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Effect of Adriamycin on DNA, RNA and Protein Biosyntheses in Mouse Tissues, in Connection with Its Cardiotoxicity

Yasuyuki Sazuka, Hisayuki Tanizawa and Yoshio Takino

School of Pharmaceutical Sciences, University of Shizuoka, 395 Yada, Shizuoka 422

We examined whether the cause of the remarkable decreases in the activities of lipid peroxidation-preventive enzymes in the heart of adriamycin (ADR)-treated mice might be related to inhibition of DNA, RNA or protein biosynthesis. It was found that biosyntheses of DNA, RNA and protein in the heart, liver and kidney of mice were markedly inhibited by ADR (15 mg/kg, ip). The inhibitory effects of ADR on each type of biosynthesis were particularly marked in the heart among the tissues examined. Strong correlations between the percentage inhibition of DNA and protein biosynthesis by ADR, and the percentage decrease in the activities of lipid peroxidation-preventive enzymes were observed in the heart, liver, kidney and lung, especially for the decrease of glutathione peroxidase activity and the inhibition of DNA and protein biosyntheses. We also found that marked decreases of DNA, RNA and protein biosyntheses in ADR-treated mice occurred not only in the heart but also in tumor tissues. From these results, we conclude that the increment of cardiac lipid peroxide in ADR-treated mice, which is closely related to the cardiotoxicity of ADR, results from inhibition of DNA, RNA and protein biosyntheses after the distribution of ADR.

Key words: Adriamycin — Aclacinomycin — Protein biosynthesis

Adriamycin (ADR) is one of the most effective antitumor agents currently available for cancer chemotherapy.[1] However, it is well known that ADR produces severe cardiotoxicity.[2] Myers *et al.* [3,4] demonstrated in experimental mice that this ADR-induced cardiotoxicity was closely associated with an increase of lipid peroxide (LPO) in the myocardium. The increment of LPO induced by ADR is generally considered to be caused by production of active oxygen radicals through the redox cycle of ADR, on the basis of *in vitro* experiments.[5–9] However, we found that there was a time lag between the increment of LPO in mouse tissues, and attainment of the peak tissue concentration of ADR after its administration.[10,11] Therefore we thought that the results obtained *in vitro* were not directly applicable to our findings[10] obtained *in vivo*. We have already reported that the increment of LPO in the hearts of mice treated with ADR is attributable to decreases in the cardiac activities of lipid peroxidation-preventive enzymes, in particular glutathione peroxidase (GSHpx).[12–14] The mechanism of the antitumor action of ADR is said to be based on the inhibition of both DNA polymerase and RNA polymerase after formation of an ADR-DNA complex.[15,16] This action is clearly desirable in tumor tissues but not in normal tissues. There are some reports describing the inhibitory effects of ADR on DNA biosynthesis in normal tissues, such as the liver and heart of rats. However, there has been no detailed study on the heart[17–20] taking into account that the increment of LPO is correlated with the cardiotoxicity of ADR.

In this study, we have examined the inhibitory effects of ADR on the biosyntheses of DNA, RNA and protein in the tissues of normal and Ehrlich ascites carcinoma-bearing mice, in comparison with other anthracyclines such as aclacinomycin (ACM), which has weaker cardiotoxicity than ADR,[21] and daunomycin (DAM), which has a cardiotoxicity intermediate between those of ADR and ACM.[22]

MATERIALS AND METHODS

Animals Male CDF_1 mice, 6 weeks old and weighing 20–25 g, obtained from Japan SLC Inc., Hamamatsu, were used. The animals were housed in a room maintained at $25 \pm 1°C$ and $55 \pm 5\%$ relative humidity and given free access to standard laboratory feed and water.

