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Effect of Adriamycin on Cultured Mouse Embryo Myocardial Cells

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Adriamycin (ADM) inhibited the beating of cultured mouse embryo myocardial cells dose- and time-dependently and subsequently lysed the cells. Fifty percent inhibition of beating cell number was observed when cultures were exposed to 430—520 μM ADM for 0.5 h, 70—100 μM ADM for 4 h and 3.5 μM for 36 h. When cultures were exposed to 3.5 μM ^3H -ADM, the radioactivity in the cells reached almost the maximum within 8 h, and about 90% was recovered as ADM itself even after 72 h. However, the beating cell number and the beating rate did not appreciably decrease within 8 h, then decreased to 78.5 and 67.1% at 24 h, and 5.6 and 5.2% at 72 h, respectively. Lysis of the cells was conspicuous at 48 h or later. It was also supported with the decrease in cellular proteins and the release of lactate dehydrogenase. These results indicate that ADM stimulates lysis of the cytoplasmic membrane following inhibition of beating. When cultures were exposed to 70 μM ADM for 4 h and then incubated in ADM-free medium, about 50% of ADM in cells was released within 24 h. However, inhibition of beating and lysis of such cells still advanced at about the same rate as in continuously exposed cells. ADM also decreased the cellular adenosine triphosphate (ATP) content in the same fashion as antimycin A, which inhibited beating in advance of ATP reduction. These results suggested that the beating state of myocardial cells is a sensitive and reliable parameter in this system for studies on the cardiotoxicity of ADM. The system may be useful for studies not only on ADM but also on agents having effects on the heart.

Keywords—adriamycin; antimycin A; ATP; rotenone; beating; mouse embryo; lactate dehydrogenase; cardiac cell culture

Adriamycin (doxorubicin, ADM) is known to produce various adverse effects on the heart, as well as such side-effects as myelosuppression and anorexia, that are commonly observed with antineoplastic agents. In particular, the cumulative dose-dependent cardiomyopathy due to ADM is a serious problem that limits the dose and application of ADM in cancer therapy.²⁾

ADM has been reported to induce a number of biochemical changes, most of which relate to (a) mitochondrial dysfunction,^{3,4)} (b) accumulation of oxygen radicals or lipid peroxides,^{5,6)} (c) inhibition of nucleic acid synthesis,^{7,8)} (d) membrane perturbation,^{9,10)} or (e) disturbance of electrolyte balance.^{11,12)} However, the interrelations between such biochemical changes and the cardiotoxicity of ADM have not been clarified. If some of these biochemical abnormalities cause the cardiotoxicity, they must develop before or in parallel to the structural and functional alterations of myocardial tissue by ADM. In order to follow the biochemical changes and the functional alterations caused by ADM in the heart simultaneously, cultured heart cells appear to be a simple and convenient material, because they retain the structural integrity of the heart cell and its ability to beat autorhythmically.

In this study, the inhibitory effect of ADM on beating of cultured mouse cardiac cells was investigated in order to establish a basic system to elucidate the biochemical mechanisms resulting in the ADM cardiotoxicity.

Experimental

Materials— $^3\text{H}(\text{G})$ -Labelled ADM (198.2 $\mu\text{Ci}/\text{mg}$) and unlabeled ADM were kindly donated by Kyowa Hakko Co., Chiyoda-ku, Tokyo, Japan. Other materials were purchased from the following sources: Nunc dishes from Japan Inter-Med Co., Minato-ku, Tokyo, Japan; trypsin (1:250) from Difco Laboratories, Detroit, Mich., U.S.A.; collagenase (type I) and firefly lantern extract (FEL-50) from Sigma Chemical Co., St. Louis, Mo., U.S.A.; Eagle's MEM, Ham's F-12 medium and Dulbecco's phosphate buffered saline (PBS) from Nissui Seiyaku, Tokyo, Japan; calf serum from Commonwealth Serum Laboratories, Victoria, Australia; ACS-II from Amersham Co., Arlington Heights, Ill., U.S.A.. The other agents were of analytical reagent grade.

