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Aneuploidy induction by benzo[*a*]pyrene and polyploidy induction by 7,12-dimethylbenz[*a*]anthracene in Chinese hamster cell lines V79-MZ and V79

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We found that different ploidy effects were induced in four Chinese hamster-derived cell lines (V79-MZ, V79, CHL and CHO-K1) treated through two cell cycles with polycyclic aromatic hydrocarbons in the absence of a metabolic activation system. 5-Bromodeoxyuridine was used to investigate cell cycle delay and sister chromatid exchanges (SCE) induced by the chemicals. Benzo[*a*]pyrene (BP) induced aneuploidy at 2.5–10 μg/ml in V79-MZ cells. 7,12-Dimethylbenz[*a*]anthracene (DMBA) induced polyploidy at 3.125–6.25 and 6.25–12.5 μg/ml in V79-MZ and V79 cells respectively. Higher concentrations caused cell cycle delay and, therefore, did not affect ploidy. BP and DMBA did not induce a significant increase in SCE frequency at the above doses. 3-Methylcholanthrene tested up to its solubility limit (10 μg/ml) did not induce numerical aberrations in any cell line. The clastogen mitomycin C, tested up to 0.01 μg/ml, did not produce numerical aberrations but did significantly increase SCE frequency in all cell lines. The spindle poison colchicine, tested up to 0.1 μg/ml, induced ploidy changes in the four cell lines that showed different sensitivities. Four cell lines showed no arylhydrocarbon hydroxylase activity, and V79-MZ, but not the other cells lines, showed high glutathione *S*-transferase activity. Aneuploidy induction by BP and polyploidy induction by DMBA in the absence of S9 mix *in vitro* have not been described before, and the finding might be due to the effect on tubulin. Due to their specificity and high sensitivity, the V79-MZ and V79 cell lines might be good systems for detecting aneuploidogens.

Introduction

A variety of mammalian cell lines have been used in gene mutation assays (Aaron *et al.*, 1994; Glatt, 1994), chromosomal aberration tests (Galloway *et al.*, 1987; Ishidate *et al.*, 1988) and other *in vitro* cytogenetic assays (Galloway *et al.*, 1985). In general, an exogenous metabolic activation system is needed because the established cell lines do not adequately convert indirect mutagens to direct mutagens. In such cell lines, benzo[*a*]pyrene (BP) and 7,12-dimethylbenz[*a*]anthracene (DMBA) are genotoxic only in the presence of an exogenous metabolic activation system.

Aneuploidy has been implicated in sterility, abortions, still births, congenital abnormalities and carcinogenesis (Barrett *et al.*, 1985; Oshimura and Barrett, 1986). The EC Aneuploidy Project reported on the detection of aneuploidogens in a special issue of *Mutation Research* (Parry and Natarajan, 1993). Many reports have been published on the mechanisms of action of potential aneuploidogens and the establishment of methods for detecting and assessing chemicals capable of inducing numerical chromosome aberrations (Galloway and Ivett, 1986; Önfelt, 1986, 1987a,b; Oshimura and Barrett, 1986; De Ferrari *et al.*, 1988; Önfelt *et al.*, 1992; Surrallés *et al.*, 1996; Parry *et al.*, 1996). However, test systems for detecting aneuploidy are not well established.

While investigating the usefulness of genetically engineered cell lines expressing human CYPs as a tool for screening genotoxic chemicals in the *in vitro* chromosomal aberration test, we accidentally found that BP induced aneuploidy in the cell line expressing human CYP2E1. To confirm that the effect was due to the expression of CYP2E1 in the cell line, we studied the effect of BP in the parental cell line, V79-MZ. Finally, as shown in the present study, we found that BP and DMBA induced aneuploidy and polyploidy respectively, without an exogenous metabolic activation system. Jensen *et al.* (1993) reported that BP showed a statistically significant increase in the frequency of c-mitoses at 10^{-6} M in V79 cells in the absence of S9 mix. Ellard *et al.* (1991) reported that BP elevated the ratio of kinetochore-positive micronuclei in the *in vitro* micronucleus test with V79 cells in the presence of S9 mix. We showed direct aneuploidy induction by BP in the absence of S9 mix in the present study.

Materials and methods

Cells

All cell lines were established from Chinese hamster. V79-MZ cells were subcloned by Glatt *et al.* (1987) from V79 cells supplied by Arlett (MRC Cell Mutation Unit, University of Sussex, UK). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco 31600-034, Grand Island, NY) supplemented with 5% fetal bovine serum (Flow Laboratories, Rockville, MD). The doubling time was ~12 h and the modal chromosome number was 22.

V79 cells, established by Elkind and Sutton (1960), were supplied from Japan Cell Resources Bank (JCRB0603; Tokyo, Japan). They were maintained in the same medium as V79-MZ cells. The doubling time was ~12 h and the modal chromosome number was 22.