Reagents ADR injection, 10 mg/vial (Adriacin), DAM injection, 20 mg/vial (Daunomycin), and ACM injection, 20 mg/vial (Aclacinon), were purchased from Kyowa Fermentation Inc., Tokyo, Meiji Seika Co. Ltd., Tokyo and Yamanouchi Pharmaceutical Co. Ltd., Tokyo, respectively. These agents were each thawed and diluted with sterile isotonic saline to obtain a 1.0 mg/ml solution. ^{14}C-Thymidine, ^{14}C-orotic acid and ^{14}C-leucine were purchased from Amersham International plc, Buckinghamshire, England. Trichloroacetic acid and Scintisol Ex-H, as a scintillation cocktail, containing xylene, DPO, Bis-MSB and non-ionic detergent, were purchased from Wako Pure Chemical Industries Co. Ltd., Tokyo. Protosol, as a tissue and gel solubilizer, was obtained from NEN Research Products, Boston, USA. The other chemicals used in this study were of the highest purity available.

Apparatus The liquid scintillation counter used was an Aloka model 661.

Animal experiments Male CDF_1 mice were divided into several groups each consisting of 5–6 mice. ADR, DAM or ACM at a dose of 15 mg/kg was injected intraperitoneally into the mice. Control animals were injected with the same volume of sterile isotonic saline alone. On the 4th day of the treatment, ^{14}C-thymidine, ^{14}C-orotic acid or ^{14}C-leucine was injected intraperitoneally at a dose of 0.1 mCi/kg. The animals were then killed by cervical dislocation 3 h later. The liver, kidney and heart were rapidly dissected out, washed in ice-cold isotonic saline, blotted and weighed to obtain the wet weight. The tissue samples were then homogenized in 20 vol (v/w) of isotonic saline at 4°C in a glass Potter-Elvehjem homogenizer with a Teflon pestle.

In the case of carcinoma-bearing mice, Ehrlich ascites carcinoma (5×10^5 cells/animal) was inoculated into the back skin of CDF_1 male mice. ADR or ACM was administered intraperitoneally to the carcinoma-bearing mice at a dose of 15 mg/kg 12 days after tumor inoculation. On the 4th day after this treatment, ^{14}C-thymidine, ^{14}C-orotic acid or ^{14}C-leucine was injected intraperitoneally at a dose of 0.1 mCi/kg. The subsequent experiments were carried out in a similar manner to those for normal animals.

Determination of radioactivity in DNA, RNA and protein fractions Experiments were carried out according to the Schmidt-Thannhauser-Schneider method.[23-25] Briefly, 5% homogenate solution (0.5 ml) was added to 0.5 ml of cold 5% trichloroacetic acid (TCA) solution and mixed well. Then the mixture was centrifuged (4°C, 3000 rpm, 15 min) and 2.0 ml of cold 5% TCA solution was added to the precipitate. The solution was then mixed and centrifuged (4°C, 3000 rpm, 15 min) again. After repeating this washing process twice, the precipitate was dissolved in 1 *N* KOH solution (0.5 ml) followed by incubation at 37°C for 20 h to hydrolyze the RNA. After the hydrolysis. 6 *N* HCl-5% TCA (0.1 ml) was added to the reaction mixture. Then the mixture was centrifuged (3000 rpm, 15 min) to precipitate the DNA and protein. The precipitate obtained was washed with 5 ml of 1% TCA solution, and the combined supernatant was used as the RNA fraction. On the other hand, 2 ml of 5% TCA solution was added to the precipitate again and the mixture was heated in a boiling water-bath for 5 min. After ice-cooling for 15–20 min, this mixture was centrifuged (4°C, 3000 rpm, 15 min). The supernatant and the precipitate were used as the DNA fraction and the protein fraction, respectively. The protein fraction was dissolved in 2 ml of Protosol by incubation at 50–60°C overnight. Then, Scintisol EX-H was added to each of the DNA, RNA and protein fractions and their radioactivities were determined. The ratio of inhibition in each drug-administration group relative to the control group for DNA, RNA and protein biosyntheses was then calculated.

Statistical analysis Statistical significance of differences was evaluated by using Student's *t* test.

RESULTS

The inhibitory effects of ADR, DAM and ACM on the biosyntheses of DNA, RNA and protein on the 4th day after administration were examined in normal mice. The results are shown in Fig. 1.