Mouse Embryo Cardiac Cell Culture—The cardiac cell culture was prepared by a modification of Goshima's method¹³⁾ as described below. The basal medium in Goshima's method, *i.e.*, Eagle's MEM containing 10% calf serum and 20 mM *N,N'*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), was replaced by Ham's F-12 medium which was supplemented with 10% calf serum and buffered at pH 7.3 with sodium bicarbonate. Such a modification allowed the cultures to keep beating so longer. BES somewhat depressed the beating of cells when the incubation period was extended over 6 h.

Hearts from 14- to 16-d-old mouse embryos (ICR strain) were minced and digested with 5 ml of 0.125% trypsin–0.025% collagenase solution at 37 °C for 20 min. The dispersed myocardial cells were collected by centrifugation at about $200 \times g$ for 10 min, and then resuspended in Ham's F-12 medium. The cell suspension was adjusted to a density of $2\text{--}4 \times 10^6$ cells/ml. Viable cells counted by a trypan blue exclusion method¹⁴⁾ always amounted to 80% or more of the total cells in the suspension. Nunc dishes (35 mm i.d., Cat. No. 153066) containing 1 ml of Ham's F-12 medium were seeded with 0.1 ml of the cell suspension, and incubated at 37 °C in a humidified environment of 5% CO_2 –95% air. The medium was renewed every 48 h thereafter. The treatment of cultures with the test agents was begun 1–2 d after the cells were seeded, at which time they were beating rhythmically, and was ended before the cells formed into a monolayer sheet.

Measurement of Beating—The beating status of cultured cardiac cells was monitored with an inverted phase-contrast microscope at magnification of 150 to 400 in a chamber controlled at 37 °C. In each culture dish, about twenty cells or clusters, which were beating regularly in the range of 33–171 beats/min, were selected for each experiment, and their beating rates, shapes and locations in the dish were recorded before application of the test agents. Beating of each cell or cluster was usually counted for 60 s at the time indicated. Each experiment was repeated at least three times by using separately prepared cell cultures, and the results were averaged.

Determination of Uptake and Metabolites of ADM— $^3\text{H}(\text{G})$ -ADM instead of unlabeled ADM was dissolved in Ham's F-12 medium and added to the culture. At the time indicated, the cultured cells were rinsed 3 times with 0.5 ml of PBS, and then dissolved in 0.5 ml of 0.1 M sodium hydroxide solution for 30 min. Two thirds of the solution was transferred to a scintillation vial and mixed with 12 ml of ACS-II. The radioactivity was measured with a Packard Tri-Carb 460 liquid scintillation counter. The remaining part of the solution was used for the measurement of protein content by the method of Lowry *et al.*¹⁵⁾ For analysis of ADM metabolites, labelled compounds were extracted with methanol from the cells burst by freezing, and divided to fractions of ADM, adriamycinone and the remaining eluate by a high-performance liquid chromatography (HPLC) as described by Haneke *et al.*¹⁶⁾ The radioactivities were determined with a scintillation counter. The HPLC column was Finepak C₁₈₋₅ (5 μm particles, 250 $\times$ 4.6 mm i.d.) and the flow rate was 0.8 ml/min.

Measurement of ATP—The ATP content of cultured cells was determined by a modified luciferase method.¹⁷⁾ ATP in adherent cells was extracted with chilled 0.6 M perchloric acid for 30 min. The extract was neutralized with 3 M potassium hydroxide and then centrifuged at $350 \times g$ for 5 min at 4 °C. The supernatant was used as a sample solution for the ATP assay. One milliliter of the reaction mixture in a quartz cell contained 0.5 ml of 0.1 M sodium arsenate–40 mM magnesium sulfate at pH 7.4, 0.2 ml of the supernatant, and 0.25 ml of water. Diluted firefly lantern extract (0.05 ml) was then added to the reaction mixture, and bioluminescence intensity was measured with a CHEM-GLOW photometer. Counts accumulated during 60 s were compared to those from the standard solution of ATP.