CHL cells were established from the lung of a female newborn Chinese hamster by Koyama *et al.* (1970) and cloned by Ishidate and Odashima (1977). The cells were maintained in Eagle's minimum essential medium (MEM; Gibco 61100-061) supplemented with 10% heat-inactivated calf serum. The doubling time of the cells was ~15 h and the modal chromosome number was 25.

CHO-K1 cells established by Kao and Puck (1967) were obtained from American Type Culture Collection (CCL 61) and maintained in F-12 nutrient mixture (Ham's F-12; Gibco 21700-075) supplemented with 10% fetal bovine serum. The doubling time was ~13 h and the modal chromosome number was 20.

Chemicals

BP (CAS No. 50-32-8), DMBA (57-97-6) and 3-methylcholanthrene (3-MC; 56-49-5) were purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan) and dissolved in dimethyl sulfoxide (DMSO). Mitomycin C (MMC; 50-07-7), purchased from Kyowa Hakko Kogyo Co. (Tokyo, Japan) and colchicine (COL; 64-86-8) from E.Merck (Darmstadt, Germany), were dissolved in physiological saline.

Chemical treatment

Cells were seeded at a density of 1.5×10^5/plate (60 mm in diameter), two plates/dose. After 17 h, they were treated with the test chemical for

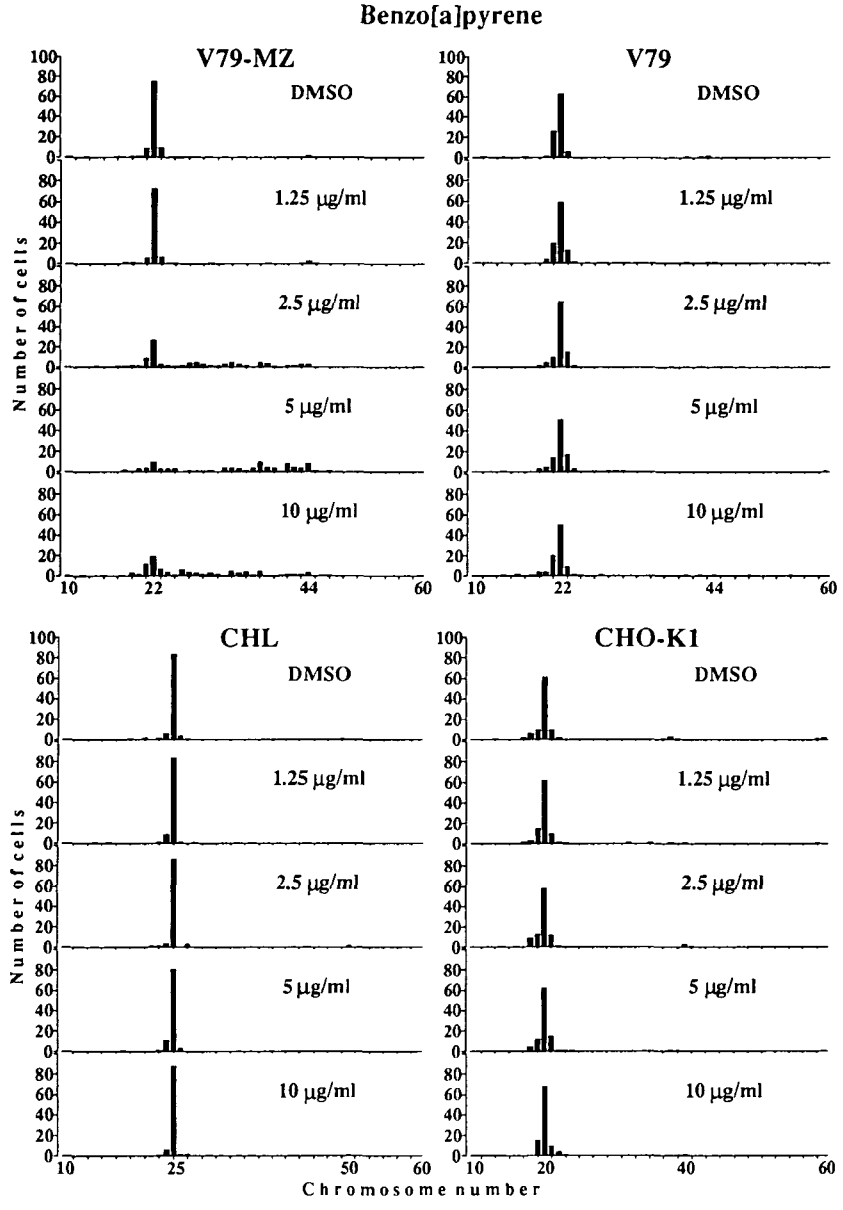

Fig. 1. Distribution of chromosome number in cells treated with benzo[*a*]pyrene.

approximately two cell cycles (24 h for V79-MZ and V79 cells, 26 h for CHO-K1 cells and 30 h for CHL cells) and harvested immediately. 5-Bromodeoxyuridine was added to one of the duplicate plates to a final concentration of 5 μM just after addition of the test chemical.