The amounts of ^{14}C-thymidine, ^{14}C-orotic acid and ^{14}C-leucine incorporated into the DNA, RNA and protein fractions in the heart tissue of normal mice were 17.37 ± 1.41, 7.49 ± 1.04 and 51.34 ± 6.60 cpm/mg wet tissue/3 h, respectively. Those in the liver were 23.71 ± 1.88, 65.39 ± 2.96 and 293.40 ± 19.06 cpm/mg wet tissue/3 h, respectively.

The inhibitory effects of ADR on the biosyntheses of DNA and protein in the tissues determined were the highest, followed by those of DAM and ACM in that order. In particular, the inhibitory effects of ACM on DNA, RNA and protein biosyntheses were weak (below 15%), except for the effect on RNA biosynthesis in the

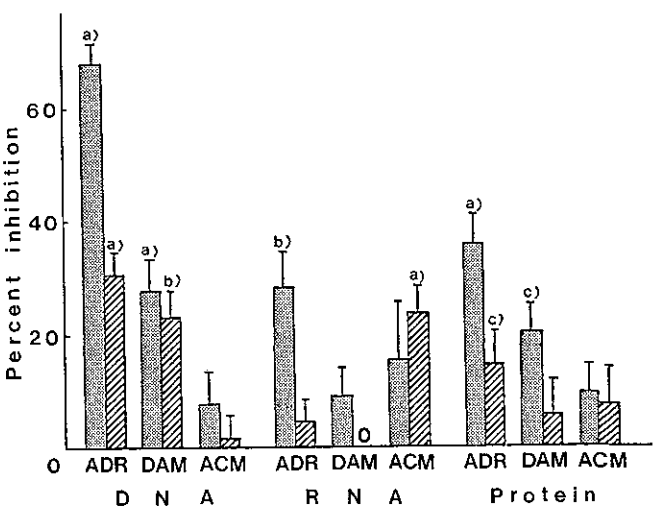

Fig. 1. Effects of ADR, DAM and ACM on DNA, RNA and protein biosyntheses in the heart and the liver of CDF_1 mice. ADR or DAM or ACM was administered intraperitoneally to mice at a dose of 15 mg/kg. On the 4th day of treatment, ^{14}C-thymidine or ^{14}C-orotic acid or ^{14}C-leucine was injected intraperitoneally at a dose of 0.1 μCi/g. Radioactivities in the tissues of mice were determined at 3 h after injection. Each value represents the mean ± SD of 5 mice. Significantly different from the control value, a) $P<0.001$, b) $P<0.01$, c) $P<0.05$. ▒, heart; ▨, liver.

liver (23.2%). The inhibitory effects of all drugs used on each type of biosynthesis were stronger in the heart than in the liver, except for the effect of ACM on RNA biosynthesis. In particular, the inhibitory ratios of ADR on DNA and protein biosyntheses in the heart were 67.2% ($P<0.001$) and 35.1% ($P<0.001$), respectively, these figures being the highest among the tissues determined. The inhibitory effects of the drugs on each type of biosynthesis in the kidney were approximately similar to those in the liver, i.e., the ratios of inhibition of DNA, RNA and protein biosyntheses for ADR were 34.3%, 14.1% and 11.6%, respectively, and those for ACM were 13.1%, 10.5% and 2.5%, respectively (not shown).

Next, the effect of ADR or ACM in mice inoculated with Ehrlich ascites carcinoma into the back skin was similarly examined. In the case of Ehrlich ascites carcinoma inoculated on the backs, the tumor could be removed from 7 days until 21 days after inoculation. ADR or ACM was administered to carcinoma-bearing mice 12 days after inoculation. The tumor weight in the control mice on the 16th day after inoculation was 0.549 ± 0.075 g (n=8). On the other hand, that in ADR- or ACM-treated mice was 0.228 ± 0.093 (n=8) or 0.322 ± 0.097 g (n=8), respectively, being significantly reduced in comparison with the control mice ($P<0.001$). The inhibitory effects of ADR and ACM on the biosyntheses of DNA, RNA and protein in the heart, liver, kidney and lung were similar to those found in normal mice, i.e., the inhibitory effects of ADR on DNA and protein biosyntheses in all tissues were stronger than those of ACM. The inhibitory ratios of DNA and protein biosyntheses in the heart of ADR-treated mice were 59.1% ($P<0.001$) and 33.5% ($P<0.001$), respectively, these percentages being the highest among the tissues examined.

