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REDUCTION BY FUMARIC ACID OF SIDE EFFECTS OF MITOMYCIN C

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Abstract—The toxic symptoms in ICR mice given mitomycin C (either successive daily i.p. doses of 1 mg/kg or two large doses of 4 mg/kg) were reduced by the concurrent administration of fumaric acid (40 mg/kg). Fumaric acid did not reduce the antitumor activity of mitomycin C against either the solid or the ascitic form of the Ehrlich tumor. Fumaric acid reduced the lethal and hematologic toxicities of mitomycin C, and studies on the nucleic acids of animal tissues indicated that mitomycin C inhibited selectively DNA synthesis of liver and kidney, whereas fumaric acid exerted an enhancing effect, antagonistic to mitomycin C, on DNA synthesis of these tissues.

In our pharmacological studies on the extract of *Capsella bursa-pastoris* (Cruciferae) [1–7], fumaric acid was isolated and identified as the component of the herb responsible for inhibiting the growth of Ehrlich tumors in mice or gastric ulceration in rats [1, 2]. Recently, we found that toxic symptoms in the mice under treatment with mitomycin C, a potent but highly toxic antitumor agent [8], were markedly reduced by fumaric acid. The effects of fumaric acid on the haematological system and on nucleic acid metabolism following exposure to mitomycin C are now reported (see also refs. 9–13).

MATERIALS AND METHODS

Treatment of animals. The animals used were male ICR mice obtained from CLEA Japan, Tokyo, Japan. They were maintained on a semisynthetic diet CE-2 [14], also obtained from CLEA Japan. At 4–5 weeks of age, they were inoculated with 2×10^6 Ehrlich tumor cells into either the subcutaneous tissue of the left inguinal region or the abdomen of the animals, and then divided into groups. At specified days after the inoculation, groups of mice were given i.p. injections of 0.2 ml of 0.9% NaCl in which either mitomycin C or both mitomycin C and fumaric acid was dissolved. Control mice were given 0.9% NaCl.

Incorporation of [^{3}H]*thymidine into tissue DNA.* Each mouse received an i.p. injection of [methyl-^{3}H]thymidine (23 Ci/mmole) at a dose of 1 mCi/8ml 0.9% NaCl/kg, and was killed by cervical dislocation 2 hr later. The liver and kidneys were quickly removed and chilled in ice-cold 0.32 M sucrose buffer containing 3 mM $MgCl_2$ and 1 mM potassium phosphate (pH 7.0). One gram of liver and both kidneys from each mouse were weighed, minced with scissors in 3 ml buffer, and transferred to a Dounce homogenizer. The minced tissue was homogenized with 10 strokes of a loose pestle, and the homogenate was transferred to a 15-ml centrifuge tube with 6 ml buffer. The preparation was centrifuged at 2500 rpm in a refrigerated centrifuge. The supernatant fraction was discarded, and the precipitate was suspended with 2 ml buffer. The suspension was mixed with 3 ml of 1 M $HClO_4$, and centrifuged. The precipitate was washed twice with 0.2 M $HClO_4$, suspended with 2 ml water, dissolved by the addition of 3 ml of 0.6 M KOH, incubated at 37° for 1 hr, cooled on ice, acidified with 4 ml of 1 M $HClO_4$, and centrifuged. The supernatant fraction was discarded, and the precipitate was suspended in 3 ml of 1 M $HClO_4$, heated at 70° for 20 min, cooled on ice, and centrifuged. The final supernatant fraction was used to determine the quantity and radioactivity of DNA. For the determination of radioactivity, an aliquot of the supernatant fraction was taken into a vial, made alkaline with 2 M NaOH, and neutralized with 0.125 M HCl. Phenolphthalein was used as the indicator. A scintillation fluid, aquazol-2 (New England Nuclear, Boston, MA), was added and the radioactivity was measured in Beckman LS-150 Liquid Scintillation System. The extent of quenching was estimated by the internal standardization technique, using [^{3}H]toluene as a standard.