Slide preparation

Chromosome preparations were made as follows: colcemid (final concentration 0.2 μg/ml) was added to the culture 2 h before harvesting. The cells were trypsinized and incubated in hypotonic solution for 20 min at room temperature. The cells were fixed three times with ice-cold fixative (glacial acetic acid: methanol, 1:3). A drop of the fixed cell suspension was placed on a clean glass slide and air-dried. The cells were stained with Giemsa solution for chromosome counting.

A fluorescence-plus-Giemsa (FPG) technique (Perry and Wolff, 1974) was used for sister chromatid differentiation (SCD) staining. The slides were stained for 20 min with Hoechst 33258 (5 μg/ml), mounted in 2× sodium chloride/sodium citrate (SSC), and exposed to 'blacklight' (15W, FL 15 BL-B, National, Osaka, Japan) for 20 min at room temperature. The slides were warmed at 50°C for 20 min. Finally, the cells were stained with Giemsa and air-dried.

All experiments were performed at least twice, and all slides were coded.

Chromosome counting

Chromosome number was counted for 100 metaphases that had ≥10 chromosomes. Chromosome counting was shared between three scorers. Two scorers counted 33 metaphases each and the other scorer counted 34 metaphases. Solvent-treated cells served as a negative control.

Scoring the second metaphase cells and sister chromatid exchanges per cell

The number of second metaphase (M2) cells showing differentially stained sister chromatids was scored on 100 metaphases. Sister chromatid exchanges (SCEs) were scored on 25 metaphases in cells showing the modal chromosome number (*m* cells). Centromeric SCEs were indistinguishable from centric twists and were not scored. Solvent-treated cells served as a negative control. SCE data were statistically analysed using the Mann–Whitney *U*-test (the normal, two-tailed version).

Activity of arylhydrocarbon hydroxylase and glutathione S-transferase

Arylhydrocarbon hydroxylase (AHH) activity was determined by modification of the method of Watanabe and Konno (1975) using BP as substrate. Glutathione *S*-transferase (GST) activity was measured spectrophotometrically using 1-chloro-2,4-dinitrobenzene as substrate (Habig *et al.*, 1974). The activities were measured in sonicated cell preparations. The liver S9 fraction prepared from polychlorinated biphenyl (Kanechlor-500) treated BALB/c mice was used as a positive control for enzyme activity.

Results

The distribution of chromosome number in the treated cells are shown in Figures 1–6. The data were reproducible except

