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Comparison of Chromosome Aberrations in CHL/IU and CHO-WBL Cells Treated with Mitochondrial Inhibitor, Antimycin A

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We investigated the clastogenic potential of antimycin A, which is well known as a mitochondrial inhibitor (Complex III blocker), utilizing two cultured cell lines, CHL/IU and CHO-WBL. Antimycin A induced chromosome structural aberrations in CHL cells in the presence of S9 mix. However, no chromosome structural aberrations were induced under three different treatment conditions in CHO cells with or without S9 mix. Instead, it induced polyploidy, including endoreduplicated cells, in CHO cells with or without S9 mix. By conducting the comet assay, we found that the antimycin A has no potential to induce DNA damage in these cells. Thus, the chromosome aberrations induced in these cells might be caused by non-DNA-reactive mechanism. Based on these findings, it is suggested that some of the differences between the congenital characteristics of these cells play an important role in the differences of the clastogenic response.

Key words: chromosome aberration, CHL/IU, CHO-WBL, antimycin A

Introduction

The chromosome aberration test has been widely used for evaluating the genotoxic potential of chemicals and has been designated as one of the standard batteries for many registration guidelines in the world. However, the results of the tests seem to differ among laboratories where various kinds of cell lines are used. Some elaborate works have been done to elucidate the factors which contribute to these differences, using CHL/IU and CHO-WBL cells (1,2). It was concluded that differences in the test protocols, such as the sampling time and treatment time, are the main factors affecting the discrepancies of the results, and negative results for some chemicals in the test using CHO-WBL cells turned to positive when using the same protocol with CHL/IU cells. However, the study was performed with a very limited set of chemicals.

Antimycin A is well known as a mitochondrial inhibitor (Complex III blocker) (3,4), and carcinogenicity of

this compound is unknown. It is not mutagenic in Ames test (unpublished results, by Izumi Ogawa), nor in mouse lymphoma *Tk* assay (5). However, no reports are available about the clastogenicity in CHL/IU or CHO-WBL cells for this compound. So, in the present study, we conducted chromosome aberration tests using these two cell lines and found that the results were different between these two cell lines. We examined whether these differences were caused by different test conditions, or by different congenital characteristics of these cells, using various test protocols. In addition, we investigated whether antimycin A has potential to directly interact with DNA by using the comet assay to assess the genotoxicity of this compound.

Materials and Methods

Chemicals: Antimycin A (Cas No.: 1397-94-0) was purchased from Sigma-Aldrich Chemicals Inc. (St. Louis, USA). Dimethyl sulfoxide (DMSO) and demecolcine (hanks' balanced solution, 10 µg/mL) were purchased from Wako Ltd. (Osaka, Japan). Regular (GP-42) agarose and low melting point (LGT) agarose were purchased from Nacalai Tesque Co., Ltd., (Kyoto, Japan).

Cell lines and their treatments with chemicals: CHL/IU cells were originally established from the lung of a female newborn Chinese hamster. The cells were cloned by Dr. M. Ishidate Jr and Dr. Udaoki and maintained at the National Institute of Health Sciences, Tokyo, Japan (NIHS). We obtained the cells from NIHS in 1985. Since then, the cells have been maintained and re-cloned several times. The chromosome modal number was 25 and the doubling time was about 14 h. Cultures were grown in Eagle's minimal essential medium (MEM) (Nissui Pharmaceutical Co., Ltd.

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Table 1. Protocols for chromosome aberration test

	CHL/IU	CHO-WBL		
Culture medium	Eagle's MEM, 10% calf serum	McCoy's 5A 10% fetal calf serum		
		Protocol I	Protocol II	Protocol III
Treatment medium	Eagle's MEM 10% calf serum	McCoy's 5A without serum	McCoy's 5A 10% fetal calf serum	McCoy's 5A 10% fetal calf serum
Treatment/recovery time (h)	6/18	3/23	6/18	24/0
S9 mix (%)	16.7	5.0	16.7	—
Composition of S9 mix	S9, 30%: HEPES, 0.67 mM: KCl, 5.5 mM: MgCl ₂ , 1.33 mM: G-6-P, 0.83 mM: NADPH, 0.67 mM: NADH, 0.67 mM	S9, 40%: KCl, 1.5 mM: G-6-P, 5.9 mM: NADP ⁺ , 0.34 mM	Same as those for CHL	—

