Probable Immune System Mediation of the Antitumor Activity of the 2,6-Dimethoxybenzo-p-Semiquinone Radical

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Abstract. **We recently reported that the antitumor activity of an immobilized oxidase-peroxidase system, which can produce radical species as intermediates, is dependent on the presence of an intact and functioning immune system in the host. A number of recent investigations have indicated that the bioactive form of many quinone-type anticancer agents may be the semiquinone-type radical. To investigate if the antitumor activity of some of the quinone-type anticancer agents may also be dependent on a fully functioning immune system in the host, we used as a simple, well-defined model the 2,6-dimethoxybenzo-p-semiquinone radical. This free radical has a potent antitumor activity against Ehrlich ascites tumors in mice, as first described by Szent-Gyorgyi's group. In the present study, we investigated its antitumor activity in immunocompromised BALB/c mice.**

We found that the antitumor activity of the semiquinone radical is completely abolished in mice that had previously been subjected to whole body γ irradiation, indicating a possible involvement of one or more cells of the host's defense system. The antitumor activity was not abolished in mice with dysfunctional macrophages induced by a severe selenium deficiency. However, the radical was inactive in mice whose immune system was suppressed with cyclosporin A and in nude (*nu/nu*) mice.

These results suggest that the dimethoxybenzo-semiquinone radical, rather than acting primarily as a cytotoxic agent as previously reported, exerts its antitumor activity mainly via a more complex mechanism in which T lymphocytes appear to play a major role. [P.S.E.B.M. 1995, Vol 208]

We recently reported that the antitumor activity of an immobilized oxidase-peroxidase system is ineffective in immunocompromised mice (1). Thus, in contrast to normal mice, mice that had received a sublethal γ-ray whole body irradiation, mice that were treated with cylosporin A, and mice that were severely selenium deficient, as well as athymic mice, did not respond to treatment with an immobilized mixture of glucose oxidase and horseradish peroxidase (2) following a challenge with Ehrlich ascites tumor cells. These results suggested that, rather than promoting a direct cytotoxic or cytostatic effect on the tumor cells, part of the host's immune system may play an essential role in the mechanism of action of the peroxidase system.

In evaluating possible mechanisms for this peroxidase-mediated antitumor effect, we considered the fact that peroxidases, in the presence of hydrogen peroxide or an H_2O_2-generating system such as an oxidase, produce free radical species as intermediates in the oxidation of a large variety of organic substrates (see Ref. 3 for an example). We also considered a number of observations during the past decade which have

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indicated that many of the clinically used quinone-type anticancer drugs undergo oxidation-reduction reactions *in vivo*, thereby becoming "bioactivated". In a number of instances this bioactivation involves the *in vivo* one-electron oxidation or reduction of the drug to a free radical. For example, 9-OH-ellipticine and some of its analogs have to be activated by hydrogen peroxide and a peroxidase in order to have anticancer activity, whereas analogs that cannot be oxidized by H_2O_2 are inactive as anticancer drugs (4, 5). Also, before expressing its anticancer activity against melanomas, 4-hydroxyanisole is believed to be "activated" by the tyrosinase in the melanoma cells (6). Other phenolic and quinone-type anticancer drugs that presumably act as "site-specific free radicals" include adriamycin (7–10), daunorubicin (10, 11), carminomycin (10, 11), rubidazone (11), nogalamycin (11), aclacinomycin A (11), steffimycin (11), mitomycin C (12), streptonigrin (11), actinomycin D (13), vinblastin (14), CCNU (15), etoposide (16, 17), and thio-TEPA (18, 19). Several reviews on this subject have recently appeared (20–22).

