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Induction of micronuclei in BALB/c-3T3 cells by selected chemicals and complex mixtures

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Summary

The genotoxicity of benzo[*a*]pyrene, cyclophosphamide, 2-aminoanthracene, 2-nitrofluorene, nitrosated coal-dust extracts, and cigarette-smoke condensate were tested with the micronucleus assay using an established mammalian cell line. The results showed that all chemicals and complex mixtures studied induced micronuclei in BALB/c-3T3 cells. These results indicate that BALB/c-3T3 cells are capable of activating certain promutagens and procarcinogens. It seems, therefore, that in addition to cell transformation, the micronucleus assay in BALB/c-3T3 cells without an exogenous activation system may be useful for in vitro studies to detect genotoxic chemicals and complex mixtures.

The established Chinese hamster cell line V79 has been used for in vitro micronucleus (MN) assay for the detection of cytogenetic effects induced by a variety of chemical agents. Results reported in the literature indicate that the micronucleus assay in V79 cells provides a useful short-term test for the detection of mutagens, clastogens and spindle damaging agents (Lasne et al., 1984). However, V79 cells have limited capacity for metabolic activation. Many genotoxic agents require metabolic activation to express their genotoxic potential (McCann et al., 1975). This has resulted in an activation system becoming an essential component in genetic toxicology testing. The most commonly used activation system is the liver S9 post-mitochondrial fraction from Aroclor 1254-pretreated rats (Ames et al., 1973).

It is desirable to use metabolically active target cells for short-term in vitro assay. The mouse embryo-derived BALB/c-3T3 cell line, clone A31-1-1, was first established in the laboratory of Dr. Kakunaga, Osaka University, Japan (Kakunaga, 1973). The cells are known to possess benzo[*a*]pyrene (BaP) metabolizing capacity (Schechtman, 1985). Recently, the metabolic activation ability of BALB/c-3T3 cells for 3-methyl-

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cholanthrene, 7,12-dimethylbenz[*a*]anthracene, aflatoxin, benzidine and some complex mixtures has been demonstrated in cell transformation studies (Cortesi et al., 1983; Wu et al., 1990).

The purpose of the present study was to determine whether several selected mutagens/carcinogens and complex mixtures can induce micronuclei in BALB/c-3T3 cells and to substantiate the ability of BALB/c-3T3 cells to activate indirect-acting genotoxic agents. In addition, the role of exposure time in the induction of MN was studied.

Materials and methods

Benzo[*a*]pyrene and cyclophosphamide were purchased from Sigma Chemical Co. (St. Louis, MO), 2-aminoanthracene and 2-nitrofluorene from Aldrich Chemical Company (Milwaukee, WI).

Nitrosated coal dust extracts were prepared as follows: Sub-bituminous coal dust from New Mexico was extracted first with dichloromethane and then with acetone plus methanol (1 : 1 ratio). The extracts were combined and reacted with nitrite at pH 3.5 (Whong et al., 1983).

Cigarette-smoke condensate was obtained by trapping the smoke from 4 burning cigarettes in cold acetone. The cigarettes were a commercial filtered brand. The acetone was evaporated and the residue redissolved in 1 ml dimethyl sulfoxide (Ong et al., 1986). All tested chemicals and mixtures were dissolved in dimethyl sulfoxide.

Cell cultures

The BALB/c-3T3 cell line subclone A31-1-13 used in these experiments was kindly provided by Dr. E.J. Matthews (Hazleton Laboratories, Kensington, MD). Cell stock was maintained in liquid nitrogen. Cell cultures were initiated from a stock by rapid thawing in a 37°C water bath, and grown in Eagle's Minimum Essential Medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U penicillin/ml, 100 μg streptomycin/ml at 37°C in a humidified incubator containing 5% CO_2 in air. The average number of chromosomes is 62.3 ± 9.68 per cell. The doubling time at exponential growth phase is about 19 h.

Micronucleus assay procedure

Cells were seeded into 100-mm culture dishes with a density of about 10^6 cells per dish 16–18 h before treatment. 1 day-old BALB/c-3T3 cultures were exposed to the chemicals for various lengths of time. The concentrations were selected on the basis of cytotoxicity data from literature and our previous experiments (Nachtman and Wolff, 1982; Cortesi et al., 1983; Silva et al., 1985; Wu et al., 1990). After treatment, the test chemical was removed and cells were rinsed twice with phosphate-buffered saline (PBS) and reincubated in fresh medium for an additional period dependent on experiment design. The harvested cells were subjected to hypotonic treatment with 0.075 M KCl at 37°C for 20–30 min. Cytogenetic slides were prepared, fixed with methanol, and stained with Diff-Quik Stain (Dage Diagnostics, Aguada, Puerto Rico). 2000 interphase cells with visible intact cytoplasm were scored per treatment in most experiments. The criteria used to score micronuclei were previously reported as follows: (1) staining intensity equal to that of the nucleus, (2) diameter less than one-fifth that of the nucleus, (3) location in cytoplasm, and (4) no contact with nucleus (to distinguish from nuclear blebs) (Lasne et al., 1984). The results are expressed as the mean number of cells with MN per 1000 cells ± S.D.