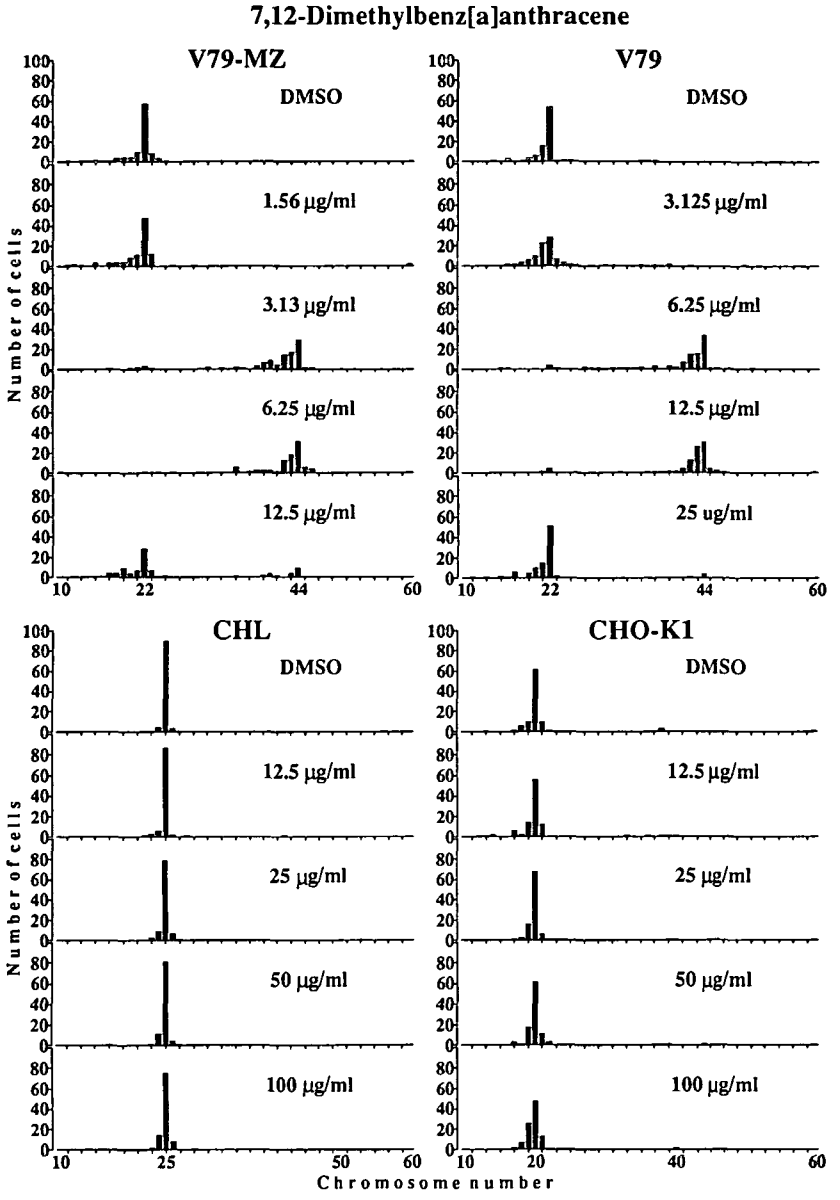

Fig. 2. Distribution of chromosome number in cells treated with 7,12-dimethylbenz[*a*]anthracene.

for COL treatment in CHL cells. Data from a representative experiment are shown in Figures 1–4. In Figure 5, four cell lines were treated with COL at the same time. The average frequency of *m* cells in the solvent controls was 56.3% for V79-MZ cells, 50.2% for V79 cells, 86.7% for CHL cells and 62.6% for CHO-K1 cells, during the present study.

The frequency of M2 cells and the mean number of SCE per cell after treatment are shown in Table I. The data were obtained at the same time as the data shown in Figures 1–5. Baseline SCE frequencies for V79-MZ, V79, CHL and CHO-K1 cells were 5.21 ± 2.32 (range 1–11), 7.98 ± 3.03 (range 1–22), 10.1 ± 3.77 (range 3–20) and 10.32 ± 3.14 (range 4–20) per cell respectively.

BP decreased the frequency of *m* cells from 75 to 27% at 2.5 µg/ml and to 10% at 5 µg/ml in V79-MZ cells (Figure 1). The distribution of chromosome number spread broadly from diploid to tetraploid range at those doses. Thus, we concluded that BP induced aneuploidy in V79-MZ cells. No remarkable changes, however, were observed in the distribution of chromosome number in the other cell lines similarly treated. Any decrease in the frequency of M2 cells was not shown up to the highest dose of 10 µg/ml (near the solubility limit) in CHL and CHO-K1 cells (Table I). However, a statistically significant increase in SCE per cell was observed in CHL cells.

DMBA clearly shifted the distribution of chromosome number to tetraploid in V79-MZ cells at 3.125–6.25 µg/ml and in V79 cells at 6.25–12.5 µg/ml (Figure 2). The distribution, however, returned to diploid range at the highest doses in both cell lines. As shown in Table I, no M2 cells were apparent at the highest doses in those cell lines, indicating that they were arrested at the first metaphase. DMBA did not change the distribution of chromosome number in CHL and CHO-K1 cells at any dose that still permitted M2 cells. SCEs per cell increased with statistical significance at 12.5 and 25 µg/ml in both CHL and CHO-K1 cells.

3-MC administered up to its solubility limit (10 µg/ml) did not affect the distribution of chromosome number in any cell line tested except V79-MZ cells, in which a small increase of