Unfortunately, these compounds possess rather complex chemical structures, which make it extremely difficult to accurately determine all the chemical interactions that may take place in a tumor-bearing host following the administration of one of these compounds. However, a clearly defined model of some simple, chemically well-characterized semiquinone radicals that also act as antitumor agents was presented in 1983 by Szent-Gyorgyi and his colleagues, who reported that a mixture of ascorbic acid and various substituted benzoquinones exerted a potent anticancer activity in mice bearing Ehrlich ascites tumor (23). Neither ascorbate nor the quinones alone demonstrated anticancer activity, indicating that the reaction product, the semiquinone radical, was the active agent. This was confirmed with electron spin resonance studies, which were done simultaneously to establish the formation of the radicals and measure their half-life. Their experiments further indicated that the semiquinones with a longer half-life were also the more effective anticancer agents. The most dramatic results were obtained using a mixture of 750 m*M* ascorbate and 3 m*M* 2,6-dimethoxy-p-benzoquinone. This mixture, when injected daily into tumor bearing mice for 7 days, resulted in a complete inhibition of tumor growth in more than 90% of the animals. No mention was made of any adverse side effects, leaving the impression that the action of the semiquinone radical was rather specifically directed toward the tumor cells.

Further *in vitro* experiments by Szent-Gyorgyi and his colleagues indicated that the 2,6-dimethoxybenzo-semiquinone radical can interact with a membrane-bound, sulfhydryl-containing enzyme present in the tumor cell membrane, which could cause the demise of the tumor cell (24). It is unlikely, however, that this enzyme is exclusively present in tumor cells. Therefore, this observation may not completely explain the specificity of the radical toward tumor cells *in vivo*.

In view of our results obtained with the immobilized oxidase-peroxidase system and the possibility that a free radical intermediate may be involved in that system, we decided to investigate if the host's immune system does participate in the antitumor activity of Szent-Gyorgyi's semiquinone radical. Our results indicate that this is indeed the case and, more specifically, it appears that the T lymphocytes may be primarily involved in the mechanism of action of this agent.

Materials and Methods

Materials. L-Ascorbate, NADPH, and glutathione reductase were purchased from Sigma Chemical Co. (St. Louis, MO). Cyclosporin A (Sandimmune) was obtained as a solution of 50 mg/ml from the University Medical Center pharmacy and 2,6-dimethoxy-p-benzoquinone was generously supplied by Dr. G. Fodor, West Virginia University (Morgantown, WV).

BALB/c and nude mice were purchased from Sasco, Inc. (Omaha, NE) and were housed in the TTUHSC vivarium throughout the experiments. All experiments reported here were approved by the TTUHSC Animal Care and Use Committee (Approval AR85069).

The wild-type Ehrlich ascites tumor was kindly provided by Dr. L. M. Slater of the University of California at Irvine (Irvine, CA). The tumor was maintained by weekly passage of 0.2 ml ascites fluid intraperitoneally into two or three healthy BALB/c mice.

Experimental Protocol. Six- to eight-week-old BALB/c mice were each inoculated by intraperitoneal injection with 0.2 ml ascites fluid which was freshly drawn from a donor mouse and contained 2–3 $\times$ 10^8 viable tumor cells. On the day following the tumor injection the animals were randomized into groups of 10 and the experimental group received a 7-day course of twice-daily intraperitoneal injections of a quinone/ascorbate mixture, consisting of 0.25 ml 3 m*M* 2,6-dimethoxy-p-benzoquinone and 0.25 ml 750 m*M* ascorbate. The sterile solutions were injected using a two-barreled syringe, in which the solutions were mixed in the needle hub immediately before passage through the needle. Control animals received either saline injections or injections of only one of the components.

On the 8th day the animals were euthanized by cervical dislocation and their peritoneal cavities were exposed and examined for the presence of ascites. Control animals routinely contained several milliliters

of ascites. Only if no ascitic fluid could be detected were animals scored as tumor free.