Tokyo, Japan) supplemented with 10% bovine calf serum (Morgate Co., Ltd. Brisbane, Australia). The CHO-WBL cell line was originally cultured from the ovary of a Chinese hamster and cloned in the laboratory of Dr. A. Bloom, Columbia University, and re-cloned in the laboratory of Dr. S. Galloway, Litton Bionetics, Inc., USA (2). We obtained this cell line from the Hatano Research Institute Food and Drug Safety Center in 1999. Cultures were grown in McCoy's 5A with L-glutamine, 10% fetal bovine serum (Morgate Co., Ltd., Brisbane, Australia), penicillin (100 U/mL) and streptomycin (100 µg/mL). The chromosome modal number was 21 and doubling time was about 14 h.

For treatment of cells with test chemicals, their DMSO solutions were added at the amount of 1% of medium volume.

Chromosome aberration tests in CHL cells: Two days before the treatment, cell suspension was plated at $4.8 \times 10^4/4$ mL in 60 mm disposable plastic dishes. On the treatment of the cells in logarithmic phase, medium was replaced by fresh MEM medium with calf serum, with or without S9 mix (Table 1).

Chromosome aberration tests in CHO cells: A day before the treatment, cell suspension was plated at $2 \times 10^5/4$ mL in 60 mm disposable plastic dishes. On the treatment of the cells in logarithmic phase, medium was replaced by fresh McCoy's 5A medium. For antimycin treatment, we used three protocols, protocol I, II and III. Protocol I is same as the standard protocol for CHO except for extension of recovery time. In protocol II, the treatment time, and composition and concentration in the treatment medium of S9 mix were modified to that used for CHL cells. In protocol III, continuous treatment (24 h) was conducted using McCoy's 5A medium with 10% fetal calf serum. The major differences in the experimental protocols are summarized in

Table 1. S9 purchased from Oriental Yeast Co. Ltd (Tokyo, Japan) was prepared from the livers of male Sprague-Dawley rats treated with phenobarbital and 5,6-benzoflavone. The protein concentration was 30 mg/mL. Maximum treatment dose for antimycin A was selected at below 50% cell confluence measured by counting the tripan blue excluded viable cells when harvesting. In all the tests, demecolcine (0.2 µg/mL in final concentration) was added to the culture medium 2 h before harvesting. The cells were harvested using the trypsin method, and the cytotoxicity was evaluated as the relative cell growth by counting the viable cells in the cell suspension that was sampled from each dish. In protocol II without S9 mix, the toxicity was demonstrated by the relative mitotic index as well as the relative cell growth because some cell cycle delays seemed to occur. All treatments were performed using duplicated dishes. Chromosome preparations were made using the conventional air-drying technique. The frequencies of structural aberration and polyploidy in the treated cells were compared with the concurrent solvent control (100 cells per dish). If the aberrations significantly increased, as assessed by Fisher's exact test ($p < 0.05$), without steep and heavy toxicity, the result was considered positive.

Comet assay: The composition and concentration in the treatment medium of S9 mix were the same as those used in the chromosome aberration test for CHL cells. In addition, the treatment medium contained the serum that was used for the respective cell cultures. The maximum treatment dose was selected by taking into consideration the result of the chromosome aberration test, as well as recommendation by IWGT that the dose of 70–80% cell confluence should be the maximum (6). After 3 h treatment, cells were harvested by exposure to trypsin-EDTA solution (trypsin 0.05%, EDTA 4Na 1 mg/mL) for 3 min followed by gentle pipetting. Immediately after, the cells were layered in the agarose