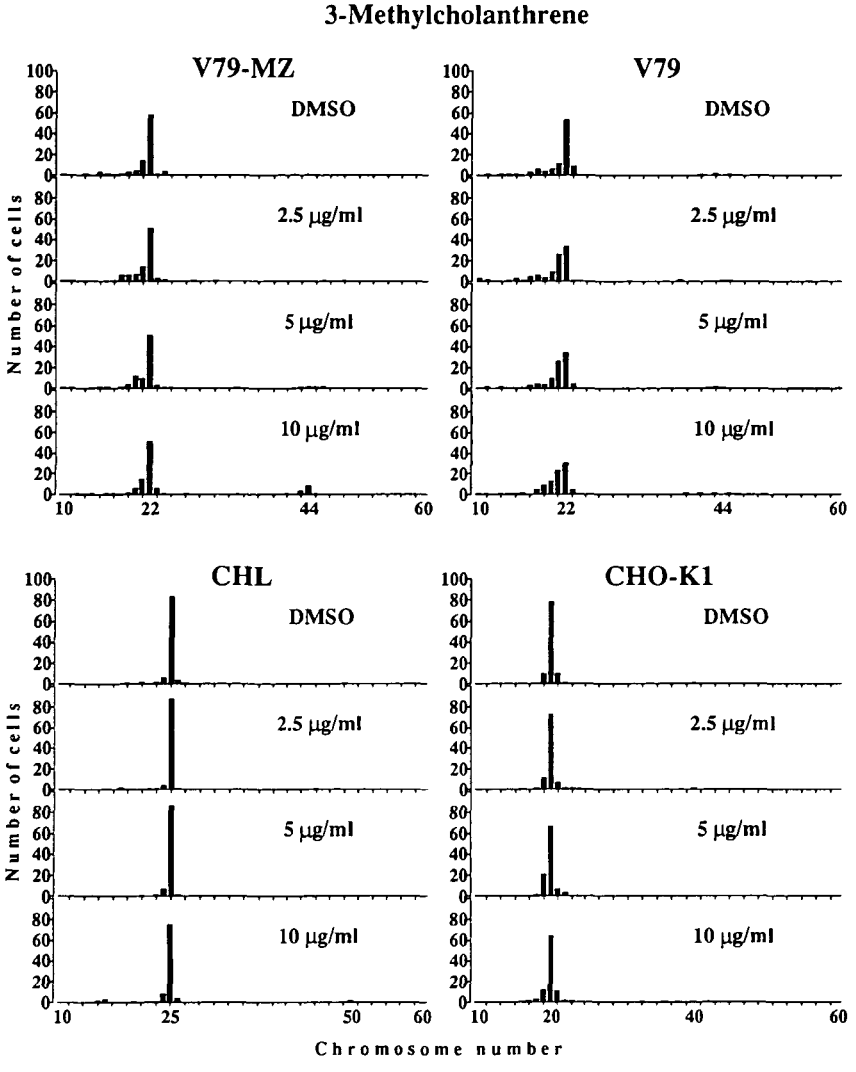

Fig. 3. Distribution of chromosome number in cells treated with 3-methylcholanthrene.

tetraploids was observed at 10 μg/ml (Figure 3). The frequency of M2 cells did not decrease, but SCEs per cell showed a statistically significant increase in V79, CHL and CHO-K1 cells (Table I).

MMC up to 0.01 μg/ml (the highest concentration tested as higher concentrations cause structural aberrations, which interfere with chromosome counting) did not induce any numerical aberrations in any of the cell lines tested. It did induce a high SCE frequency at the highest dose in all cell lines, indicating that MMC was incorporated into the cells and interacted with DNA.

COL changed the distribution of chromosome number from the diploid to the tetraploid range in all cell lines, except for CHL cells (Figure 5). In another experiment, CHL cells showed a major shift of the chromosome distribution peak to tetraploid (Figure 6). The frequency of M2 cells decreased with dose in the same order as in polyploidy induction, but number of SCEs/cell did not increase. The M2 frequency was 14% at the highest dose in CHL cells in the experiment that showed the ploidy shift (Figure 6). V79-MZ cells were the most sensitive to polyploid induction.

Relatively high frequencies of M2 cells, 72 and 60% respectively, were shown following DMBA- and COL-treatment in V79 cells. Since a large portion of the SCD cells consisted of $2m$ cells, SCEs could not be counted.

Activity of AHH and GST

To search for the reason for the difference among cell lines in numerical aberration induction by BP and DMBA, we measured AHH and GST activity. AHH activity was below the detection limit in all cell lines. GST activity was detected in V79-MZ cells, and was equivalent to 13.5% of mouse S9, confirming the high GST activity of V79-MZ cells previously reported by Glatt *et al.* (1987). GST activity was not detected in the other cell lines.

Discussion

Aneuploidy induction was discussed with respect to carcinogenesis by Oshimura and Barrett (1986), who recommended that the cells selected for detecting aneuploidogens should have four properties: (i) easily distinguishable chromosomes; (ii) a stable karyotype; (iii) a short generation time; and (iv) a high cloning efficiency. The cells used in this study satisfied those properties. Since changes in the distribution of chromosome number may occur during passage *in vitro*, established cell lines are considered to be unsuitable for the detection of