Gamma Irradiation. BALB/c mice were irradiated for 0.47 min in groups of 10, receiving a total of 500 rads of radiation during this time period, with a J. L. Shepherd Model 143 Blood Irradiator containing a ^{137}Cs source. Irradiated mice were challenged with tumor cells and then treated with the semiquinone system following the protocol described above. Injection with tumor cells was done either 1 day or 3 days after irradiation.

Induction of Selenium Deficiency. BALB/c mice were fed selenium-free chow for at least 4 weeks prior to tumor induction. The defined diet was the Schwartz-Fredga Se-Deficient chow, containing 50 IU/kg of vitamin E, and was purchased from Dyet, Inc. (Catalog no. 116014: Bethlehem, PA). Water was given *ad libitum* during this period. We had previously established that this diet regimen was sufficient to decrease liver glutathione peroxidase activity to almost undetectable levels (less than 2% of normal values).

Assays for Glutathione Peroxidase. BALB/c mice were sacrificed by cervical dislocation and their livers were removed and carefully blotted dry. Approximately 1 g of tissue was then excised from each liver and perfused in 0.15 *M* KCl for 10–20 min. The tissue was homogenized at 4°C in a total of 4 ml 0.25 *M* sucrose and centrifuged for 10–20 min at 20,000 rpm. Assays were done on the supernatants, using livers of three healthy mice and three Se-deficient mice.

Assays were done spectrophotometrically by coupling the glutathione peroxidase reaction to the oxidation of NADPH with glutathione reductase:

$$2GSH + H_2O_2 \longrightarrow GSSG + 2H_2O$$

$$GSSG + 2NADPH \longrightarrow 2GSH + 2NADP^+$$

Assay cuvettes contained the following ingredients: 2 m*M* reduced glutathione, 0.25 m*M* NADPH, 1 m*M* Na-azide (to inhibit any catalase activity), 1 unit/ml glutathione reductase and 20–200 μl of the crude liver extract in a total of 2 ml 50 m*M* phosphate buffer, pH 7.0, containing 1.5 m*M* EDTA. The reaction was started with the addition of hydrogen peroxide to a final concentration of 0.25 m*M*. Activity was determined by measuring the decrease in absorbance of NADPH at 340 nm over a 5-min period, using a Model 8450A Hewlett Packard UV/VIS spectrophotometer.

Statistical Analysis. Tumor incidences between the treatment groups were evaluated by chi-square analysis. Results in the tables are expressed as the number of mice bearing tumor ascites from the total number of mice in each group, following the 7-day regimen of twice-daily injections of the indicated compounds.

Results

Activity of the Dimethoxybenzo-Semiquinone Radical Against Ehrlich Ascites Tumor *In Vivo*. In the first experiment we administered a mixture of 750 m*M* ascorbate and a saturated solution of 2,6-dimethoxy-p-benzoquinone (~3 m*M*) to normal, Ehrlich ascites tumor-bearing BALB/c mice, using the basic protocol of Pethig *et al.* (23), as described in Materials and Methods. This resulted in a complete inhibition of tumor growth in more than 90% of the animals during the time of the experiment (Table I), thus confirming the results reported by Szent-Gyorgyi and his colleagues (23). Differences in tumor incidence were significant ($P < 0.005$) between mice which received the complete ascorbate/2,6-dimethoxy-p-benzoquinone treatment and mice which received the individual components or saline as controls. In general, no significant differences ($P > 0.05$) in tumor incidence were observed between the control groups, which indicates that neither ascorbate nor the dimethoxybenzoquinone alone were effective in inhib-

Table I. Antitumor Effect of 2,6-Dimethoxybenzo-Semiquinone Radical in BALB/c Mice Exposed to Sublethal γ Radiation

Administered compound(s)	Tumor Incidence		
	No radiation[a]	1 Day post x-ray	3 Days post x-rays
2,6-Dimethoxy-p-benzoquinone and Ascorbate[b]	0/10	2/10	9/10
2,6-Dimethoxy-p-benzoquinone[c]	9/10	—	10/10
Ascorbate only	6/10	—	9/10
Saline only[d]	10/10	8/10	10/10

[a] Significant differences ($P < 0.005$) in tumor incidence were demonstrated between mice which received the complete ascorbate/2,6-dimethoxy-p-benzoquinone treatment and mice which received the individual components or saline as controls. In general, no significant differences ($P > 0.05$) in tumor incidence were observed between the control groups. The lone exception was a difference in tumor incidence between mice administered only ascorbate and those given saline ($P < 0.05$).