Measurement of Lactate Dehydrogenase (LDH)—LDH activity released into the culture medium was determined spectrophotometrically by a modified Wroblewski-LaDue method.¹⁸⁾ The reaction mixture (1.17 ml total) in a quartz microcell contained 0.9 ml of 50 mM potassium phosphate buffer at pH 7.5, 0.1 ml of 23 mM sodium pyruvate, 0.05 ml of a sample (the culture medium) and 0.12 ml of water. The reaction mixture was brought to 25 °C before the assay. An aliquot of 0.03 ml of 6.12 mM reduced nicotinamide adenine dinucleotide (NADH) containing 59.5 mM sodium bicarbonate was added to the reaction mixture and mixed immediately. The change in extinction at 340 nm during 4–5 min was measured with a Parkin-Elmer Model 551 UV-VIS double-beam spectrophotometer. The LDH activity was expressed as the rate of oxidation of NADH by the sample medium, in nmol/min/ml.

Determination of Protein Content—Cellular proteins were solubilized with 0.1 M sodium hydroxide solution from the cells attaching on the growing surface of the dish and determined by the method of Lowry *et al.*¹⁵⁾

Results

Inhibitory Effect of ADM on Beating Cells

On cultivation for one day, trypsin-dispersed heart cells became attached and spread out on the surface of culture dishes. Two different cell types, myocardial cells and fibroblast-like cells, could be relatively easily distinguished under a phase contrast microscope, as previously noted by Goshima and Tonomura¹⁹⁾: the fibroblast-like cells were very thin, well-spread cells. Because they were so thin, they were transparent and poorly refractile under phase optics. The myocardial cells were thick, highly refractile cells. The percentage of myocardial cells in our culture prepared from fetal mouse heart was about 80% and most of them exhibited spontaneous beating (Table I).

Table II shows the average beating status of mouse myocardial cells, which served for this series of ADM experiments. In the basal medium, almost all the cells selected at initial stage continued beating rhythmically during incubation for up to 72 h, and their mean beating rate increased gradually up to 147.1% of the initial rate during the period of 72 h. In the presence of 3.5 μ M ADM, the number of beating cells and their beating rates started decreasing concomitantly with a time lag of about 24 h, and decreased to about 5% of the initial values of 72 h. Most cells acquired irregular beating and/or structural alterations within this period.

Figure 1 shows morphological changes of cardiac cells under the same conditions as in Table II. Most cells in the presence of 3.5 μ M ADM did not show marked morphological changes up to 24 h (Fig. 1A, B). After 48 h of incubation, however, they tended to lyse and to become detached from the surface (Fig. 1C). Many of the cells disappeared from the surface

TABLE I. Percentage of Myocardial Cells

Cultivation time (h)	Myocardial cells among attached cells (%)	Beating cells among myocardial cells (%)
48	80 $\pm$ 4	96 $\pm$ 3
72	73 $\pm$ 7	88 $\pm$ 9
96	81 $\pm$ 6	—

The values are means $\pm$ SE of 6 experiments. A total of 780—1230 cells was counted for each value.

TABLE II. Inhibitory Effects of Adriamycin on the Beating Cell Number and the Beating Rate of Cultured Mouse Myocardial Cells

Incubation time (h)	Beating cell number (%)		Beating rate (%)	
	None	3.5 μ M ADM	None	3.5 μ M ADM
0	88/88 (100)	107/107 (100)	100 ^{a)}	100 ^{b)}
4	60/60 (100)	—	111 $\pm$ 8	—
8	55/60 (92)	—	106 $\pm$ 9	—
24	74/88 (84)	84/107 (79)	134 $\pm$ 16	67 $\pm$ 5 ^{d)}
48	81/85 (95)	13/107 (29) ^{c)}	150 $\pm$ 11	28 $\pm$ 5 ^{d)}
72	46/48 (96)	6/107 (6) ^{c)}	147 $\pm$ 15	5 $\pm$ 2 ^{d)}