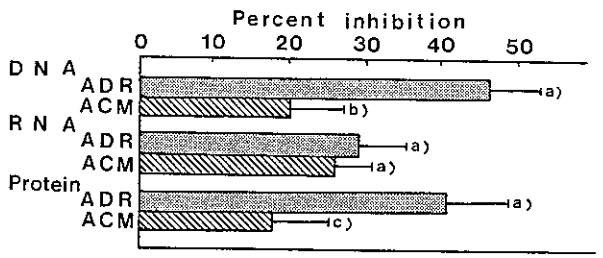

Fig. 2. Effects of ADR and ACM on DNA, RNA and protein biosyntheses in the tumor of Ehrlich carcinoma-bearing mice. Ehrlich ascites carcinoma (5×10^5 cells/animal) was inoculated into the backs of CDF_1 mice (6 weeks old, weighing 20–25 g). ADR or ACM was administered intraperitoneally to mice at 12 days after inoculation at a dose of 15 mg/kg. On the 4th day of treatment, ^{14}C-thymidine or ^{14}C-orotic acid or ^{14}C-leucine was injected intraperitoneally at a dose of 0.1 μCi/g. Radioactivities in the tissues of mice were determined at 3 h after injection. Each value represents the mean$\pm$SD of 5 mice. Significantly different from the control value, a) $P<0.001$, b) $P<0.01$, c) $P<0.05$.

The inhibitory effects of ADR and ACM on DNA, RNA and protein biosyntheses in the tumor are shown in Fig. 2. The inhibitory ratio of DNA biosynthesis for ADR in the tumor was even larger than that in the heart. However, the inhibitory effects of ADR on RNA and protein biosyntheses in the tumor were the largest among the tissues examined. Furthermore, those of ACM on DNA, RNA and protein biosyntheses in the tumor were also the largest. The inhibitory effects of ADR on each type of biosynthesis in the tumor were greater than those of ACM.

DISCUSSION

We have previously reported that the lipid peroxide level in the heart of ADR-treated mice peaked on the 4th day,[10] whereas the concentration of ADR in the heart reached a maximum within 3 h after administration.[12] Therefore, it appeared that there was a time lag between the lipid peroxide increment and attainment of the peak tissue concentration of ADR. Furthermore, we[14] clarified that the lipid peroxide increment in the heart of mice after ADR administration was caused by marked decreases in the activities of cardiac lipid peroxidation-preventive enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GSHpx) and catalase, at days 3–5 after ADR administration. In particular, the cardiac level of GSHpx activity decreased markedly to about 40–50% of the normal level within 1–5 days. This remarkable decrease of each enzyme activity in the heart of ADR-treated mice was considerd to be due to inhibition of DNA and RNA biosyntheses, followed by inhibition of enzyme protein biosynthesis, and in the present study we examined these aspects.

The inhibitory effects of ADR on DNA, RNA and protein biosyntheses in normal tissues of experimental animals have been reported previously.[17–20] However, the experimental conditions described in those reports were greatly different from ours with regard to the dose of ADR, the experimental animals used, and the length of the time course determined. For these reasons, the previous results could not be used to explain the mechanism of the lipid peroxide increment induced by ADR. In the present study, we examined the inhibitory effect of ADR on DNA, RNA and protein biosyntheses on the 4th day after ADR (15 mg/kg, ip) administration, when the increment of the lipid peroxide level in the tissues had reached a maximum, using CDF_1 male mice, which had been observed to show a marked increase in the cardiac lipid peroxide level due to ADR.[6]

Biosyntheses of DNA, RNA and protein was markedly inhibited by ADR in the heart, liver and kidney of mice,

