treated with superoxide dismutase had 19% long-term survivors (more than 90 days); the saline-treated group had 0% long-term survivors.

Similar experiments with Ehrlich ascites carcinoma cells produced little effects on tumor growth, but the animals treated with superoxide dismutase survived longer. Preliminary evidence indicates that this is because of better encapsulation of the tumor in the group treated with superoxide dismutase, with less metastasis resulting. Thus, it appears that natural CuZnSOD does have an effect, although it is small.

If we could find a compound with superoxide dismutase activity that could penetrate the cell, our results might be more dramatic. For this reason, we investigated the copper coordination compounds that have been studied by Dr. John Sorenson for many years because of their antiarthritic and antiulcer activities (32); these compounds have high superoxide dismutase activity (33,34). Recently we synthesized copper coordination compounds with high penetrability. Our preliminary evidence indicates that these compounds have a dramatic effect on tumor growth. Figure 3 shows the results of the first experiments. Tumors were induced in CBA mice by intramuscular injection of 5×10^6 Ehrlich ascites tumor cells. These tumors were then treated by an injection of 1 mg of copper coordination compound at 1 hr, 1 day, 2 days, 3 days, and 4 days. Three compounds were tested: copper(II)$_2$(aspirinate)$_4$(DMSO)$_4$, copper(II)$_2$(aspi-

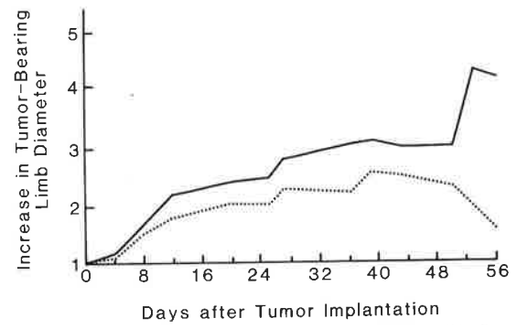

Fig. 1. Effect of superoxide dismutase treatment on tumor growth. Average tumor size was measured as a function of time after tumor implantation. Sarcoma 180 cells (4×10^6) were injected intramuscularly into male CF1 mice; CuZnSOD (---) or saline (—) was given intramuscularly 1 hr after implantation of the tumor. Each experimental group contained 16 animals.

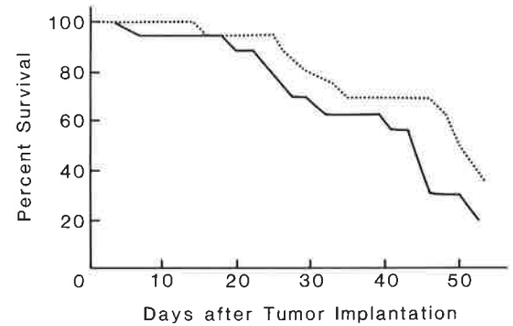

Fig. 2. Effect of superoxide dismutase treatment on cumulative mortality of tumor-bearing mice. Animal survival was monitored as a function of time after tumor implantation. Sarcoma 180 cells (4×10^6) were injected intramuscularly into male CF1 mice; SOD (---) or saline (—) was given intramuscularly 1 hr after implantation of the tumor. Each experimental group contained 16 animals. Survival time of the animals treated with superoxide dismutase was significantly greater than that of the saline-treated animals, as evaluated by the Smirorov test ($p = 0.05$).