Beating cell number and beating rate are given as the ratio of beating cells at the indicated time to that at 0 h. Figures in parentheses show the percentage of beating cell number. Beating rates are expressed as averaged beating rates (mean $\pm$ SE) of individual cells. a) 99 $\pm$ 5 beats/min at 37 °C, mean $\pm$ SE (n =88). b) 104 $\pm$ 6 beats/min at 37 °C, mean $\pm$ SE (n =107). Values with superscripts c) and d) are significantly different from the corresponding control (values at 0 h) with p <0.001 by the χ^2 -test and unpaired t -test, respectively.

Fig. 1. Morphological Changes of Cardiac Cells Incubated with $3.5 \mu\text{M}$ Adriamycin

A, B, C and D are phase contrast micrographs of cells observed at a magnification of 400 at 0 h, 24, 48, and 72 h after the addition of $3.5 \mu\text{M}$ ADM, respectively. The scale shows $50 \mu\text{m}$. E and F show phase contrast micrographs, which were observed at a magnification of 100, of the cultures incubated without and with $3.5 \mu\text{M}$ ADM for 48 h, respectively. The scale shows $200 \mu\text{m}$.

after 72 h (Fig. 1D). Such morphological changes could be seen more clearly at a lower magnification as shown in the micrographs of two groups of cells grown with and without $3.5 \mu\text{M}$ ADM at 48 h in Fig. 1E and F.

Lysis of cells during incubation with ADM was also supported by the decrease in the total cellular protein content and the increase in the release of LDH, a cytosolic enzyme. Table III shows the total protein content of the cells attached to the growing surface. The protein content of cells in the presence of $3.5 \mu\text{M}$ ADM decreased gradually to about half the initial content during cultivation for 72 h, while that of parallel control cells increased to twice the initial content at 72 h.

Table IV shows the amounts of LDH released from cells during cultivation for 72 h. ADM increased the release of LDH to about twice that of the parallel control. These results indicate that ADM stimulates lysis of the cytoplasmic membrane following inhibition of beating. Figure 2 shows the effects of various doses of ADM on beating cells. The decrease in

TABLE III. Effect of Adriamycin on Protein Content of Cardiac Cells

Incubation time (h)	Protein content (%)	
	None	3.5 μ M ADM
24	114 $\pm$ 9 (13)	83 $\pm$ 12 (9)
48	170 $\pm$ 12 (13)	77 $\pm$ 17 (9)
72	190 $\pm$ 18 (13)	54 $\pm$ 6 (13)

Protein contents are expressed as mean percentage $\pm$ SE of the initial content. Figures in parentheses are the number of experiments. The initial protein content of cardiac cells was $22.0 \pm 2.2 \mu$ g per dish.

TABLE IV. The Release of Lactate Dehydrogenase from Cardiac Cells by Adriamycin

Incubation time (h)	LDH activity in culture medium (nmol/min/ml)	
	None	ADM 3.5 μ M
24	16.9 $\pm$ 6.2	36.0 $\pm$ 9.9
48	38.8 $\pm$ 7.7	68.2 $\pm$ 14.5 ^{a)}
72	83.2 $\pm$ 8.5	195.5 $\pm$ 17.0 ^{b)}

The data on the LDH activity are expressed as mean $\pm$ SE of 11 experiments. Values with superscripts a) and b) are significantly higher than the corresponding controls with $p < 0.05$ and $p < 0.001$, respectively.