Table 2. Chromosome aberrations induced by antimycin A in CHL and CHO cells

Cell	Compound	Concentration μg/mL	S9 mix	Frequency of cells with structural aberrations (%)							Frequency of polyploid cell (%)*	Relative cell growth (%)†		
				gap	ctb	cte	csb	cse	oth	total (– gap)				
CHL	Antimycin A	0	+	0.0	0.5	0.0	0.0	0.0	0.0	0.5	0.5	100		
		30	+	0.0	1.0	1.0	0.0	0.0	0.0	2.0	1.0	67		
		40	+	3.0	0.5	2.0	0.0	0.0	0.0	2.5	0.0	58		
		50	+	2.5	5.0	11.5	1.0	0.0	0.0	15.0‡	0.0	48		
	B(a)P	10	+	0.0	14.0	12.0	0.0	0.0	0.0	34.0‡	0.0	66		
	Antimycin A	0	–	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	100		
		0.625	–	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	67		
		1.25	–	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	58		
		2.5	–	0.0	1.0	0.0	0.0	0.0	0.0	1.0	0.0	43		
	MMC	0.1	–	0.0	8.0	48.0	0.0	0.0	0.0	56.0‡	0.5	94		
	CHO	Protocol I	Antimycin A	0	+	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100
				10	+	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.5 (3.0)‡	62
20				+	0.0	0.0	0.0	0.5	0.0	0.0	0.5	3.5 (2.0)§	51	
40				+	0.0	0.5	4.0	0.0	0.0	0.0	4.0§	0.0	25 \$	
CP			3	+	0.0	10.0	72.0	18.0	0.0	0.0	90.0‡	0.0	51	
Antimycin A			0	–	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100	
			0.25	–	0.0	0.0	0.0	0.0	1.0	0.0	1.0	1.0 (0.5)	73	
			0.5	–	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0 (0.5)	47	
MMC			0.1	–	0.0	16.0	38.0	4.0	0.0	0.0	54.0‡	2.0 (2.0)	58	
Protocol II		Antimycin A	0	+	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.0	100	
			10	+	1.0	0.0	0.0	0.0	1.0	0.0	1.0	6.0 (1.0)	59	
			20	+	0.0	0.0	0.0	0.0	0.0	0.0	0.0	9.0 (4.0)‡	50	
			40	+	1.0	0.0	1.0	0.0	0.0	0.0	1.0	5.0 (2.5)	50	
		B(a)P	10	+	0.0	16.0	18.0	2.0	4.0	0.0	34.0‡	1.0	45	
		Antimycin A	0	–	0.0	0.0	0.0	0.0	1.0	0.0	1.0	0.5	100	
			0.5	–	0.0	1.0	0.0	0.0	0.0	0.0	1.0	9.0 (0.5)‡	63 (68)	
			1	–	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.0§	61 (35)	
			2	–	0.0	0.0	1.0	0.0	0.0	0.0	1.0	3.5	65 (33)	
		MMC	0.1	–	2.0	12.0	40.0	0.0	0.0	0.0	50.0‡	4.0	66 (84)	
Protocol III		Antimycin A	0	–	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.0	100	
			0.125	–	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.5	56	
			2	–	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.0	48	
		MMC	0.03	–	0.0	12.0	24.0	2.0	2.0	0.0	42.0‡	1.0	67	

ctb: Chromatid break cte: Chromatid exchange csb: Chromosome break cse: Chromosome exchange oth: others

CP: cyclophosphamide MMC: mitomycin C B(a)P; 3,4-benzopyrene.

^{*}: The values in parentheses show percentage of endoreduplicated cells.

[†]: The values in parentheses show relative mitotic index.

‡, §: Significantly different from the control (0 concentration) at the $p < 0.01$, $p < 0.05$, respectively (Fisher's exact test).

||: Positive control.