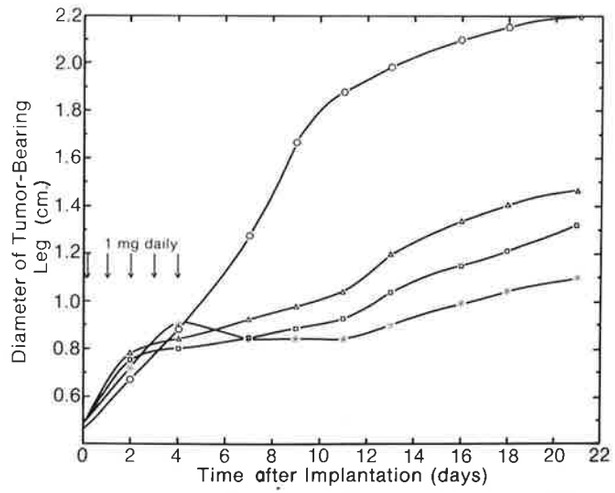

Fig. 3. Effect of copper coordination compounds on the growth of Ehrlich ascites tumor cells in CBA mice. Average tumor size in centimeters was determined as a function of time after tumor implantation. Ehrlich ascites tumor cells (5×10^6) were injected intramuscularly into male CBA mice. A 10% solution of Tween 80 in saline or 1 mg of copper coordination compound in Tween 80 was given intramuscularly at 1 hr, 1 day, 2 days, 3 days, and 4 days after tumor implantation; 0.2 ml was injected in each case. Each experimental group contained four animals. Key: ○, 10% Tween 80–saline; △, Cu(II)$_2$(aspirinate)$_4$(DMSO)$_4$; □, Cu(II)$_2$(aspirinate)$_4$(pyridine)$_4$; *, Cu(II)(3,5-diisopropyl salicylate)$_2$.

rinate)$_4$(pyridine)$_4$, copper(II)(3,5-diisopropyl salicylate)$_2$. The three compounds have quite different degrees of lipid solubility, the copper(II)$_2$(aspirinate)$_4$(DMSO)$_4$ being the least soluble and copper(II)(3,5-diisopropyl salicylate)$_2$ being the most soluble. The degree of inhibition of tumor growth follows the same order as the degree of lipid solubility, the aspirinate–DMSO compound being the least effective and diisopropyl salicylate the most effective. Histological studies indicate that these compounds function not by killing cells but by inhibiting cell division. Furthermore, there was no evidence of an inflammatory infiltrate in treated or control tumors. We have not established the mechanism of action of these compounds, but we can say that compounds with superoxide dismutase activity that penetrate the cell appear to inhibit tumor cell growth.

The idea that copper coordination compounds can be used as antitumor agents is not new; many other copper compounds have been shown to have antitumor activity (35–37). However, all of these studies have centered on compounds that are cytotoxic; copper coordination compounds that are not cytotoxic but only inhibit cell division have not been studied in detail (38). Compounds that are not cytotoxic must be administered continuously, whereas cytotoxic compounds can be given only intermittently. This represents a fundamentally new approach to cancer therapy.

It appears that this type of therapy has distinct possibilities for cancer treatment. However, all of these compounds exhibit toxicity at high doses (32), so chronic long-term administration may also have deleterious side effects. For this reason, it is still very desirable to use naturally occurring superoxide dismutase, which is metabolized by natural processes. Thus, encapsulating superoxide dismutase in liposomes may ultimately lead to the best treatment of cancer.

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DISCUSSION

FRIDOVICH: Congratulations on your exciting work. Have you considered that the Warburg phenomenon, that is, lactate production by tumor cells (even under aerobic conditions), might be explained by the lack of MnSOD from tumor mitochondria? Thus, tumor mitochondria would suffer self-inflicted damage due to unscavenged O_2^- generated within the mitochondria. The tumor cell would then have to rely on the cytoplasmic fermentation (Embden–Meyerhof pathway) for its energy supply.

OBERLEY: We have considered this possibility and believe that it is very likely that the high glycolytic rate of tumors is caused by mitochondrial damage. We are at present examining tumors with varyious rates of glycolysis, and our results show that the degree of mitochondrial damage and the level of MnSOD correlate well with the rate of glycolysis. Thus, tumors with a high glycolytic rate show a low level of MnSOD and significant mitochondrial damage.

ŌYANAGUI: I have observed the existence of electron release to an unknown compound in the mitochondria of a variety of liver tumor cells at the cytochrome oxidase site. Is there a possibility of O_2^- involvement at this site? Also, what is the superoxide dismutase content in the cultured cells of normal origin?

OBERLEY: Normally, electrons are not donated singly to oxygen at the cytochrome oxidase site. However, tumor cell mitochondrial membranes may be damaged, so that leakage from the cytochrome chain may occur; thus, your observation could be consistent with my observations on the lack of MnSOD in tumor cells. In answer to your second question, the one study that was done indicated that tissue culture cells of normal origin had both CuZnSOD and MnSOD. The content of each was about the same as in the *in vivo* cells.