Statistical analysis

For a comparison of mean values, the Chi square test was used. The dose–response of BALB/c-3T3 cells to the chemicals tested was estimated using linear regression and trend test analysis.

Results

The relative frequencies of MN in BALB/c-3T3 cells induced by the chemicals or complex mixtures tested at various concentrations are presented in Tables 1–6. The p values are indicated for each group versus respective solvent control. All the chemicals and complex mixtures tested induced micronuclei in BALB/c-3T3 cells. For BaP, cyclophosphamide, 2-aminoanthracene and 2-nitrofluorene, an increase in the micronucleus frequency was observed at concentrations which

TABLE 1

RESULTS OF THE MICRONUCLEUS ASSAY FOR BENZO[*a*]PYRENE IN BALB/c-3T3 CELLS [a]

Concentration (μg/ml)	Treatment time (h)	Micronucleated cells/ 1000 cells scored (M ± SD)
0 [b]	8	21.5 ± 2.12
0.25	8	35.5 ± 0.71 **
0.50	8	54.0 ± 1.41 **
1.00	8	59.5 ± 0.71 **
0 [b]	24	16.5 ± 2.12
0.25	24	58.0 ± 1.41 **
0.50	24	45.0 ± 2.83 **
1.00	24	46.0 ± 1.41 **

[a] The cells were fixed after 48 h incubation from the beginning of treatment.
[b] Dimethyl sulfoxide (5 μl/ml) was used for solvent control (0 μg/ml groups). A total of 2000 cells were scored for each treatment.
* Significantly different from control at $p < 0.05$ by Chi square test.
** Significantly different from control at $p < 0.01$ by Chi square test.

TABLE 2

RESULTS OF THE MICRONUCLEUS ASSAY FOR CYCLOPHOSPHAMIDE IN BALB/c-3T3 CELLS [a]

Concentration (μg/ml)	Treatment time (h)	Micronucleated cells/ 1000 cells scored (M ± SD)
0	8	30.5 ± 3.54
1	4	44.5 ± 0.71 *
2	4	53.0 ± 11.31 **
4	4	53.0 ± 1.41 **
1	8	59.5 ± 2.12 **
2	8	58.5 ± 0.71 **
4	8	67.0 ± 9.90 **

[a] See footnote in Table 1.

TABLE 3

RESULTS OF THE MICRONUCLEUS ASSAY FOR 2-AMINOANTHRACENE IN BALB/c-3T3 CELLS [a]

Concentration (μg/ml)	Micronucleated cells/ 1000 cells scored (M ± SD)
0	16.0 ± 2.83
1.25	21.5 ± 0.71
2.50	25.0 ± 4.24 *
5.00	51.5 ± 3.54 **

[a] See footnote in Table 1.
The treatment time was 48 h.

TABLE 4

RESULTS OF THE MICRONUCLEUS ASSAY FOR 2-NITROFLUORENE IN BALB/c-3T3 CELLS [a]

Concentration (mM)	Micronucleated cells/ 1000 cells scored (M ± SD)
0	16.0 ± 0.00
30	35.0 ± 0.00 **
60	49.5 ± 4.95 **
120	56.5 ± 7.78 **

[a] See footnote in Table 1.
The treatment time was 48 h.

TABLE 5

RESULTS OF THE MICRONUCLEUS ASSAY FOR NITROSATED COAL DUST EXTRACT IN BALB/c-3T3 CELLS

Concentration (mg/ml) [a]	Number of cells scored	Micronucleated cells/ 1000 cells scored (M ± SD)
0	2000	16.0 ± 0.00
3.75	2000	32.5 ± 0.71 **
7.50	1000	31.0 ± - **
15.00	toxic	-

[a] Expressed in original amount of the coal dust. The treatment time was 48 h, see footnote in Table 1.

did not induce cytotoxicity, and at almost all the concentrations tested, the differences were statistically significant between treated groups and respective solvent control except for cigarette-smoke condensate and nitrosated coal-dust extracts.