[b] No difference in tumor incidence was demonstrated between nonirradiated (normal) and irradiated mice when challenged with tumor 1 day after irradiation and administered complete ascorbate/2,6-dimethoxy-p-benzoquinone therapy ($P > 0.05$). At tumor challenge 3 days after irradiation, a significant difference in tumor incidence between nonirradiated and irradiated mice was observed ($P < 0.005$).

[c] No significant differences in tumor incidence were observed between nonirradiated and irradiated mice administered ascorbate ($P > 0.05$) or dimethoxy-p-benzoquinone ($P > 0.05$) only, with tumor challenge at 3 days post irradiation, or administered saline at all indicated experimental conditions ($P > 0.05$).

[d] A significant difference in tumor incidence was observed between saline treatments and treatments with the complete system with tumor challenge one day after irradiation ($P < 0.010$), but with tumor challenge 3 days after irradiation no significant differences were observed between treatments ($P > 0.05$).

iting tumor growth. The partial antitumor effect of ascorbate, observed in the experiment reported in Table I in which 4 of the 10 animals were tumor-free, was not observed in subsequent experiments.

Effect of Whole Body γ Irradiation. In order to determine whether or not the host's immune system plays a role in the antitumor activity, we first investigated the effects of exposing the experimental animals to whole body γ irradiation. At the dose used in our experiments (500 rad) about 96% of the animal's stem cells are destroyed (25). Dysfunctional cellular immunity rapidly ensues as lymphocytes and, to a lesser extent, granulocytes fail to adequately respond to a tumorigenic challenge.

When BALB/c mice were irradiated with 500 rads of γ radiation prior to challenge with tumor cells, the anticancer effect of the ascorbate/dimethoxybenzoquinone mixture decreased drastically, as indicated in Table I. When tumor challenge was done within 24 hr after irradiation, some anticancer activity of the semiquinone was still evident; however, when 3 days were allowed to elapse between the irradiation and the start of the experiment, the ascorbate/dimethoxybenzoquinone mixture was found to be totally ineffective in preventing tumor growth. These results indicate that the suppression of the host's cellular immune system via stem cell destruction and decreased cellular responsiveness by γ irradiation eliminates the antitumor activity of the ascorbate/dimethoxy-benzoquinone treatment system. These results thus suggested that one or more of the host's immune cells may be intimately involved in the inhibition of tumor cell growth by this treatment system.

Extreme care was taken to prevent any exposure of the irradiated mice to conditions that might cause infections. In this experiment, none of the 40 experimental mice died prior to the end of the experiment. The nonirradiated animals used in the previous experiment served as controls for this experiment.

Antitumor Effect of the 2,6-Dimethoxybenzo-Semiquinone Radical in Selenium-Deficient Mice. Several reports have appeared in the literature showing that some of the quinone-type anticancer agents are able to stimulate macrophages to the tumoricidal state *in vivo* (26–29). Given the results described above, it appeared likely to us that macrophages were involved in this model system. Studies by other investigators have previously established that the normal functioning of granulocytes and lymphocytes is greatly impaired in selenium-deficient animals (liver glutathione peroxidase <5% of normal). Baker *et al.* (30) and Arthur and Boyne (31) showed that neutrophils are functionally incapacitated when the levels of the selenium-containing glutathione peroxidase become very low. Glutathione peroxidase is also abundantly present in macrophages, where it serves an important role in their phagocytic function, as well as in lymphocytes. Serfass and Ganther showed that the glutathione peroxidase levels of peritoneal macrophages are severely depressed in Se-deficient mice (32) and that in selenium-deficient animals having a liver glutathione peroxidase level of less than 5% of normal, polymorphonuclear phagocytes are unable to kill ingested bacteria (33). In contrast, however, Spallholz *et al.* (34) found that a severe selenium deficiency leads to an increased production of hydrogen peroxide and superoxide by peritoneal macrophages. Finally, Fishbach *et al.* (35) showed that a selenium deficiency makes T cells unresponsive to antigenic stimulation, but Meeker *et al.* (36) showed that the cytotoxic activity of T-lymphocytes is not impaired in selenium-deficient mice.