Incorporation of [^{14}C]*orotic acid into tissue RNA.* Each mouse received an i.p. injection of [6-^{14}C]orotic acid (57 Ci/mole) at a dose of 50 μCi/4 ml 0.9% NaCl/kg, and was killed by cervical dislocation 2 hr later. A 10% homogenate of each tissue was prepared as described above. One millilitre of homogenate was transferred to a 15-ml centrifuge tube, mixed with 5 ml of 0.5 M $HClO_4$, and centrifuged. The precipitate was washed twice with 0.2 M $HClO_4$, hydrolysed with 2 ml of 0.3 M KOH at 37° for 1 hr, cooled on ice, acidified with 1.25 ml of 1 M $HClO_4$, and centrifuged. The supernatant fraction was used to determine the quantity and radioactivity of RNA. The extent of quenching was estimated with the use of [^{14}C]toluene as a standard.

DNA and RNA syntheses by isolated liver nuclei. Isolation of liver nuclei was made by a modification [15] of the method of Dingman and Sporn [16]. DNA syntheses by isolated liver nuclei was measured by a slight modification of the method of Lynch *et al.* [17, 18]. The reaction mixture (0.5 ml) contained 0.1 M Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 16 mM KCl, 2 mM ATP, 8×10^{-2} mM each of dATP, dGTP

and dCTP, 1.6×10^{-2} mM [^{3}H]dTTP (1 Ci/mmole), and the suspension of isolated liver nuclei (containing about 100 μg DNA). The preparation was incubated at 37° for 10 min. Reaction was stopped by the addition of 5 ml of ice cold 0.5 M $HClO_4$. Two milligrams of bovine albumin dissolved in 0.1 ml of water was added as carrier. After thorough mixing, the acid-insoluble material was sedimented by centrifugation at 3500 rpm for 10 min. The precipitate was dissolved in 1 ml of 1 M NaOH, heated at 70° for 50 min, cooled on ice, acidified with 5 ml of 1 M $HClO_4$, and centrifuged. The final precipitate was dissolved in 0.3 ml of 0.25 M NaOH, heated over boiling water for 60 min, neutralized with 0.125 M HCl, and transferred with 10 ml of aquazol-2 into a vial for the determination of radioactivity. RNA synthesis by isolated liver nuclei was measured in the systems under activation by Mg^{2+} at low ionic strength and Mn^{2+} at high ionic strength with ammonium sulfate according to the procedure of Widnell and Tata [19].

Chemicals. Orotic acid[6-^{14}C], [4-^{14}C]UTP and [methyl-^{3}H]thymidine were purchased from the Radiochemical Center, Amersham, U.K.; [methyl-^{3}H]dTPP was from New England Nuclear, Boston, MA; the unlabelled ribonucleoside triphosphates were from Böehringer GmbH, Mannheim, F.R.G.; and the unlabelled deoxyribonucleoside triphosphates were from Sigma Chemical Co., St. Louis, MO, U.S.A. Mitomycin C was a product of Kyowa Hakko Kogyo Co., Tokyo, Japan.

The quantitative analysis of DNA and RNA were made by the methods of Giles and Myers [20] and Ceriotti [21], respectively. The 50 per cent lethal dose was determined by the method of Litchfield and Wilcoxon [22].

