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Elevation of Serum Lipid Peroxide Level Associated with Doxorubicin Toxicity and Its Amelioration by [dl]-α-Tocopheryl Acetate or Coenzyme Q_{10} in Mouse (Doxorubicin, Toxicity, Lipid Peroxide, Tocopherol, Coenzyme Q_{10})

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Summary. *Elevations of serum lipid peroxide levels were demonstrated in mice after an equitoxic dose of doxorubicin. When BDF_1 mice were injected with doxorubicin (20 mg/kg body weight, IP), lipid peroxide levels in sera were elevated 1 day after the injection and the levels declined on subsequent days. 5-Fluorouracil (400 mg/kg body weight, IP) never changed the peroxide levels in serum. Furthermore, it was found that the co-administration of [dl]-α-tocopheryl acetate or coenzyme Q_{10} IM strongly inhibited the doxorubicin-induced elevation of lipid peroxides in serum.*

The effectiveness of [dl]-α-tocopheryl acetate or coenzyme Q_{10} in reducing the lethality of doxorubicin in mice was also confirmed.

These results indicate that the measurement of serum 2-thiobarbituric acid-reacting substances provides a useful measurement of lipid peroxide levels, which may be involved in some way with doxorubicin toxicity, and that the administration of antioxidants provide protection against some of the side effects of doxorubicin.

Introduction

The anthracyclic antitumor antibiotic doxorubicin is one of the most clinically useful cancer chemotherapeutic agents against a wide spectrum of human tumors [2, 3, 5]. However, cardiotoxicity is a major dose-limiting complication of doxorubicin therapy [4, 11, 12]. Doxorubicin-induced cardiac damage has also been observed in various species of animals, and was believed to be the cause of lethality of this drug [10, 13, 18, 22]. However, the pathogenesis of the doxorubicin-induced cardiomyopathy remains unclear at present. Recently, Myers and his group reported that doxorubicin-induced cardiac toxicity was associated with peroxidation of cardiac lipid in mice and showed that α-tocopherol, a free radical scavenger, ameliorated cardiac damage caused by doxorubicin [14, 15].

In this report, we describe attempts to find an appropriate parameter to indicate doxorubicin toxicity. Lipid peroxide levels in sera were increased by the administration of doxorubicin to mice, and co-administration of antioxidants such as α-tocopherol and coenzyme Q [19] inhibited the elevation of serum lipid peroxide levels and prevented animal deaths.

Materials and Methods

Chemicals

Sources of the drugs used for experiments were as follows: doxorubicin and 5-fluorouracil (5-FU) were obtained from Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan. [dl]-α-Tocopheryl acetate (Vit E) and coenzyme Q_{10} (CoQ) were from Eisai Co., Ltd., Tokyo, Japan (50 mg/ml Vit E or 10 mg/ml CoQ was dissolved in a solution containing 70 mg polyoxyethelene (60) hydrogenated castor-oil derivative/ml). Doxorubicin was also supplied by Drug Research and Development, Chemotherapy, NCI, Maryland. Bovine serum albumin (BSA) was obtained from Sigma Chemical Co., St. Louis, Mo. All other chemicals were of analytical grade.

Measurement of Lipid Peroxide Levels in Serum

Lipid peroxide in serum was measured in terms of the formation of 2-thiobarbituric acid (TBA)-reacting substances, presumed to be malondialdehyde, by means of a fluorometric assay described by Yagi [20] with slight modifications. Sample serum was diluted with 0.5 ml 0.8% NaCl solution containing 2 mg/ml BSA, and 4.0 ml N/12

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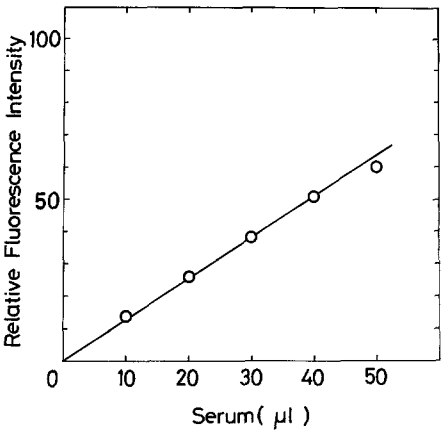

Fig. 1. Relationship between fluorescence intensity of TBA-reacting substance and serum volume. The sera of normal BDF_1 mice were obtained by centrifugation of blood collected from cervical blood vessels. Each amount of serum was diluted to 0.5 ml 0.8% NaCl solution containing 2 mg BSA/ml, and 4.0 ml N/12 H_2SO_4 was added as described in 'Materials and Methods.' Relative fluorescence intensity was measured at 553 nm emission with 515 nm excitation by means of a Hitachi MPF-3 spectrofluorometer, calibrated with quinine sulfate