Fig. 2. Dose and Time Dependencies of the Inhibitory Effect of Adriamycin on Beating of Cultured Myocardial Cells

Beating cell numbers were counted at the indicated times after the addition of various concentrations of ADM. Open circles, open triangles and closed circles show incubation periods of 0.5, 1.0 and 4.0 h, respectively.

the beating cell number by ADM was dose- and exposure time-dependent. Fifty percent cessation of beating of cells was observed on incubation with 430–520 μ M ADM for 0.5 h, 280 μ M ADM for 1 h, 70–100 μ M for 4 h or 3.5 μ M ADM for 36 h.

Incorporation of $^3\text{H}(\text{G})\text{-ADM}$

Table V shows the amounts of $^3\text{H}(\text{G})\text{-ADM}$ incorporated into the cells under the same conditions as in Table II. The radioactivity in cells reached almost the maximum within 8 h, and more than 90% was from ADM itself even 72 h after the addition, as confirmed by HPLC analysis.

Table VI shows the relationship between the amount of ADM incorporated into cells and the inhibitory effect on beating. When cultures were incubated with 70 μ M $^3\text{H}(\text{G})\text{-ADM}$, uptake of the isotope by the cells was 13.4 pmol/ μ g protein at 4 h and reached the maximum level at 24 h, while the beating cell number was 53.5% of the control at 4 h and decreased as the incubation time was prolonged. When cultures were incubated with 70 μ M ADM for 4 h

TABLE V. Uptake and Metabolism of Adriamycin by Cardiac Cells
(in the presence of 3.5 μM adriamycin)

Incubation time (h)	Total uptake of ^3H -ADM (pmol ADM eq/ μg protein)	ADM found (% of total uptake)
0.5	1.42 ± 0.05 (6)	—
1	1.52 ± 0.04 (6)	—
2	2.03 ± 0.10 (6)	—
4	2.30 ± 0.12 (6)	—
8	2.80 ± 0.15 (6)	—
24	2.72 ± 0.21 (28)	95.9 ± 8.64 (7)
48	3.43 ± 0.17 (28)	90.2 ± 4.72 (7)
72	2.97 ± 0.17 (28)	96.2 ± 4.46 (7)

Radioactivity of ^3H (G)-ADM used in the experiments was 198.2 $\mu\text{Ci}/\text{mg}$. The data are expressed as mean $\pm$ SE and figures in parentheses are the number of experiments. The total uptake of ^3H -ADM was estimated by measuring radioactivity of the isotope incorporated in cells. ADM found in cells was determined by HPLC as described in the text.

TABLE VI. Uptake of Adriamycin by Cardiac Cells (in the presence
of 70 and 140 μM adriamycin)

ADM (μM)	Incubation time (h)	Cellular radioactivity ^{a)} (pmol ADM eq/ μg protein)	Beating cell number (% ^{b)})
70	4	13.4 ± 0.61 (45)	61/114 (53.5)
	24	24.1 ± 1.96 (12)	—
	28	—	19/114 (16.7)
	48	18.7 ± 2.25 (12)	—
	52	—	1/114 (0.9)
	72	14.7 ± 1.10 (6)	—
	76	—	0/114 (0)
70	4 (24) ^{c)}	6.37 ± 0.35 (40)	23/114 (20.2)
	(48)	4.94 ± 2.25 (12)	6/114 (5.3)
	(72)	7.23 ± 0.68 (6)	3/114 (2.6)
140	1	11.0 ± 1.92 (17)	38/ 54 (70.4)
	4	19.3 ± 3.08 (17)	23/ 54 (42.3)

a) The total cellular radioactivity from ^3H (G)-ADM was calculated in ADM eq and is expressed as the mean $\pm$ SE, figures in parentheses are the number of experiments. b) Ratio of beating cell number at the indicated incubation time to that before ADM treatment; figures in parentheses show the percentage of the beating cell number. c) The cells were incubated with ^3H (G)-ADM for 4 h and then incubated in the basal medium (ADM-free) after being washed three times with the basal medium. Figures in parentheses show the incubation time after the removal of ADM.