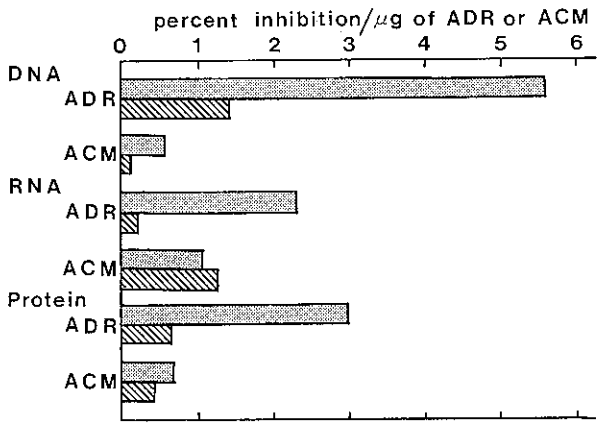

Fig. 3. Comparisons of inhibitory effects of distributed ADR and ACM on DNA, RNA and protein biosyntheses in the heart and liver of mice. Values were calculated using the following equation: % inhibition/μg of ADR or ACM = % inhibition at 4th day after ADR (ACM) administration/Cmax of ADR (ACM). ▨, heart; ▧, liver.

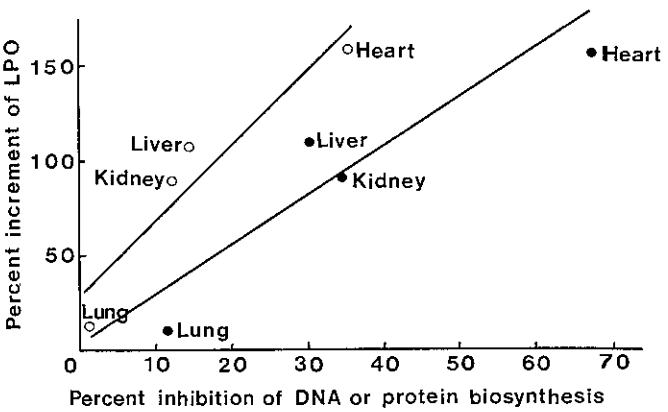

Fig. 4. Correlation between inhibition percent of DNA or protein biosynthesis and percent increment of lipid peroxide levels by ADR. ●, DNA; ○, protein.

in which decreases in the activities of lipid peroxidation-preventing enzymes together with an increment of lipid peroxide were seen.[10] The inhibitory effects of ADR on each type of biosynthesis were the largest in the heart among all the tissues examined. The percentage levels of inhibition produced by 1 μg of ADR distributed at peak tissue concentration on DNA, RNA and protein biosynthesis in the heart and liver were calculated and are shown in Fig. 3.

The percentage levels of inhibition of DNA, RNA and protein biosyntheses per 1 μg of ADR in the heart were 4.0, 12.1 and 4.4 times larger than those obtained in the liver, respectively. These results show that ADR exerts a much more marked effect in the heart than in the liver. The difference between the two tissues is considered to be due to the fact that the nuclei of cardiac myocytes exhibit a very low rate of DNA biosynthesis in comparison with that in hepatocyte nuclei.[18]

Next, the relationships between the percentage inhibition of DNA, RNA and protein biosyntheses by ADR, and the percentage increment of lipid peroxide levels induced by ADR in the heart, liver, kidney and lung of mice were investigated and the results are shown in Fig. 4. In these tissues, good correlations were observed between the percentage inhibition of DNA and protein biosyntheses by ADR, and the percentage increment of lipid peroxide levels. The correlation coefficients (r) were 0.928 for DNA and 0.929 for protein. There was a low correlation between the percentage inhibition of RNA biosynthesis by ADR and the percentage increment of lipid peroxide level.

In the same way, the relationship between the percentage inhibition of DNA and protein biosyntheses by ADR, and the percentage decrease in the activities of lipid peroxidation-preventive enzymes was examined. The best correlations were observed between the decrease of GSHpx activity and the inhibitory effect on DNA (r = 0.976) and protein (r = 0.980) biosyntheses. Therefore, the decreases in the activities of lipid peroxidation-preventive enzymes, especially GSHpx, caused by ADR resulted in an increment of lipid peroxide in the heart. It seems clear that the decreases in these enzyme activities were due to the inhibitory effect of ADR on both DNA and protein biosyntheses.