\$: Heavy toxicity.

slides. The cytotoxicity was evaluated by counting the viable cells in the cell suspension, which was sampled from each dish. We followed Dr. Sasaki's method for preparing the comet slides (7,8). The slides were placed in a chilled lysing solution (pH 10) and kept at 0°C in the dark for 60 min to lyse the cell/nucleus membrane, and subsequently placed on a horizontal gel electrophoresis platform, covered with a chilled alkaline solution (pH 13), and left in the dark at 4°C for 20 min to unwind DNA strands. The DNA underwent electrophoresis for 30 min at 1 V/cm (250–300 mA). The slides were gently rinsed with 400 mM Trizma-HCl (pH 7.5) to

neutralize the excess alkali. Each slide was stained with 50 μL of ethidium bromide solution (20 $\mu\text{g/mL}$). Fifty nuclei per dish were examined at $\times 200$ magnification using a fluorescence microscope. The comet images were scanned into an image analyzer (SCG32; Keiotoshi Industries, Ltd., Tokyo, Japan) to measure the tail moment. The average DNA tail moment of the treatment group was compared with the concurrent solvent control by Dunnett's multiple comparison test.

Table 3. Tail moment induced by antimycin A

Cell	Compound	Concentrations ($\mu\text{g/mL}$)	S9 mix	Tail moment	Relative cell growth
				Mean $\pm$ SEM	
CHL	Antimycin A	0	+	0.25 ± 0.035	100
		6.25	+	0.28 ± 0.080	102
		12.5	+	0.30 ± 0.040	75
		25.0	+	0.19 ± 0.030	83
		50	+	0.32 ± 0.030	68
	B(a)P*	30	+	$0.66 \pm 0.065^\dagger$	102
CHO	Antimycin A	0	+	0.17 ± 0.050	100
		62.5	+	0.14 ± 0.045	100
		125	+	0.11 ± 0.000	95
		250	+	0.29 ± 0.090	14§
	B(a)P*	30	+	$0.73 \pm 0.015^\dagger$	94
	Antimycin A	0	—	0.11 ± 0.005	100
		0.5	—	0.12 ± 0.015	84
		1.0	—	0.11 ± 0.015	64
		2.5	—	0.14 ± 0.010	52§
	MMS*	80	—	$1.26 \pm 0.060^\dagger$	79

B(a)P: 3,4-benzopyrene MMS: methyl methanesulfonate.

*: Positive control.

 $^\dagger, \ddagger$: Significantly different from the control (0 concentration) at the $p < 0.01$, $p < 0.05$, respectively (student's t test).

§: Heavy toxicity.

Results

Induction of chromosome structural aberrations in CHL cells: The structural aberration frequency increased by 15% when the cells were treated with $50 \mu\text{g/mL}$ antimycin A, in the presence of S9 mix. However, there was no increase in aberration frequency with any doses without S9 mix. Polyploidy did not increase significantly at any doses with or without S9 mix (Table 2).

Induction of polyploidy but no structural aberrations in CHL cells: Although we have routinely used 17 h as the recovery time for the conventional CHO protocol, severe mitotic delays were seen in the cells treated with antimycin A after 17 h (data not shown). Therefore, we delayed harvesting until 23 h after treatment in protocol I. By protocol I, structural aberration frequencies did not increase significantly at any doses with or without S9 mix, except the highly toxic dose (relative cell growth of 25%) where the frequencies were slightly increased by 4%. The frequency of polyploidy increased significantly when S9 mix was present, by 4.5% and 3.5% at 10 and $20 \mu\text{g/mL}$ antimycin A, respectively, at which moderate cytotoxicity appeared. More than half of these cells with polyploidy were endoreduplicated cells (Table 2).

In protocol II, structural aberration frequencies did not increase significantly at any doses either with or without S9 mix. The frequencies of polyploidy increased significantly in the treatments with or without S9 mix. With S9 mix, the frequency of polyploidy reached 9.0%

at $20 \mu\text{g/mL}$ antimycin A. These polyploid cells also contained some amount of endoreduplicated cells. Without S9 mix, the frequency of polyploidy also increased by 9% at $0.5 \mu\text{g/mL}$. However, these cells contained only 0.5% of endoreduplicated cells. In the treatment without S9 mix, the toxicity demonstrated by the relative mitotic index was higher than that demonstrated by the relative cell growth, which revealed that some cell cycle delays seemed to occur during the treatment (Table 2).

No increases in the frequencies of cells with structural aberration nor polyploidy were shown by protocol III (Table 2).