These facts were used in the design of the next experiment in which a group of 80 BALB/c mice were fed the selenium deficient chow for at least 4 weeks. This resulted in a reduction in the level of liver glutathione peroxidase to less than 2% of normal. Presumably, the macrophages and neutrophils in these animals were thus no longer able to carry out their normal biological function, or were greatly impaired in doing so. When the antitumor activity of the dimethoxybenzosemiquinone radical was evaluated using these selenium-deficient mice, the ascorbate/dimethoxybenzoquinone system was shown to be as effective in promoting the regression of the Ehrlich ascites as it was in normal mice (Table II). These results tend to suggest that macrophages may not be involved in the observed antitumor effect. Normal, nondeficient mice were used as control animals, but only the semiquinone and saline injections were done in an effort to minimize the number of animals used.

Effect of Cyclosporin A. In an attempt to determine if T lymphocytes may be involved we investigated whether or not the antitumor activity of the ascorbate/dimethoxy-benzoquinone system would be less effective in animals in which the development of T cells had been suppressed by the administration of cyclosporin A. It has been well established that cyclosporin acts by preventing the T cells from maturing into cytotoxic T cells (37). Cyclosporin A at 30 mg/kg was administered to the experimental animals on Day 0, 2, 4, and 6 following the inoculation with tumor cells. Otherwise, the same protocol was followed as that described for the irradiated animals. The results, also presented in Table II, indicated a complete loss of the antitumor activity of the semiquinone radical. The results obtained with the cyclosporin-treated animals thus suggest (but do not prove) that the antitumor activity of this semiquinone radical is mediated by functional T lymphocytes.

Table II. Antitumor Effect of the 2,6-Dimethoxybenzo-Semiquinone Radical in Selenium-Deficient and Cyclosporin-Treated BALB/c Mice and in Nude Mice

Administered compound(s)	Tumor Incidence			
	Normal	Se-deficient[a]	Cyclosporin-treated[b]	Nude mice[c]
2,6-Dimethoxy-p-benzoquinone and Ascorbate	0/10	0/8	10/10	10/10
2,6-Dimethoxy-p-benzoquinone only	—	8/9	—	10/10
Ascorbate only	—	8/9	—	10/10
Saline only	10/10	8/9	9/10	9/10

[a] No significant differences in tumor incidence were observed between normal and selenium-deficient mice treated with the complete system ($P > 0.05$) and with saline ($P > 0.05$). Regarding normal mice, significant differences in tumor incidence were observed between the complete and the saline therapies ($P < 0.005$). Regarding selenium-deficient mice, significant differences were present between the complete therapy and all control therapies ($P < 0.005$). Furthermore, no significant differences in tumor incidence were observed between the selenium deficient control groups ($P > 0.05$).
[b] Tumor incidence between normal and cyclosporin A-treated mice that received the complete system was significantly different ($P < 0.005$). No difference ($P > 0.05$) was observed between normal and cyclosporin A-treated mice that received saline only. Tumor incidence in normal mice was significantly different ($P < 0.005$) between the mice receiving the complete system and those receiving saline only, whereas there was no significant difference ($P > 0.05$) between the same groups in the cyclosporin A-treated mice.
[c] No significant differences ($P > 0.05$) in tumor incidence exist between the groups as a result of the different treatments.