and then with ADM-free medium for 24 to 72 h, the ADM content of the cells decreased to less than half that of the cells exposed continuously to ADM. However, inhibition of beating of such rinsed cells still progressed at about the same rate as that of the cells exposed continuously to 70 μM ADM, resulting in lysis of cells. When cultures were incubated with 140 μM ^3H (G)-ADM, the amount of the isotope incorporated in the cells within 1 h was almost the same as that within 4 h at 70 μM ADM. The uptake of ADM at 4 h increased further and reached twice that at 1 h. At the same time, the beating cell number decreased rapidly from 70.4% of the initial beating cells at 1 h to 42.3% at 4 h. These results suggest that not the metabolites of ADM but ADM itself produces the inhibitory effect on the beating of myocardial cells and that this effect may result from some biochemical changes induced by ADM, since the time lag was prolonged as the dose of ADM was lowered.

TABLE VII. Inhibitory Effect of Adriamycin on ATP Content of Cardiac Cells

Incubation time (h)	ATP content (pmol/ μ g protein)	
	None	3.5 μ M ADM
24	37.5 $\pm$ 0.90	36.6 $\pm$ 1.67
48	37.7 $\pm$ 0.82	25.4 $\pm$ 0.92 ^{a)}
72	39.7 $\pm$ 1.23	17.5 $\pm$ 0.49 ^{a)}

Values show the mean $\pm$ SE in 8 experiments. The values with superscript *a*) are significantly lower than those from the parallel control (none) with $p < 0.001$ by the unpaired *t*-test.

TABLE VIII. Inhibitory Effects of Antimycin A and Rotenone on ATP Content and Beating of Myocardial Cells

Inhibitor (μ M)		ATP content (pmol/ μ g protein)	Beating cell number (%)
None	—	26.7 $\pm$ 0.47	100
Antimycin A	18	27.5 $\pm$ 0.76	97
	55	25.7 $\pm$ 1.14	57
	91	16.9 $\pm$ 1.07 ^{b)}	3
	128	9.83 $\pm$ 0.63 ^{b)}	—
Rotenone	13	29.6 $\pm$ 1.98	90
	25	23.8 $\pm$ 0.78 ^{a)}	47
	51	23.4 $\pm$ 0.80 ^{a)}	3
	127	21.1 $\pm$ 1.38 ^{a)}	—

ATP content was determined after the cells were incubated in the medium with the inhibitors for 0.5 h. Values of ATP content are the mean $\pm$ SE of 9 experiments. Beating cell numbers are shown as percentages of 30–52 beating cells at the start of the incubation. Values with superscripts *a*) and *b*) are significantly lower than the control (none) with $p < 0.01$ and $p < 0.001$ by the unpaired *t*-test, respectively.

Effect of ADM on the ATP Content of Cells

Table VII shows the relationship between the inhibitory effect of ADM on beating and the ATP level of cardiac cells under the same conditions as in Table II. ADM depressed ATP level in the cells at incubation periods of longer than 48 h. This reduction in ATP content by ADM, however, developed more slowly than the decreases in the number of beating cells and the beating cell rate, that is, about 24 h later.

Table VIII shows the inhibitory effects of antimycin A and rotenone, potent inhibitors of mitochondrial electron transport, on beating and ATP content. The concentrations of 55 μ M antimycin A and 25 μ M rotenone caused about 50% reduction in the number of beating cells. Reduction of ATP content by antimycin A, however, was caused only by higher concentrations than 91 μ M. In addition, rotenone at concentrations up to 127 μ M did not much reduce the ATP content.