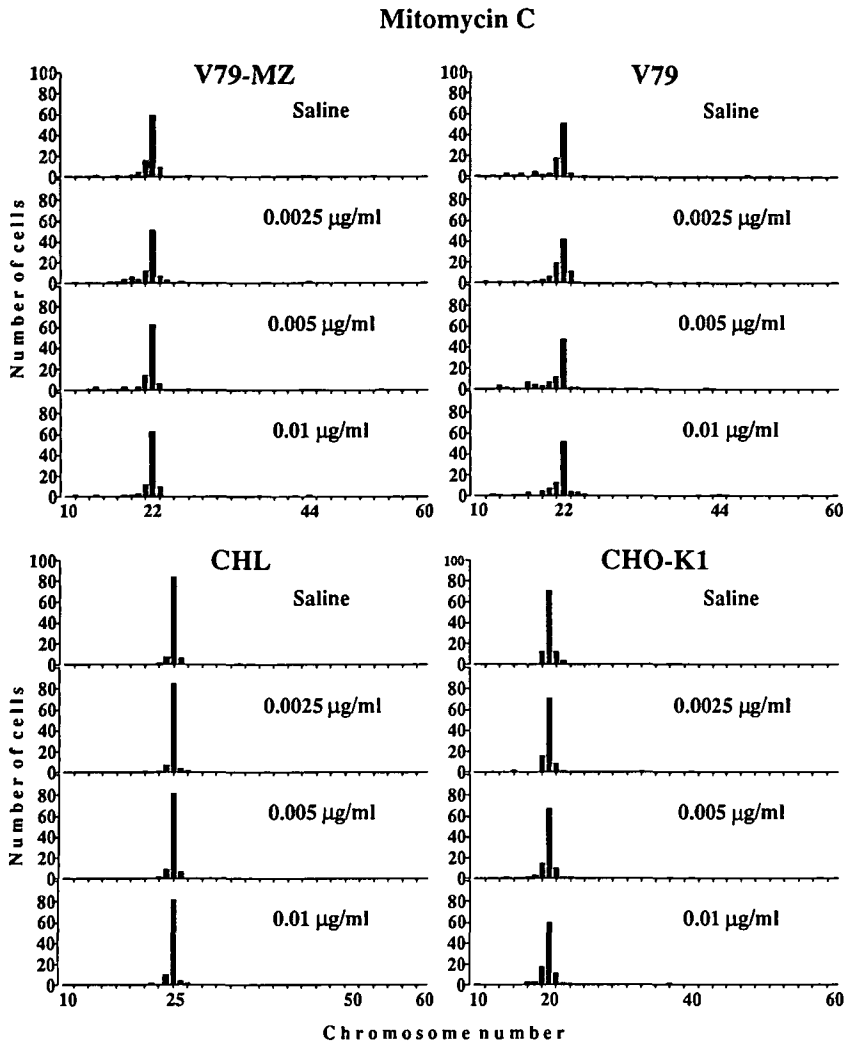

Fig. 4. Distribution of chromosome number in cells treated with mitomycin C.

aneuploidy and so aneuploidy induction has generally been estimated from observations of polyploidy. However, we have demonstrated in this study that it is possible to detect a chemical aneuploidogen from a change in the distribution of chromosome number, although it is difficult to distinguish the specific chromosomes relating to the aneuploidy. The mean frequencies of *m* cells in V79-MZ and V79 cells in control cultures were 56.3 and 50.2% respectively. Weak aneuploidy induction would not be detectable in cells with such a low frequency of *m* cells. Furthermore, such cells might easily undergo unspecific change if culture conditions are not optimal. Cloning of cells would be needed for further studies.

Aneuploidy and polyploidy induction coincided with a moderate to dramatic decrease in the frequency of M2 cells (Table I) in the present study. At doses where aneuploidy or polyploidy induction was observed, the frequency of M2 cells was always <73% of that in concurrent controls. This observation clearly shows the relationship between mitotic disturbance resulting in cell cycle delay, and numerical aberration induction. On the other hand, numerical aberration induction was observed independently of the SCE induction. No significant increase in SCEs per cell was observed with the chemical–cell line combination that induced numerical aberration. No numerical aberration was observed with the chemical–cell line combination that induced a significant increase in SCEs per cell. This observation indicates that numerical aberration induction did not follow from effects on DNA.

BP mutagenicity is supposed to be detectable in the cell lines only in the presence of exogenous metabolic activation. Ellard *et al.* (1991) reported aneuploidy induction by BP in V79 cells in the presence of S9 mix. Here, BP induced aneuploidy without a metabolic activation system in V79-MZ cells, which was the only cell line that showed GST activity. BP is reportedly oxidized in a pathway involving CYP1A1 to a 4,5-oxide, which undergoes glutathione conjugation catalysed by GST, or to a 7,8-diol-9,10-epoxide that binds to DNA. But V79-MZ did not show CYP1A1-catalysed AHH activity in our study, and unmetabolized BP is not a substrate for GST. It is not clear whether BP itself acted on the cell components. As well as the AHH system, prostaglandin endoperoxide synthase (PES)-dependent co-oxidation of polycyclic aromatic hydrocarbons to genotoxic forms has been reported. This has been suggested for BP and DMBA in a Chinese hamster cell line, Don 6, in SCE induction (Abe *et al.*, 1986), and for 7,8-dihydro-7,8-dihydroxybenzo[*a*]pyrene in a bacterial mutation test (Marnett *et al.*, 1978) and a cell transformation assay (Boyd *et al.*, 1982). PES may also conjugate chemicals with glutathione as has been reported for styrene (Stock *et al.*, 1986).