Antitumor Activity of the Semiquinone Radical in Nude Mice. The results obtained with the cyclosporin A treatment prompted us to test the anticancer effect of the dimethoxybenzo-semiquinone radical in nude (*nu/nu*) mice. Nude mice are athymic, resulting in a lack of functional T lymphocytes. We found again that the semiquinone was without effect against the Ehrlich ascites tumor in the nude mice (Table II). Hence, functional T lymphocytes do represent at least one factor essential for the semiquinone to exert its anticancer activity.

Discussion

In an attempt to elucidate the mechanism of the antitumor activity of the 2,6-dimethoxybenzo-semiquinone radical, Szent-Gyorgyi and his colleagues investigated the action of the radical on tumor cells in cell culture. Their studies revealed that a pyridine nucleotide-dependent enzyme that contains a sulfhydryl group and is present in the tumor cells appeared to be the target of the semiquinone radical (24, 38). They concluded from their results that the antitumor activity may be the result of a direct cytotoxic or cytostatic attack by the semiquinone on an essential enzyme in the tumor cells. Our observations that the semiquinone radical is ineffective in immunocompromised mice does establish that such a direct cytotoxic effect cannot be the major pathway for the tumoricidal activity *in vivo*.

Our results obtained following full body γ irradiation strongly indicate that one or more cells of the host's defense system are intimately involved in the antitumor activity of the dimethoxybenzo-semiquinone radical. However, these experiments do not allow a more precise interpretation, because the stem cell is the parent cell of most of the cells that constitute the defense system. Destroying the stem cells will thus affect the populations of most, if not all, types of immune cells. The fact that a more pronounced effect is observed after a waiting period of 3 days is consistent with the notion that it takes a few days following irradiation for the full debilitating effect(s) on existing mature defense cells to be expressed.

The experiments with the selenium-deficient mice indicated that the antitumor activity of the dimethoxybenzo-semiquinone radical was not compromised in these animals. If any of the phagocytic cells were intimately involved in the antitumor effect, one would expect to have observed at least a diminished antitumor activity. Although the results of this experiment as such are not conclusive, they suggest the exclusion of macrophages and neutrophils as candidates for the action of this semiquinone free radical.

The mechanism of action of cyclosporin A has been established quite firmly as a specific inhibition of the maturation of T cells to the cytotoxic state (37). The fact that cyclosporin A administration inhibits the anticancer activity of the dimethoxybenzo-semiquinone thus suggests that the T lymphocyte may play a dominant role in the observed inhibition of tumor development. The results obtained with the nude mice add further support to this interpretation. Moreover, since macrophage and natural killer cell functions are somewhat enhanced in nude mice, these results provide additional evidence for the noninvolvement of macrophages in the observed antitumor effect.

Our results thus point toward a direct involvement of T lymphocytes in the antitumor activity of the dimethoxybenzo-semiquinone radical. This may indicate that T lymphocytes are subject to activation to the tumoricidal state by certain semiquinone radicals, and perhaps by other free radical species as well. Alterna-

tively, the semiquinone radical could stimulate lymphokine release by T lymphocytes to enhance cellular communication and activation of other responsive immune or phagocytic cells. Experiments are presently being conducted in our laboratory to identify the T lymphocyte subgroup(s) that is (are) affected by this dimethoxybenzo-semiquinone radical.