H_2SO_4 was added. After mixing, 10% phosphotungstic acid (0.5 ml) was added with shaking. After 5 min, the mixture was centrifuged at 3000 rpm for 10 min. The sediment was mixed with 2.0 ml N/12 H_2SO_4 and 0.3 ml 10% phosphotungstic acid and shaken. After centrifugation at 3000 rpm for 10 min, the precipitate was suspended in 4.0 ml distilled water and was mixed with 1 ml 1% TBA solution containing 50% of glacial acetic acid. The mixture was incubated at 95° C for 60 min. The reaction was terminated by cooling and 5 ml of *n*-butanol was added to extract the fluorescent pigment. After vigorous shaking, the mixture was centrifuged for 20 min at 3000 rpm. The fluorescence intensity at 553 nm in the butanol layer was measured with 515 nm excitation by means of a Hitachi MPF-3 spectrofluorometer calibrated with quinine sulfate. 1,1,3,3,-Tetraethoxypropane was used as the standard solution. The amount of fluorescence intensity increased linearly with addition of serum up to at least 40 μl of serum (Fig. 1), and 20 μl serum was sufficient to detect TBA-reactive substance formation by means of this assay. Doxorubicin does not interfere with this assay method.

Treatment of Mice with Doxorubicin and with or without Vit E or CoQ

BDF_1 male mice with an average weight of 25 g were used (one group: 10 mice). All mice treated with doxorubicin received 20 mg/kg body weight IP. In the groups that were treated with Vit E or CoQ (Vit E: 5, 50, or 500 mg/kg body weight, CoQ: 50 mg/kg body weight) the treatment was administered in four doses (IM) as follows with respect to the time of doxorubicin treatment: first, 24 h before; second, 2 h before; third, 2 h after; and fourth, 24 h after. For the measurement of lipid peroxide levels in serum, each group of mice (one group: 5 mice) was sacrificed on the days indicated and blood was taken from cervical blood vessels.

Results

Elevation of Serum Lipid Peroxide Levels by Doxorubicin

Figure 2 shows the change in serum lipid peroxide levels after the administration of doxorubicin. The basal levels of lipid peroxides in sera were as low as about 6 nmol/ml. When doxorubicin was administered as an equitoxic dose (a single dose of 20 mg/kg body weight IP), three mice out of five exhibited 3–7 times the serum lipid peroxide levels of control mice after 1 day. The serum lipid peroxide levels then declined gradually over 3 days after doxorubicin administration. The differences between doxorubicin treated and control groups after 1 and 3 days of doxorubicin administration were significant ($P < 0.05$). An equitoxic dose of 5-FU (a single dose of 400 mg/kg body weight IP) [17] did not raise the level of serum lipid peroxides, as shown in Table 1. Thus the elevation of serum lipid peroxide seems to result from a specific effect of doxorubicin rather than from a nonspecific degeneration of tissues.

Inhibition of the Doxorubicin-induced Elevation of Lipid Peroxide Levels by Vit E or CoQ

The effect of Vit E or CoQ was examined on lipid peroxide levels in sera of doxorubicin-treated mice. As shown in Fig. 3, co-administration of Vit E (500 mg/kg body weight IM four times, to give a total dose of 2,000 mg/kg body weight) with the equitoxic dose of doxorubicin did not produce an elevation of lipid peroxide levels in serum. CoQ (50 mg/kg body weight IM four times, to give a total dose of 200 mg/kg body weight) was also effective in inhibiting the formation of lipid peroxide induced by doxorubicin. Vit E or CoQ alone low-

Table 1. Effect of 5-FU on serum lipid peroxide levels

	Malondialdehyde formation (nmol/ml serum)
Control	6.9 ± 0.9[a]
5-FU	6.3 ± 0.4

[a] Mean ± SE

BDF_1 male mice, average weight 25 g (one group: 5 mice) received 400 mg/kg body weight 5-FU IP at time 0. Lipid peroxide levels in sera of 5-FU treated mice were compared with those of non-5-FU treated mice after 24 h of 5-FU administration. The serum of each mouse was obtained by centrifugation of blood collected from cervical blood vessels and 20 μl serum was used for each measurement. A fluorometric assay method was used for the measurement of lipid peroxides, as described in 'Materials and Methods,' and the value was represented as nmol of malondialdehyde, tetraethoxypropane being used as the standard