RESULTS

Following the findings that fumaric acid inhibited the solid growth of Ehrlich tumor [2], an examination was made of the combined effect of fumaric acid with mitomycin C (Table 1). From 3 days after the subcutaneous inoculation of Ehrlich tumor cells, mice were given i.p. injections of mitomycin C, fumaric acid, or both agents in combination for 14 days. The daily dose of mitomycin C was 1 mg/kg and that of fumaric acid was 40 mg/kg. On the day following the last injection all the animals were killed, and tumor lumps were removed and weighed. The growth of tumor nodules was inhibited by the administration of either fumaric acid ($P < 0.02$, with respect to control mice) or mitomycin C ($P < 0.01$). However, the toxic effects of mitomycin C on the host mice were severe. Loss in body weight, bleeding at the sites of injection and in the abdomen, and congestion in such tissues as intestine and testes were noted, and 8 of 15 mice were dead before the end of the experiment. In the animals given mitomycin C and fumaric acid in combination, such toxic symptoms were markedly reduced, while the antitumor activity of mitomycin C was not retarded by the concurrent administration. Then the lethal and hematologic assays were performed on the mice which had been given i.p. injections of mitomycin C, alone or in combination with fumaric acid (40 mg. kg^{-1} day^{-1}), for 14 days (Table 2). The 50 per cent lethal dose of mitomycin C was 1.07 (0.96–1.20) mg. kg^{-1} day^{-1}, calculated by the method of Litchfield and Wilcoxon [22]. The hematologic assays made on the mice given daily doses of 1 mg/kg of mitomycin C indicated that this treatment decreased the counts of leucocytes and platelets in the peripheral blood. The administration of fumaric acid in combination elevated the LD_{50} of mitomycin C to 1.91 (1.74–2.10) mg. kg^{-1} day^{-1} and retarded the decreases of leucocytes and platelets.

Another experiment was made on the ascitic growth of Ehrlich tumor; this killed all animals within 2–3 weeks and was insensitive to treatment with fumaric acid (Fig. 1). Mice were inoculated i.p. with tumor cells and divided into 3 groups. At 1 and 3 days after the inoculation, the first group was given i.p. injections of 0.9% NaCl, the second group was given mitomycin C at the dose of 4 mg/kg, and the third group was given fumaric acid at the dose of 40 mg/kg in combination with mitomycin C. In the control group, the ascitic growth of tumor killed all

Table 1. Antitumor activities of mitomycin C and fumaric acid against solid growth of Ehrlich tumor, and inhibition by fumaric acid of death and loss of body weight in mice under successive doses of mitomycin C*

Dose (mg kg^{-1} day^{-1})		No. of mice			
MC	FA	Initial	Final	Body wt (g)	Tumor wt (g)
0	0	15	15	31.3 ± 2.3‡	1.97 ± 1.07‡
0	40	15	15	32.0 ± 2.7‡	1.04 ± 0.66‡
1	0	15	7	24.6 ± 3.3	0.26 ± 0.22
1	40	15	15	28.1 ± 2.9†	0.26 ± 0.19

* Mice, 20 g average body wt. were inoculated with tumor cells into the subcutaneous tissue of the left inguinal region, and divided into four groups. At three days after the inoculation, each group of 15 mice were treated for 14 days with i.p. injections of 0.2 ml of 0.9% NaCl, in which mitomycin C (MC) and fumaric acid (FA) were dissolved, and killed on the following day. Each value of body and tumor weight represents the mean ± S.D. of a group of animals killed at the end of the experiment.

† $P < 0.05$, with respect to mice treated with mitomycin C.

‡ $P < 0.01$, with respect to mice treated with mitomycin C.

Table 2. Effect of fumaric acid on lethal and hematologic toxicities of mitomycin C*

Treatment†	LD_{50} (mg kg^{-1} day^{-1})	Counts of blood cells: Leucocytes ($10^3/mm^3$)	Counts of blood cells: Platelets ($10^5/mm^3$)
Control	—	8.04 ± 3.28‡	9.35 ± 2.30‡
MC	1.07 (0.96–1.20)	4.47 ± 0.91	4.07 ± 1.40
MC + FA	1.91 (1.74–2.10)	6.68 ± 2.22‡	6.96 ± 1.52‡

* The 50 per cent lethal dose (LD_{50}) was determined by the method of Litchfield and Wilcoxon [22]. Groups of ten mice were given i.p. injections of mitomycin C, either alone or in combination with fumaric acid, for 14 days. The number of mice that were dead during the injections and in the subsequent 14 days was counted. The values in the parentheses are the confidence limits at $P = 0.05$. The hematologic assays were made on the groups of mice that had been treated with i.p. injections for 14 days. The dose of mitomycin C was 1 mg. kg^{-1} day^{-1} and that of fumaric acid was 40 mg. kg^{-1} day^{-1}. On the following day, ten animals of each group had the end portions of their tails cut off, and blood samples were taken from the cut ends for the hematologic assays. Each value represents the means ± S.D. of ten animals.