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1. Combs SH, Morgan CD, Everse J. The antitumor activity of an immobilized oxidase-peroxidase system against murine Ehrlich ascites is mediated by the immune system. Proc Soc Exp Biol Med **204**:306–311, 1993.
2. Everse KE, Lin H, Stuyt EL, Brady JC, Buddingh F, Everse J. Antitumour activity of peroxidases. Br J Cancer **51**:743–746, 1985.
3. Everse J, Everse KE, Grisham MB, Eds. Peroxidases in Chemistry and Biology. Boca Raton: CRC Press, Vol. I and II, 1991.
4. Auclair C, Paoletti C. Bioactivation of the antitumor drugs 9-hydroxyellipticine and derivatives by a peroxidase-hydrogen peroxide system. J Med Chem **24**:289–295, 1981.
5. Meunier G, Bernadou J, Meunier B. Peroxidase-catalyzed oxidation of N^2,N^6-dimethyl-9-hydroxyellipticinium acetate: Evidence for the formation of an electrophilic quinone-iminium derivative. Biochem Pharmacol **36**:2599–2604, 1987.
6. Morgan BDG, O'Neill T, Dewey DL, Galpine AR, Riley PA. Treatment of malignant melanoma by intravascular 4-hydroxyanisole. Clin Oncol **7**:227–234, 1981.
7. Hasinoff BB. Self-reduction of the iron (III)-doxorubicin complex. Free Rad Biol Med **7**:583–593, 1989.
8. Turner MJ III, Everman DB, Ellington SP, Fields CE. Detection of free radicals during the cellular metabolism of adriamycin. Free Rad Biol Med **9**:415–421, 1990.
9. Sinha BK, Mimnaugh EG, Rajagopalan S, Myers CE. Adriamycin activation and oxygen free radical formation in human breast tumor cells: Protective role of glutathione peroxidase in adriamycin resistance. Cancer Res **49**:3844–3848, 1989.
10. Hoey BM, Butler J, Lea JS, Sarna T. A comparison of the free radical properties of several anthracycline anti-tumour drugs and some of their analogues. Free Rad Res Commun **5**:169–176, 1988.
11. Bachur NR, Gordon SL, Gee MV. A general mechanism for microsomal activation of quinone anticancer agents to free radicals. Cancer Res **38**:1745–1750, 1978.
12. Dusre L, Covey JM, Collins C, Sinha BK. DNA damage, cytotoxicity and free radical formation by mitomycin C in human cells. Chem-Biol Interactions **71**:63–78, 1989.
13. Flitter WD, Mason RP. The enzymatic reduction of actinomycin D to a free radical species. Arch Biochem Biophys **267**:632–639, 1988.
14. Elmarakby SA, Duffel MW, Goswami A, Sariaslani FS, Rosazza JPN. *In vitro* metabolic transformations of vinblastine: Oxidations catalyzed by peroxidase. J Med Chem **32**:674–679, 1989.
15. Raikov ZD, Zheleva AM, Raikova ET. Activation of the anticancer drug CCNU into a free radical intermediate via UV irradiation—An ESR study. Free Rad Biol Med **9**:423–426, 1990.
16. Katki AG, Kalyanaraman B, Sinha BK. Interactions of the antitumor drug, etoposide, with reduced thiols *in vitro* and *in vivo*. Chem-Biol Interactions **62**:237–247, 1987.
17. Kalyanaraman B, Nemec J, Sinha BK. Characterization of free radicals produced during oxidation of etoposide (VP-16) and its catechol and quinone derivatives. An ESR study. Biochemistry **28**:4839–4846, 1989.
18. Teicher BA, Waxman DJ, Holden SA, Wang Y, Clarke L, Sotomayor EA, Jones SM, Frei E III. Evidence for enzymatic activation and oxygen involvement in cytotoxicity and antitumor activity of N,N′,N″-triethylenethiophosphoramide. Cancer Res **49**:4996–5001, 1989.