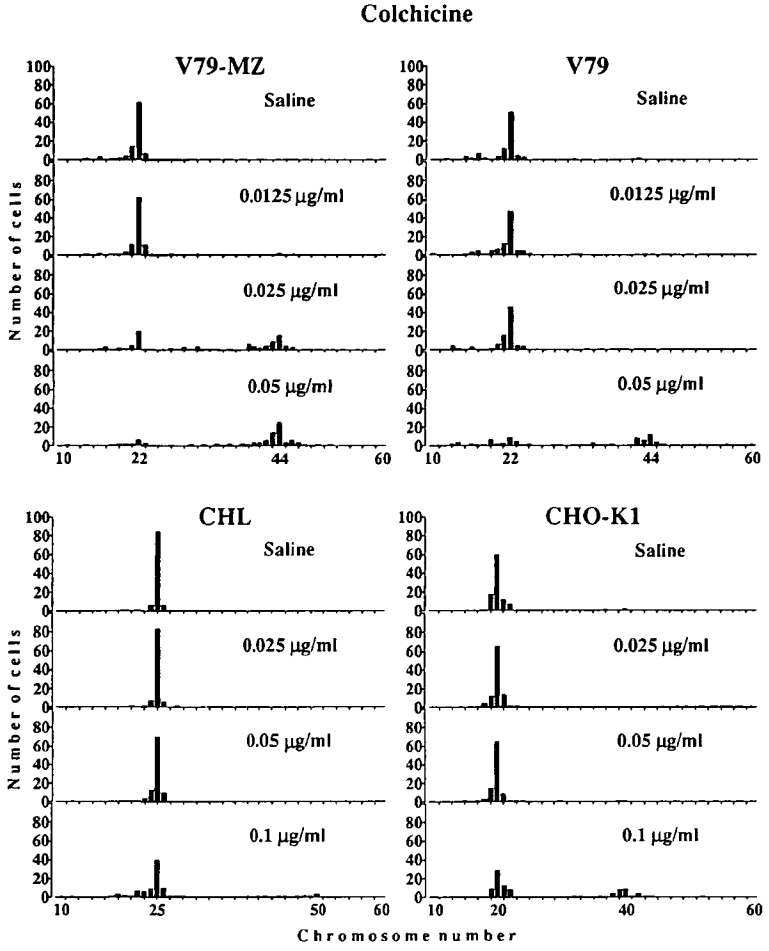

Fig. 5. Distribution of chromosome number in cells treated with colchicine.

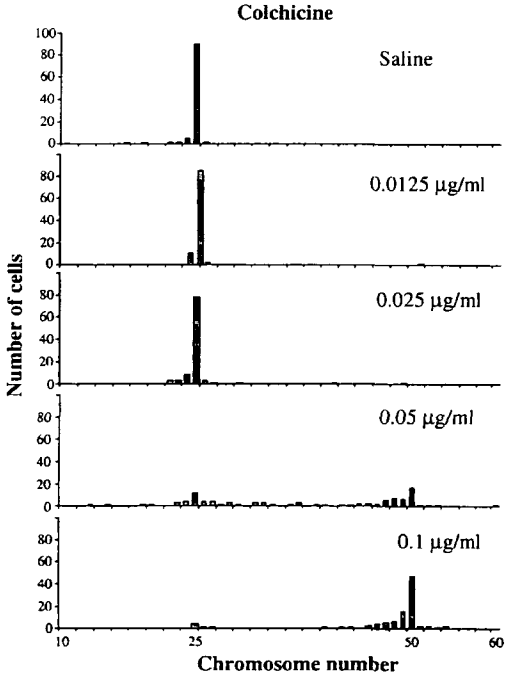

Fig. 6. Distribution of chromosome number in CHL cells treated with colchicine.

Aneuploidy induction (Liang and Brinkley, 1985; Önfelt, 1986, 1987a,b; Oshimura and Barrett, 1986; Önfelt *et al.*, 1992; Parry *et al.*, 1996) has been attributed to effects on the mitotic apparatus and to physico–chemical interactions with cellular components. In the present study, V79-MZ cells after BP treatment and V79-MZ and V79 cells after DMBA treatment that adhered to the plate became round in contrast to the fibre-like shape of the control. All cell lines did the same after COL treatment. This observation suggests that the chemicals acted on the cytoskeleton, possibly tubulin. In a preliminary micronucleus experiment with the same treatment procedure as that in the present study, a drastic increase in the number of multinucleates was observed in DMBA treatment in both V79-MZ and V79 cells (data not shown). On the other hand, a small increase in the number of multinucleates was observed in BP treatment in V79-MZ cells. These observations also support the action of the chemicals on the cytoskeleton. Furthermore, Jensen *et al.* (1993) reported that BP (10^{-6} M) was a potent inducer of c-mitoses in V79 cells. Further studies on the interactions of BP and DMBA with the tubulin of these cells are needed to explain the observed effects.

The reproducibility of the COL experiments on CHL cells was not good (Figures 5 and 6). Poor polyploidy induction was observed by chance in an experiment (Figure 5) in which four cell lines were tested simultaneously to compare their relative sensitivity to COL. At that time, a decrease in the number of *m* cells was apparent and a small number of cells with tetraploid-range chromosomes was observed in CHL cells.