† Control, given 0.2 ml of 0.9% NaCl; MC-treated, given mitomycin C; MC + FA-treated, given mitomycin C in combination with 40 mg/kg of fumaric acid.

‡ $P < 0.01$, with respect to mice treated with mitomycin C.

animals and 50 per cent death was noted at 14–15 days. The other two groups could survive longer than the control group and half of each group escaped death. The reduction of body and tissue weights by mitomycin C noted in the animals of the second group was effectively inhibited by the concurrent administration of fumaric acid (Fig. 1 and Table 3).

In an attempt to investigate the modes of action of fumaric acid in reducing the toxic side-effects of mitomycin C, the metabolic alterations in tissue nucleic acids were examined. Mice were grouped without inoculation of tumor cells and given two i.p. injections as described above (Table 4). At 2 days after the second injection the content of tissue nucleic acids, the incorporation of [^{3}H]thymidine into DNA and the incorporation of [^{14}C]orotic acid into RNA were determined. Examination of liver and kidney indicated that the effects of both mitomycin C and fumaric acid were seen selectively in DNA metabolism. Treatment with mitomycin C suppressed the incorporation of [^{3}H]thymidine into DNA and, by contrast, administration of fumaric acid in combination with mitomycin C enhanced the [^{3}H]thymidine incorporation and prevented alterations in the contents of tissue nucleic acids. No marked differences in tissue RNA content and incorporation of [^{14}C]orotic acid into RNA were noted between the three groups of mice. A further study on liver indicated that the effects of mitomycin C and fumaric acid on [^{3}H]thymidine incorporation persisted throughout a 96-hr period of study without recovery (Fig. 2). The activities for the syntheses of DNA and RNA were then determined on the isolated liver nuclei (Table 5). The assays at 2 days after dosing (fifth day) indicated that DNA synthesis in liver nuclei was also selectively suppressed by mito-

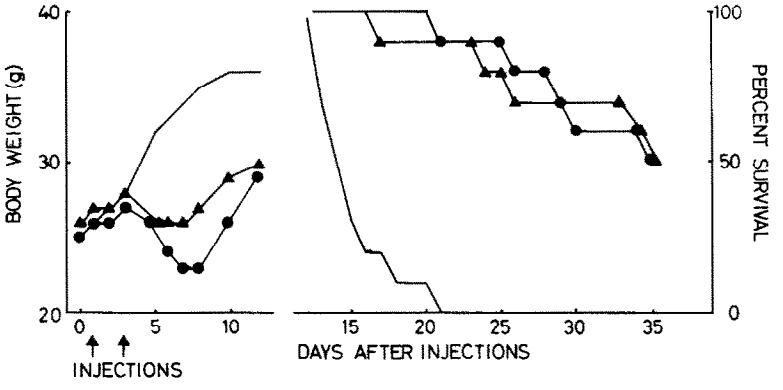

Fig. 1. Antitumor activity of mitomycin C against ascitic growth of Ehrlich tumor, and inhibition by fumaric acid of loss of body weight in mice treated with two large doses of mitomycin C. Mice were inoculated i.p. with tumor cells and divided into three groups. At 1 and 3 days after the inoculation, each group of 10 animals were treated with two i.p. injections. (——), 0.2 ml of 0.9% NaCl; (●—●), 4 mg/kg of mitomycin C; (▲—▲), 4 mg/kg of mitomycin C and 40 mg/kg of fumaric acid.