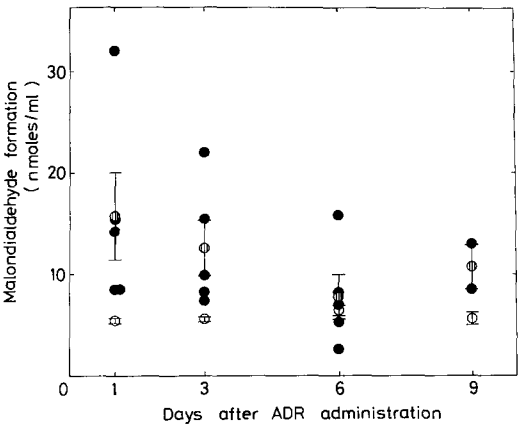

Fig. 2. Serum lipid peroxide levels of doxorubicin(ADR)-treated mice. BDF_1 male mice, average weight of 25 g (one group: 5 mice) received 20 mg ADR/kg IP at time 0. Lipid peroxide levels in the sera of ADR-treated mice were compared with those of control mice on the indicated days. Twenty microliters of serum was obtained from each mouse and lipid peroxide was measured as described in 'Materials and Methods.' The value was expressed as nmol of malondialdehyde, tetraethoxypropane being used as the standard. ●, value for a given ADR-treated mouse; Φ, represents the mean value ± SE for the ADR-treated group. Three mice of five in the group '9 days after ADR administration' died. Φ, mean value ± SE for the control group

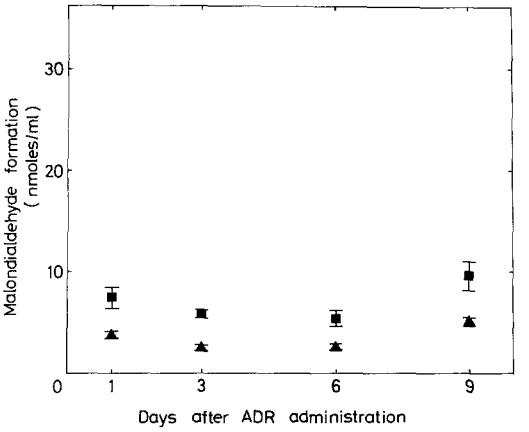

Fig. 3. Inhibition of lipid peroxide formation in sera of ADR-treated mice by Vit E or CoQ. BDF_1 male mice, average weight of 25 g (one group: 5 mice) each received 20 mg ADR/kg IP at time 0. Vit E (500 mg/kg IM each time) or CoQ (50 mg/kg IM each time) was administered four times, as described in 'Materials and Methods.' Twenty microliters of serum from each mouse was used for the measurement of lipid peroxides, as described in Fig. 2. ▲, mean value ± SE for the ADR- and Vit E-treated group; ■, mean value ± SE for ADR- and CoQ-treated group

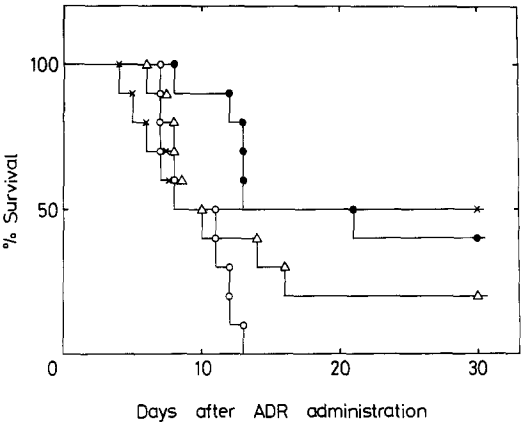

Fig. 4. Effect of Vit E on the lethality of ADR. BDF_1 male mice, average weight of 25 g (one group: 10 mice) each received 20 mg ADR/kg at time 0. The survival of ADR-treated mice and of groups of mice treated with ADR and Vit E was compared. Equal amounts of Vit E (IM) were administered four times as follows (5 mg/kg, 50 mg/kg, or 500 mg/kg each time): 24 h and 2 h before ADR treatment and 2 h and 24 h after ADR treatment. O——O, 20 mg ADR/kg; ●——●, 20 mg ADR/kg + 500 mg Vit E/kg × 4; ×——×, 20 mg ADR/kg + 50 mg Vit E/kg × 4; △——△, 20 mg ADR/kg + 5 mg Vit E/kg × 4