19. Ng S, Waxman DJ. N,N′,N″-triethylenethiophosphoramide (thio-TEPA) oxygenation by constitutive hepatic P450 enzymes and modulation of drug metabolism and clearance *in vivo* by P450-inducing agents. Cancer Res **51**:2340–2345, 1991.
20. Powis G. Free radicals in antitumor quinone. Free Rad Biol Med **6**:63–101, 1989.
21. Sinha BK. Free radicals in anticancer drug pharmacology. Chem-Biol Interactions **69**:293–317, 1989.
22. Sinha BK, Mimnaugh EG. Free radicals and anticancer drug resistance: Oxygen free radicals in the mechanisms of drug cytotoxicity and resistance by certain tumors. Free Rad Biol Med **8**:567–581, 1990.
23. Pethig R, Gascoyne PRC, McLaughlin JA, Szent-Gyorgyi A. Ascorbate-quinone interactions: electrochemical, free radical, and cytotoxic properties. Proc Natl Acad Sci USA **80**:129–132, 1983.
24. Pethig R, Gascoyne PRC, McLaughlin JA, Szent-Gyorgyi A. Enzyme-controlled scavenging of ascorbyl and 2,6-dimethoxysemiquinone free radicals in Ehrlich ascites tumor cells. Proc Natl Acad Sci USA **82**:1439–1442, 1985.
25. Till JE, McCulloch EA. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res **14**:213–222, 1961.
26. Martin F, Caignard A, Olsson O, Jeannin JF, Leclerc A. Tumoricidal effect of macrophages exposed to adriamycin *in vivo* or *in vitro*. Cancer Res **42**:3851–3857, 1982.
27. Haskill JS. Adriamycin-activated macrophages as tumor growth inhibitors. Cancer Res **41**:3852–3856, 1981.
28. Stoychkov JN, Schultz RM, Chirigos MA, Pavlidis NA, Goldin A. Effects of adriamycin and cyclophosphamide treatment on induction of macrophage cytotoxic function in mice. Cancer Res **39**:3014–3017, 1979.
29. Ogura T, Shindo H, Namba M, Yamamura Y. Tumoricidal activity of peritoneal exudate cells from rats treated with mitomycin C. GANN **71**:920–921, 1980.
30. Baker SS, Lerman RH, Krey SH, Crocker KS, Hirsh EF, Cohen H. Selenium deficiency with total parenteral nutrition: Reversal of biochemical and functional abnormalities by selenium supplementation: A case report. Am J Clin Nutr **38**:769–774, 1983.
31. Arthur JR, Boyne R. Superoxide dismutase and glutathione peroxidase activities in neutrophils from selenium-deficient and copper-deficient cattle. Life Sci **36**:1569–1575, 1985.
32. Serfass RE, Ganther HE. Defective microbicidal activity in glutathione peroxidase-deficient neutrophils of selenium-deficient rats. Nature **255**:640–641, 1975.
33. Serfass RE, Ganther HE. Effects of dietary selenium and tocopherol on glutathione peroxidase and superoxide dismutase activities in rat phagocytes. Life Sci **19**:1139–1144, 1976.
34. Spallholz JE, Boylan LM, Larsen HS. Advances in understanding selenium's role in the immune system. Ann NY Acad Sci **587**:123–139, 1990.
35. Fishbach M, Jenkinson S, Talal N, Lawrence R. Selenium deficient T-cells are unresponsive to antigenic stimulation. Clin Res **32**:232A, 1984.
36. Meeker HC, Eskew ML, Scheuchenzuber W, Scholz RW, Zarkower A. Antioxidant effects on cell-mediated immunity. J Leukocyte Biol **38**:451–458, 1985.
37. Hess AD, Tutschka PJ, Santos GW. The effect of cyclosporin A on T-lymphocyte subpopulations. In: White DJG, Ed. Cyclosporin A. Amsterdam: Elsevier Biomedical, pp209–231, 1982.
38. Pethig R, Gascoyne PRC, McLaughlin JA, Szent-Gyorgyi A. Interaction of the 2,6-dimethoxysemiquinone and ascorbyl free radicals with Ehrlich ascites cells: A probe of cell surface charge. Proc Natl Acad Sci USA **81**:2088–2091, 1984.