PIETRONIGRO: If your hypothesis concerning bleomycin antitumor effectiveness is correct, one would expect to see an oxygen effect with respect to tumor cell killing. Has this been observed?

OBERLEY: I believe that an oxygen effect has been seen in connection with *in vitro* DNA breakage, but I don't know if one has been seen in association with cell killing.

PIETRONIGRO: Since the formation of the "active" bleomycin–Fe^{2+}–O_2 complex seems independent of DNA binding, one may expect to see bleomycin damage at other cellular sites, especially those producing reducing species, including O_2^-. In this regard, do you know whether bleomycin damaged either tumor or lung mitochondria?

OBERLEY: I don't know of any data on this. Perhaps some DNA might be damaged, including both the nuclear or mitochondrial DNA.

DEL MAESTRO: Could the level of superoxide dismutase in tumor cells be a reflection of the O_2^- content in which the tumor cells are living *in vivo* rather than a genetic DNA effect?

OBERLEY: This possibility has been considered by us and others. Dr. Petkau has shown that total superoxide dismutase activity increases as it

goes from the poorly oxygenated center of mammary tumor to the well-oxygenated periphery of the tumor. However, we have observed in preliminary studies that, in H6 hepatoma tumors, the levels of MnSOD do not seem to vary much in the tumor; so the answer to your question is that it is possible, but the evidence so far does not point in that direction.

PROCTOR: This is very exciting work. Bleomycin is of particular interest in cancer chemotherapy because it is uniquely active against cells in the G_0 (nonreplicating) tumor population. Under ordinary circumstances, these are very difficult to kill chemotherapeutically. There is a tendency for anoxic tumor cells to be in the G_0 phase. Perhaps this is related to their sensitivity to bleomycin-induced radicals since the content of intracellular protective systems may be diminished. I wonder what the content of superoxide dismutase and associated enzymes is as a function of the cell cycle, since both bleomycin and adriamycin seem to be cycle-dependent cytotoxic agents.

SHEREMATA: Dr. Oberley's observations prompt me to make a comment on a recent study made in collaboration with Dr. Stanley Skoryna and Dr. K. Tanaka at McGill University. Manganese deficiency is a well-recognized cause of seizures in foraging animals. It has also been induced in a variety of laboratory animals, including rats. These observations led us to study levels of manganese and magnesium in the serum of humans with epileptic seizures. Newborn infants in status epilepticus had barely detectable levels (<5 ng/ml) of these elements. In children or adults with idiopathic epilepsy, intermediate levels (10–20 ng/ml) were observed. Others who had isolated seizures after trauma or surgery had only slightly decreased levels (20–35 ng/ml). Effective anticonvulsant therapy did not alter these levels. In children, however, there was a definite rise in manganese, possibly reflecting unrelated factors. In view of the abrupt rise in blood and tissue oxygen levels following delivery, these observations suggest that an absence of manganese-containing superoxide dismutase may result in the abrupt appearance of O_2^- and neuronal membrane damage, leading to depolarization and seizure activity. It should be mentioned that manganese serum levels bore no relationship to the presence or absence of seizures.

Our observations also relate to the fact that brain tumors frequently produce seizures. However, malignant, infiltrating tumors of brain may not. This fact does cloud the issue somewhat. We must study MnSOD in these tumors including the edematous and normal surrounding brain tissue, as well as in normal brain. The evidence nevertheless points to the importance of MnSOD deficiency in the genesis of seizures.

CUTLER: You have put forth a very interesting model suggesting that low superoxide dismutase levels may be responsible for some of the characteristics of tumors (transformed) cells. I wonder if the reverse might be true, that is, that an initial low value of superoxide dismutase could lead to transformation. According to such a model, a low superoxide dismutase level would lead to tumor formation but would not be responsible for its maintenance.

OBERLEY: The model you suggest is as plausible as the one we have suggested. Experimentally, all we know is that superoxide dismutase levels are altered. It is possible that changes in DNA lead to a loss of MnSOD or that a loss of MnSOD leads to DNA damage from oxygen-derived radicals. We have no real evidence for either model at the present time.