Table 3. Inhibition by fumaric acid of losses of body and tissue weights in mice treated with mitomycin C*

Treatment†	Body wt (g)	Tissue wt (g)			
		Liver	Lung	Kidney	Spleen
Control	32.1 ± 1.1	1.9 ± 0.3	0.22 ± 0.02	0.51 ± 0.07	0.10 ± 0.01
MC	25.1 ± 3.5§	1.5 ± 0.2‡	0.18 ± 0.02§	0.33 ± 0.05§	0.05 ± 0.02§
MC + FA	32.6 ± 1.7	1.8 ± 0.2	0.23 ± 0.03	0.46 ± 0.03	0.11 ± 0.01

* Mice, average body wt 25 g, were divided into three groups without inoculation of tumor cells. Each group of animals were treated with two i.p. injections on the first and third days, and were killed on the eighth day. Each value represents the mean ± S.D. of results from seven mice.

† Control, given 0.2 ml of 0.9% NaCl; MC-treated, given 4 mg/kg of mitomycin C; MC + FA-treated, given 4 mg/kg of mitomycin C and 40 mg/kg of fumaric acid.

‡ $P < 0.02$, with respect to control.

§ $P < 0.01$, with respect to control.

mycin C. At 4 days after dosing (seventh day), activity in the liver nuclei from the animals that had been given fumaric acid in combination with mitomycin C was restored to initial levels.

DISCUSSION

Fumaric acid was isolated and identified as an active component of the extract of *Capsella bursa-pastoris* for inhibiting either the solid growth of Ehrlich tumor or the gastric ulceration in rats [1, 2]. Mitomycin C is an antitumor antibiotic which is effective against a variety of tumors [23–25] but is also very toxic to the host animals [26, 27]. The present study indicated that fumaric acid reduced the toxic side-effects of mitomycin C, shown by loss of body and tissue weight, bleeding at the sites of injection and in the abdomen, congestion in such tissues as intestine and testes, decreases of leucocytes and platelets in the peripheral blood, and death of animals, but not the antitumor activity of mitomycin C against the growth of Ehrlich tumor. The use of fumaric acid in combination with mitomycin C is expected to increase the utility of this antitumor agent that has been widely used in cancer chemotherapy.

Selective inhibition of DNA synthesis was inferred to be associated with the modes of action of mitomycin C in its bacteriostatic and antitumor activities [9–13]. In mitomycin C-treated mice such a selective inhibition was noted for the incorporation of [^{3}H]thymidine injected i.p. into DNA of liver and kidney and for the activity of liver nuclei for synthesis of DNA. It is of interest that fumaric acid also exerted a selective effect on DNA synthesis and that, in contrast to mitomycin C, fumaric acid enhanced

Table 4. Effects of mitomycin C and fumaric acid on liver and kidney as to contents of nucleic acids and incorporation of either [^{3}H]thymidine into DNA or [^{14}C]orotic acid into RNA*

Treatment†	DNA		RNA	
	Content (mg/g tissue)	Incorporation of [^{3}H]thymidine (dpm × 10^{-3}/mg DNA)	Content (mg/g tissue)	Incorporation of [^{14}C]orotic acid (dpm × 10^{-4}/g tissue)
Liver				
Control	2.35 ± 0.17	5.04 ± 1.53	8.40 ± 1.09	3.09 ± 0.31
MC	3.52 ± 0.15§	2.46 ± 0.43§	7.90 ± 0.21	3.20 ± 0.13
MC + FA	2.88 ± 0.52‡	11.7 ± 3.3§	8.00 ± 0.70	3.37 ± 0.45
Kidney				
Control	3.70 ± 0.11	3.13 ± 0.18	4.90 ± 0.25	40.0 ± 4.2
MC	4.42 ± 0.19§	2.39 ± 0.16§	4.98 ± 0.09	50.7 ± 5.9§
MC + FA	3.89 ± 0.39	6.58 ± 2.54§	4.89 ± 0.13	46.7 ± 5.9

* Mice were divided into three groups, and each group of animals were treated with two i.p. injections on the first and third days. On the fifth day the contents of tissue nucleic acids and the incorporation of either [^{3}H]thymidine into DNA or [^{14}C]orotic acid into RNA were determined. Each value represents the mean ± S.D. of six determinations.