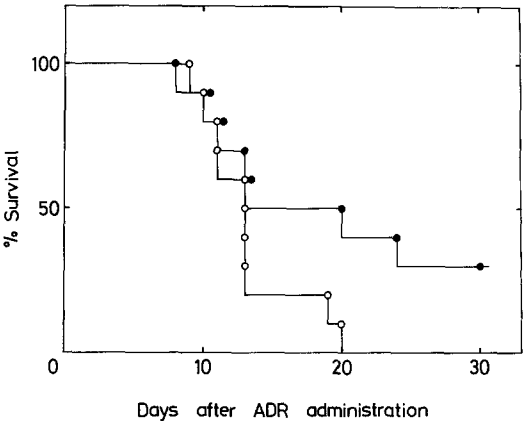

Fig. 5. The effect of CoQ on the lethality of ADR. BDF_1 male mice, average weight of 25 g (one group: 10 mice) each received 20 mg ADR/kg IP at time 0. ADR-treated mice were compared with the group of mice treated with ADR and CoQ. CoQ (50 mg/kg body weight IM) was administered four times as described in Fig. 4 for Vit E. O——O, 20 mg ADR/kg; ●——●, 20 mg ADR/kg + 50 mg CoQ/kg × 4

ered the basal level of serum lipid peroxide (data not shown).

Protective Effect of Vit E or CoQ on the Lethality of Doxorubicin

As shown in Fig. 4, the effect of Vit E on the lethality of doxorubicin was examined. The dose schedule was the same as indicated in Fig. 3. Vit E was effective to ameliorate the toxicity of doxorubicin in the group of mice treated with 500 mg/kg body weight.

Doxorubicin-associated cardiotoxicity has been reported to be effectively prevented by CoQ [1], and the above results indicate that this compound apparently inhibits the formation of lipid peroxide in serum caused

by doxorubicin administration (Fig. 3). Therefore, 50 mg CoQ/kg body weight IM was administered four times to mice, as indicated in Fig. 3, and the lethality of doxorubicin was examined (Fig. 5). The same dose schedule and amount of CoQ was shown to be effective in decreasing the toxicity of doxorubicin.

In contrast, Vit E or CoQ had no effect against the lethality of 5-FU (data not shown).

Discussion

The development of a microfluorometric assay has permitted us to determine lipid peroxides in the serum of each small animal. Normal mice exhibited relatively low levels of peroxide, and differences among individual animals were very small. In this study, after administration of doxorubicin, the lipid peroxide levels in sera were apparently elevated, but there were considerable differences in the response to doxorubicin among individual mice. The source of the variability is now under investigation. Doxorubicin is known to liberate free radicals through a microsomal NADPH-dependent electron transport system and to stimulate the formation of lipid peroxide [7, 8]. Myers et al. [14] reported that doxorubicin-induced cardiac toxicity was associated with peroxidation of cardiac lipid in mice. Therefore, it was suggested that the release of lipid peroxide from the heart increased the lipid peroxide levels in serum. An equitoxic dose of 5-FU [17], which does not stimulate free radical reaction (data not shown), never elevated the peroxide level in serum (Table 1). Aclacinomycin A, an anthracycline antibiotic discovered by Oki et al. [16], which also stimulates free radical reaction as observed with doxorubicin, increased the lipid peroxide level in serum (data not shown). These results suggest that the drugs that involve free radicals and lipid peroxidation [8, 21] also have the potential to increase serum lipid peroxide.

Administration of Vit E, which is changed to α-tocopherol in vivo [6], inhibited the elevation of serum lipid peroxide by doxorubicin (Fig. 3) and reduced the doxorubicin-induced mortality (Fig. 4). These results confirmed the results obtained by Myers et al. [14, 15]. Doxorubicin is known to be a potent inhibitor of mitochondrial CoQ enzymes [9], and CoQ was shown to be effective in decreasing doxorubicin-induced cardiotoxicity [1]. In this report, CoQ was found to inhibit the elevation of serum lipid peroxide by doxorubicin and ameliorate the lethality (Figs. 3 and 5). As CoQ is also an antioxidant [19], the results obtained in this investigation could be explained in terms of the inhibition of lipid peroxidation in vivo by CoQ. Earlier work by others showed that the antitumor effect of doxorubicin was not reduced by α-tocopherol or CoQ [1, 14]. Therefore, coadministration of antioxidants such as α-tocopherol and CoQ may be effective in preventing cardiac toxicity by doxorubicin.

As 20 µl serum is sufficient for measurement of lipid peroxide levels, the fluorometric assay method may be useful not only for animal studies but also for clinical work. Our preliminary measurements have indicated that elevation of lipid peroxide levels in sera could be detected in some patients who were receiving drugs such as doxorubicin and bleomycin, which are involved in free radical formation [8, 21].