Discussion

The incidence of ADM-induced cardiomyopathy in patients increases dramatically at cumulative ADM doses of above 550 to 600 mg/sq m.²⁰⁾ A variety of morphological changes, including loss of myofibrils, mitochondrial swelling, vacuolization, and z-band disruption, have been noted upon autopsy in the hearts of patients who had succumbed to ADM-induced congestive heart failure.²¹⁾ Similar morphological changes have been observed in various animal species.²²⁾

Some attempts to use heart cell cultures to evaluate ADM-induced toxicity have been reported,^{23–25)} though most of them did not systematically investigate the relationships between dose levels, exposure time, cellular uptake of ADM and biochemical and physiological functions of myocardial cells. Lampidis *et al.*²⁶⁾ in one such study showed that structural and functional alterations induced by ADM in cultured rat heart cells are similar to those seen *in vivo*, and can be related to ADM dose and exposure time. The present results on the inhibitory effect of ADM on mouse embryo myocardial cells are similar to their results. The doses of 3.5 and 140 μM ADM in our work seem to correspond to their low or intermediate doses and high doses, *i.e.*, the levels for so-called chronic and acute toxicities, respectively. They also suggested that the beating rate is not a reliable parameter for study since the beats/min of individual ADM-treated cultures overlapped those of untreated cultures, but the present results have proved that the beating rate and the beating cell number are sensitive and reliable parameters of ADM toxicity. This discrepancy may be due to the use of cardiac cell cultures at different stages. While we used a primary culture rich in myocardial cells after cultivation for 1 to 2 d (Table I), they used a confluent cell culture after 7 d which contained many fibroblast-like cells as well as myocardial cells. At a low dose of 3.5 μM ADM (Table II), the amount of ADM incorporated into the cells reached nearly the maximum level in 8 h, and remained unchanged till 72 h. Nevertheless, cessation of beating developed gradually after a lag time of almost one day, and lysis of cells followed, as shown by release of cellular proteins and lactate dehydrogenase (Tables III and IV). Such a time lag for inhibition of beating by ADM, though it was shortened as the ADM dose was increased, suggested that the beat inhibition may be a secondary effect of some biochemical changes induced in the cells by the addition of ADM and that the true form inducing such changes is not an ADM metabolite such as adriamycinone, but ADM itself (Table V). Exposure of myocardial cells to 70 μM ADM was lethal even if ADM was completely removed from the culture medium after 4 h (Table VI). Furthermore, beating of such cells was inhibited almost as effectively as that of cells continuously exposed to 70 μM ADM. Consequently, it appears that about 3 pmol or more of ADM per μg protein in cardiac cells results in irreversible inhibition of beating.

The myocardium is a most-energy consuming tissue, and the energy for the myocardial function is generated and supplied mainly through the mitochondrial respiratory chain, which is abundant in the heart tissue. ADM is known to produce structural and functional alterations in heart mitochondria *in vivo*.²⁷⁾ Thus, the effect of ADM on mitochondria in heart has been studied intensively. In 1974 Gosalvez *et al.*²⁸⁾ showed that ADM inhibited mitochondrial respiration. Iwamoto *et al.*²⁹⁾ revealed that ADM inhibited mitochondrial succinoxidase and NADH-oxidase activities from beef heart. We have also shown previously that CoQ₁₀ reduced the inhibitory effect of ADM on such CoQ-enzymes.³⁰⁾ Bertazzoli *et al.*³¹⁾ also reported upon the protective effect of CoQ₁₀ against cardiotoxicity of ADM in an isolated rabbit heart system. Further, the relation between mitochondrial injury and cardiomyopathy was emphasized by the observations that ADM produced a reduction in the cardiac store of ATP in the isolated perfused rat heart³²⁾ and that ADM associated strongly with cardiolipin, a phospholipid specific to the mitochondrial inner membrane.³³⁾ The present results also showed that ADM depresses ATP content in cultured cardiac cells (Table V). At a dose of 3.5 μM ADM, however, the decrease in ATP content was observed after the beat inhibition. Accordingly, the reduction in ATP content does not seem to explain the time lag. Antimycin A, a strong inhibitor of cytochrome b-c₁, however, also inhibited beating in advance of ATP reduction. Therefore, it is necessary to accumulate more evidence before the relationship between ADM cardiotoxicity and cellular respiration can be understood.

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