† Control, given 0.2 ml of 0.9% NaCl; MC-treated, given 4 mg/kg of mitomycin C; MC + FA-treated, given 4 mg/kg of mitomycin C and 40 mg/kg of fumaric acid.

‡ $P < 0.05$, with respect to control.

§ $P < 0.01$, with respect to control.

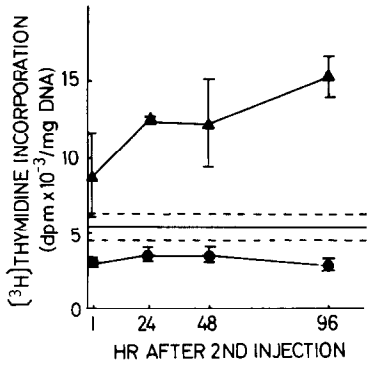

Fig. 2. Time course of effects of mitomycin C and fumaric acid on incorporation of [^{3}H]thymidine into liver DNA. Mice were divided into four groups, and a group of 16 animals were treated with two i.p. injections on the first and third days. At 1, 24, 48, and 96 hr after the second injection, four animals were taken from each group and the incorporation of [^{3}H]thymidine into liver DNA was determined. The mean value (±S.D.) of the control group that had been given i.p. injections of 0.2 ml of 0.9% NaCl was 5.40 (±0.91) × 10^3dpm/mg DNA, and this is indicated in the figure as a horizontal line. The dotted lines indicate S.D. within the control group. (●—●), 4 mg/kg of mitomycin C; (▲—▲), 4 mg/kg of mitomycin C and 40 mg/kg of fumaric acid. Each point represents the mean and range of results from four animals.

the [^{3}H]thymidine incorporation. The activity of liver nuclei for DNA synthesis, which had been reduced by mitomycin C, was made to recover earlier by the concurrent administration of fumaric acid. The striking contrast between mitomycin C and fumaric acid in their effects on DNA synthesis seems to be associated with the modes of actions of both agents [9, 28, 29], and suggests that fumaric acid would enhance the repair of mitomycin C-induced damage of tissue DNA. However, there remains the possibility that fumaric acid would transform mitomycin C into a derivative that is less toxic to the animals but has a potent antitumor activity. It is expected that the protective effect of fumaric acid would be more effective on the animals given lower doses of mitomycin C and on the delayed and chronic toxicities of mitomycin C. The present findings would indicate another aspect of side-effects of mitomycin C, and further studies in search of the substances that reduce or ameliorate the toxic side-effects of an antitumor agent are expected to contribute to cancer chemotherapy.

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Table 5. Effects of mitomycin C and fumaric acid on DNA and RNA syntheses by isolated liver nuclei*

Treatment†	DNA synthesis (pmoles dTTP/mg DNA)		RNA synthesis (pmoles UTP/mg DNA)	
			Mg^{2+}-activated	Mn^{2+}-$(NH_4)_2SO_4$-activated
	5th day‡	7th day§	5th day‡	
Control	11.1 ± 1.5	10.3 ± 1.6	742 ± 142	1439 ± 284
MC	6.3 ± 1.7‖	5.6 ± 0.7‖	589 ± 166	1383 ± 203
MC + FA	6.8 ± 1.0‖	10.0 ± 3.4	612 ± 65	1289 ± 263

* Mice were divided into three groups, and each group of animals were treated with two i.p. injections on the first and third days. On the fifth or seventh days the activities of isolated liver nuclei for DNA and RNA syntheses were determined. Each value represents the mean ± S.D. of five determinations.

† Control, given 0.2 ml of 0.9% NaCl, MC-treated, given 4 mg/kg of mitomycin C; MC + FA-treated, given 4 mg/kg of mitomycin C and 40 mg/kg of fumaric acid.

‡ Fifth day, two days after injection.

§ Seventh day, four days after injection.

‖ $P < 0.01$, with respect to control.

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