It was concluded that antimycin A induced polyploidy, but not structural aberrations, in CHO cells.

Comet assay: Comet assays were performed to clarify whether chromosome aberrations were results of DNA-reactivity of antimycin A or not. Tail moment in both CHL and CHO cells did not show any significant increase at any doses of antimycin A, although positive control, B[a]P and MMS gave higher tail moment values than those of controls (Table 3).

Discussion

We found that antimycin A induced chromosome structural aberration in CHL cells but did not in CHO cells, under the conditions widely used for CHO (protocol I). However, antimycin A induced polyploidy, including endoreduplication, in CHO cells but did not in CHL cells. These differences between these two cell

lines were also seen in protocol II for CHO cells, with the same conditions as those for CHL cells, such as longer treatment time, different sampling time and a higher concentration of S9 than those in protocol I, as routinely used in European countries and the USA. Therefore, it can be considered that the differences in the responses of chromosome aberration stem from some congenital differences between these cells. Moreover, considering the fact that antimycin A gave a negative result in the Ames test and mouse lymphoma *Tk* assay, as well as the comet assay conducted in the present study, chromosome aberrations induced by antimycin A do not seem to be caused by direct interaction of antimycin A with DNA.

Our results showed that chromosome structural aberration in CHL cells appeared with increasing cytotoxicity, which seemed to have a threshold of cytotoxicity accompanied by clastogenicity. It has been reported that chromosome aberrations that are observed only above a certain threshold dose, associated with cytotoxicity, could be caused by a non-DNA-reactive mechanism, which was not expected to follow single hit linear kinetics, as would be observed with DNA-reactive chemicals (9). However, at present the reason why structural aberrations were not induced in CHL cells without S9 mix is not known.

In CHO cells, it could be difficult to attribute the mechanism of polyploidy formation to a simple spindle poison. If antimycin A works as a typical spindle poison, polyploidy would also appear in CHL cells. However, there was no induction of polyploidy in CHL cells, and almost half of the polyploid cells were endoreduplicated cells in CHO cells. The mechanism of endoreduplication, a result of the occurrence of two successive rounds of DNA replication without intervening mitosis, is poorly understood. Recently, there have been some reports that this mechanism is related to topoisomerase II, an enzyme capable of decatenating replicated DNA strands after cell replication (10,11). When this enzyme malfunctions, it might be possible for some cells to skip anaphase and initiate a new cell cycle, leading to a further round of replication (12). Considering the result that the endoreduplication did not appear in CHL cells in this study, it can be speculated that there are some differences between these cells with regard to topoisomerase II activity. However, further work will be required to clarify this.

As discussed above, the chromosome aberrations induced in these cells may be derived from a non-DNA-reactive mechanism, such as DNA synthesis, protein synthesis or a cell cycle disorder, which is related to a certain enzymes.

Many generally-known DNA-reactive compounds used as positive controls in the chromosome aberration tests express equivalent positive responses in both cells.

In addition, nine derivatives of phenylenediamine or chlorophenol, which are mutagens or tumorigens, were reported to turn out indistinguishable positive when tested using the same protocol for the CHL and CHO cell systems (1,2). These facts also imply the prediction that the diverse responses of antimycin A for two cell lines might come from the non-DNA-reactive mechanism on the clastogenicity.

Antimycin A, a mitochondrial inhibitor, is known to interfere with ATP production, which means that non-specific inhibition of the activities of ATP-dependent enzymes could occur at any time and place. This inhibition might be related to the non-DNA-reactive mechanism for inducing chromosome aberration. It was reported that CHO-WBL cells have mutant *p53* with mutant function and CHL cells have wild type *p53* associated with abnormal function, which leads to a lack of G1 check point or G1 delay in their cell cycles (13). It is speculated that, in such cells with poor control of the cell cycle, the ATP-dependent enzyme disorders attributable to energy depletion would be enhanced much more than in normal cells.

It can be noticed that antimycin A seems to be detoxified because the concentrations in the treatment with S9 mix were higher than those without S9 mix, however, how the metabolized form is related to the expression of the clastogenicity is unknown.

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