The inhibitory effects of DAM and ACM, which possess weaker cardiotoxicity than ADR, on DNA and protein biosynthesis in tissues, were weaker than those of ADR, reflecting the order of both their cardiotoxicities[21] and effects on lipid peroxidation-preventive enzymes.[12]

The results obtained in normal tissues of Ehrlich carcinoma-bearing mice were the same as those obtained in normal mice. On the other hand, the inhibitory effects of ADR and ACM on DNA, RNA and protein biosyntheses in the tumor were greater than those in the normal tissues examined, except for the inhibitory effect of ADR on DNA biosynthesis. Thus, these results reflected the antitumor characteristics of the two drugs. However, the percentages of inhibition by ADR of DNA, RNA and protein biosyntheses in the heart, as mentioned above, approximated to those in the tumor, confirming the severe effect of ADR in the heart. Also, the inhibitory percentages of ADR on DNA, RNA and protein biosyntheses in the tumor were larger than those of ACM. The percentages of inhibition produced by 1 μg of ADR or ACM, when distributed at peak tissue con-

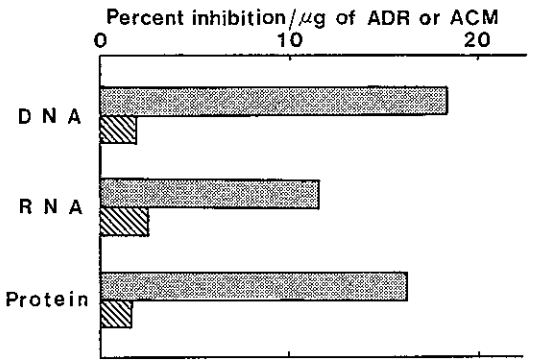

Fig. 5. Comparisons of inhibitory effects of distributed ADR and ACM on DNA, RNA and protein biosyntheses in the tumor of Ehrlich ascites carcinoma-bearing mice. Values were calculated using the following equation: % inhibition/μg of ADR or ACM=% inhibition at 4th day after ADR (ACM) administration/Cmax of ADR (ACM). ▒▒▒, ADR; ▨▨▨, ACM.

centration in the tumor, on DNA, RNA and protein biosyntheses[26] were calculated, and the results are shown in Fig. 5.

The inhibitory percentages per 1 μg of ADR on DNA, RNA and protein biosyntheses were 9.7, 4.6 and 9.5 times as large as those of ACM, respectively, showing a marked effect of ADR, and also reflecting the effects of both drugs on tumor weight; the percentage decreases in tumor weight produced by ADR and ACM were 58.5% and 41.3%, respectively. These data for both drugs are consistent with the finding that ADR has a stronger antitumor effect than ACM[21] clinically.

From these results, we consider that the mechanism responsible for the increment in lipid peroxides induced by ADR is as follows. Marked inhibitory effects of ADR on DNA, RNA and protein biosyntheses appear not only in the tumor but also in normal tissues, particularly the heart. These effects of ADR result in inhibition of the biosyntheses of lipid peroxidation-preventive enzymes, especially GSHpx.[14] Finally, an increment of lipid peroxide in the heart, which is related closely to the cardiotoxicity of ADR, occurs. Okamoto and Ogura[27] have reported that the inhibitory effect of ADR on DNA biosynthesis in Ehrlich ascites carcinoma does not decrease when antioxidants, such as α-tocopherol and riboflavin, are added *in vitro*. Therefore, it is reasonable to consider that the inhibitory effects of ADR on DNA, RNA and protein biosyntheses in the heart are not influenced by such antioxidants. In fact, the decreases in activities of lipid peroxidation-preventive enzymes which were induced by ADR were not changed by antioxidants.[14] However, antioxidants are able to inhibit the increment of lipid peroxide induced by ADR. α-Tocopherol (85 mg/kg, ip) has also been reported to decrease markedly the death rate in mice after ADR (15 mg/kg, ip) treatment.[4] Therefore, this increment of lipid peroxide seems to be very injurious and sometimes fatal. Moreover, the effectiveness of antioxidants seems to be more marked in the heart, composed of aerobic tissue, than in the tumor, composed of anaerobic tissue. Therefore, it seems to be possible to control the cardiotoxicity of ADR to some degree without influencing its antitumor effect.

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