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References

1. Bertazzoli, C., Ghione, M.: Adriamycin-associated cardiotoxicity: Research on prevention with coenzyme Q. Pharmacol. Res. Commun. **9,** 235–250 (1977)
2. Blum, R. H., Carter, S. K.: Adriamycin – A new anticancer drug with significant clinical activity. Ann. Intern. Med. **80,** 249–259 (1974)
3. Bonadonna, G., Monfardini, S., De Lena, M., Fossati-Bellani, F., Beretta, G.: Phase I and preliminary phase II evaluation of adriamycin (NSC-123127). Cancer Res. **30,** 2572–2582 (1970)
4. Carter, S. K.: Adriamycin – A review. J. Natl. Cancer Inst. **55,** 1265–1274 (1975)
5. DiMarco, A., Gaetoni, M., Scarpinato, B.: Adriamycin (NSC-123127): A new antibiotic with antitumor activity. Cancer Chemother. Rep. **53,** 33–37 (1969)
6. Gallo-Torres, H. E.: Obligatory role of bile for the intestinal absorption of vitamin E. Lipids **5,** 379–384 (1970)
7. Goodman, J., Hochstein, P.: Generation of free radicals and lipid peroxidation by redox cycling of adriamycin and daunomycin. Biochem. Biophys. Res. Commun. **77,** 797–803 (1977)
8. Handa, K., Sato, S.: Generation of free radicals of quinone group-containing anticancer chemicals in NADPH-microsome system, as evidenced by initiation of sulfite oxidation. Gann **66,** 43–47 (1975)
9. Iwamoto, Y., Hansen, I. L., Porter, T. H., Folkers, K.: Inhibition of coenzyme Q_{10}-enzymes, succinoxidase and NADH-oxidase, by adriamycin and other quinones having antitumor activity. Biochem. Biophys. Res. Commun. **58,** 633–638 (1974)
10. Jaenke, R. S.: An anthracycline antibiotic-induced cardiomyopathy in rabbits. Lab. Invest. **30,** 292–304 (1974)
11. Lefpak, E. A., Pitha, J., Rosenheim, S., Gottlieb, J. A.: A clinicopathologic analysis of adriamycin cardiotoxicity. Cancer **32,** 302–314 (1973)
12. Lenaz, L., Page, J. A.: Cardiotoxicity of adriamycin and related anthracyclines. Cancer Treat. Rev. **3,** 111–120 (1976)
13. Mettler, F. P., Young, D. M., Ward, J. M.: Adriamycin-induced cardiotoxicity (cardiomyopathy and congestive heart failure) in rats. Cancer Res. **37,** 2705–2713 (1977)
14. Myers, C. E., McGuire, W. P., Liss, R. H., Ifrim, I., Grotzinger, K., Young, R. C.: Adriamycin: the role of lipid peroxidation in

cardiac toxicity and tumor response. Science **197,** 165–167 (1977)
15. Myers, C. E., McGuire, W. P., Young, R.: Adriamycin: Amelioration of toxicity by α-tocopherol. Cancer Treat. Rep. **60,** 961–962 (1976)
16. Oki, T., Matsuzawa, Y., Yoshimoto, A., Numata, K., Kitamura, I., Hori, S., Takamatsu, A., Umezawa, H., Ishizuka, M., Naganawa, H., Suda, H., Hamada, M., Takeuchi, T.: New antibiotics, aclacinomycin A and B. J. Antibiot. (Tokyo) **28,** 830–834 (1975)
17. Rosenoff, S. H., Brooks, E., Bostick, F., Young, R. C.: Alteration in DNA synthesis in cardiac tissue induced by adriamycin in vivo-relation to fatal toxicity. Biochem. Pharmacol. **24,** 1898–1901 (1971)
18. Rosenoff, S. H., Olson, H. M., Young, D. M., Bostick, F., Young, R. C.: Adriamycin-induced cardiac damage in the mouse: A small-animal model of cardiotoxicity. J. Natl. Cancer Inst. **55,** 191–194 (1975)
19. Slater, T. F.: Free radical mechanisms in tissue injury, pp. 49–61. London: Pion 1972
20. Yagi, K.: A simple fluorometric assay for lipoperoxide in blood plasma. Biochem. Med. **15,** 212–216 (1976)
21. Yamanaka, N., Kato, T., Nishida, K., Ota, K.: Enhancement of DNA chain breakage by bleomycin A_2 in the presence of microsomes and reduced nicotinamide adenine dinucleotide phosphate. Cancer Res. **38,** 3900–3903 (1978)
22. Young, D. M.: Pathologic effects of adriamycin (NSC-123127) in experimental systems. Cancer Chemother. Rep. **6,** 159–175 (1975)

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