Table I. Frequency of M2 cells and the mean (±SD) number of SCE per cell after treatment in four Chinese hamster cell lines

Chemical	Dose (μg/ml)	V79-MZ		V79		CHL		CHO-K1	
		M2 (%)	SCE/cell	M2 (%)	SCE/cell	M2 (%)	SCE/cell	M2 (%)	SCE/cell
BP	0 (DMSO)	93	4.32 ± 2.25	82	7.36 ± 2.58	96	8.60 ± 3.57	100	10.2 ± 3.03
	1.25	88	4.72 ± 1.99	84	8.64 ± 4.07	98	11.4 ± 6.45		ND[a]
	2.5	62	4.68 ± 2.44	78	6.92 ± 2.56	96	10.5 ± 5.29	99	11.6 ± 3.56
	5	27	NA[b]	67	7.76 ± 3.29	98	11.3 ± 4.11	98	11.8 ± 3.45
	10	45	6.24 ± 1.98	66	9.32 ± 3.20	99	14.9 ± 5.28[c]	97	11.6 ± 3.98
DMBA	0 (DMSO)	95	5.44 ± 2.43	99	8.16 ± 3.61	99	11.7 ± 3.84	100	10.2 ± 3.03
	1.56	92	5.88 ± 2.37	98	8.40 ± 3.95		NT[d]		NT
	3.125	26	NA	92	10.1 ± 2.95		NT		NT
	6.25	15	NA	72	NA		NT		NT
	12.5	0	NA	44	NA	97	16.8 ± 5.59[c]	97	15.1 ± 3.80[e]
	25		Tox[f]	0	NA	97	21.9 ± 10.2[e]	97	20.8 ± 7.21[e]
	50		NT		NT	0	NA	0	NA
	100		NT		NT	0	NA	0	NA
3-MC	0 (DMSO)	95	5.48 ± 2.65	95	7.04 ± 3.16	96	8.60 ± 3.57	98	9.92 ± 3.84
	2.5	100	6.40 ± 3.30	94	10.1 ± 4.45	97	16.6 ± 4.73[e]	99	13.2 ± 4.93
	5	95	6.08 ± 2.81	96	10.4 ± 4.54	94	15.0 ± 6.02[c]	98	14.1 ± 3.93[c]
	10	95	8.04 ± 3.48	100	11.3 ± 4.70[c]	98	18.6 ± 8.92[e]	99	13.6 ± 3.51[c]
MMC	0 (saline)	99	5.64 ± 2.41	97	7.20 ± 2.72	96	12.1 ± 3.47	100	11.0 ± 4.00
	0.0025		ND		ND		ND	97	19.3 ± 6.09[e]
	0.005		ND		ND		ND		ND
	0.01	97	19.8 ± 4.66[e]	88	35.1 ± 9.09[e]	85	39.3 ± 10.3[e]	99	37.2 ± 9.41[e]
COL	0 (saline)	88	5.20 ± 2.06	94	9.36 ± 2.66	98	9.96 ± 4.04	98	10.6 ± 3.06
	0.0125	78	4.96 ± 2.72	96	8.32 ± 3.22	96	9.40 ± 2.61	96	10.4 ± 4.07
	0.025	6	NA	95	7.68 ± 3.54	95	9.64 ± 3.26	93	10.2 ± 4.71
	0.05	5	NA	60	NA	98	10.1 ± 3.43	72	9.60 ± 2.69
	0.1		NT		NT	55	9.20 ± 3.35	4	NA

[a]ND, SCE not determined.
[b]NA; the no. SCE could not be counted because of the shortage of M2 cell in *m* cells.
[c]$P < 0.001$.
[d]Not tested.
[e]$P < 0.0001$.
[f]Cytotoxicity appeared.

V79-MZ cells differed from V79 cells in their response to BP (Figure 1). V79-MZ differed from another V79 cell line (V79-UL) with respect to the spontaneous pattern of mutations at the HPRT locus (Köberle *et al.*, 1993; Helbig *et al.*, 1994). V79-MZ and V79-UL cells, however, showed a similar response to ethyl methanesulphonate in HPRT mutations, SCE/cell, and the frequency of aberrant cells (Speit *et al.*, 1994). In the present study V79-MZ and V79 cells showed a similar response to DMBA (Figure 2). Further characterization of the cell lines and how they metabolize and/or interact with chemicals are needed to explain our results.

The chromosomal constitution of BP- and DMBA-induced mouse lymphoma (Chan *et al.*, 1979; Wiener *et al.*, 1981) and of DMBA-induced rat leukaemia (Sugiyama *et al.*, 1978) was aneuploid. In conclusion, V79-MZ and V79 cells were unique in producing numerical aberrations in response to BP and DMBA in the absence of exogenous metabolic activation. Due to their specificity and high sensitivity, both cell lines might be useful not only to detect numerical aberrations, but also to investigate mechanisms of aneuploidy induction.

Acknowledgements

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