TABLE 6

MICRONUCLEUS ASSAY DATA FOR CIGARETTE-SMOKE CONDENSATE IN BALB/c-3T3 CELLS

Concentration (cig./ml) [a]	Number of cells scored	Micronucleated cells/ 1000 cells scored (M ± SD)
0	2000	30.5 ± 3.54
1	2000	59.0 ± 4.24 **
2	2000	105.5 ± 13.44 **
4	1000	40.0 ± -

[a] 50 μl cigarette-smoke condensate stock solution was added into 10 ml of medium. 1, 2 and 4 cig./ml are equivalent to the amount of smoke from 1/20, 1/10 and 1/5 cigarette per dish. The treatment time was 8 h, see footnote in Table 1.

TABLE 7

THE DOSE-RESPONSE RELATIONSHIP IN INDUCTION OF MN IN BALB/c-3T3 CELLS BY THE CHEMICALS AND COMPLEX MIXTURES TESTED

Chemicals (treatment time)	Linear regression equation	Correlation coefficient R	Trend test analysis	
			Z value	p value
BaP (8 h)	$Y = 26.0 + 38.00\ X$ (μg/ml) [a]	0.93	6.768	< 0.001
BaP (24 h)	$Y = 32.9 + 19.37\ X$ (μg/ml)	0.46	3.941	< 0.001
Cyclophosphamide (4 h)	$Y = 36.1 + 5.23\ X$ (μg/ml)	0.84	3.828	< 0.001
Cyclophosphamide (8 h)	$Y = 40.4 + 7.70\ X$ (μg/ml)	0.81	5.077	< 0.001
2-Aminoanthracene (48 h)	$Y = 12.9 + 7.31\ X$ (μg/ml)	0.96	6.822	< 0.001
2-Nitrofluorene (48 h)	$Y = 22.2 + 0.33\ X$ (mM)	0.92	7.287	< 0.001
Nitrosated coal dust extract (48 h)	$Y = 19.0 + 2.00\ X$ (mg/ml)	0.82	3.019	< 0.005
Cigarette-smoke condensate [b] (8 h)	$Y = 27.5 + 37.50\ X$ (cig./ml)	0.99	10.336	< 0.001

[a] Y, predictive frequency of cells with MN per 1000 cells scored at selected dose of chemical tested.
[b] The regression equation was calculated within the dose ranges from 0 to 2 cig./ml, the highest toxic group was excluded.

Both of the latter, at the highest concentration, exhibited toxic effects on cells; i.e., no cells, or very few, were collected and could be scored.

The dose–response relationship in the induction of MN by the chemicals and complex mixtures tested is presented in Table 7. All correlation coefficients were higher than 0.80, except that for BaP (24 h) which was 0.46. Trend test analysis showed the p values less than 0.01 at all experiments.

BaP and cyclophosphamide were tested for 2 different exposure times; 8 and 24 h, and 4 and 8 h, respectively. A positive response was found for both chemicals at both treatment times. However, the longer exposure did not result in a consistent increase in induced micronucleus frequencies (Tables 1 and 2).

Discussion

The formation of micronuclei in cells is a rare cytogenetic event. In in vivo micronucleus assay, the sampling distribution of cells with micronuclei possesses the characteristics of a negative binomial distribution or Poisson distribution (Amphlett and Delow, 1984). However, the distribution of MN in in vitro micronucleus assay, has not been reported. Based on the data for the solvent control groups, the mean is greater than variance in most of our experiments. The distribution of MN in these in vitro assays does not appear to follow a negative binominal distribution or Poisson distribution. More data is needed to determine the exact distribution pattern.

In the present paper, a tested chemical was assessed to have positive response on the basis of two criteria: (1) the test group(s) is statistically significantly different from respective solvent control, (2) the increase in MN frequency is dose-dependent, determined by trend test analysis. By these criteria, all 4 chemicals and 2 complex mixtures tested induced micronuclei in BALB/c-3T3 cells.

Activation of BaP by various transformation assay indicator cells has been reported (Schechtman, 1985). Established cell lines such as BALB/c-3T3 have only limited BaP metabolising activity when compared to primary Syrian hamster embryo (SHE) cells. 3T3 cells possess, on the average, approximately 30% of the activity of SHE cells. Different subclones of 3T3 cells, however, have been found to differ as much as 3-fold in their BaP metabolising capacity. Clone A31-1-13, used in this study, has higher than average metabolic activity. Present results indicate that the 3T3 cell line is able to activate polycyclic aromatic hydrocarbons, amino- and nitro-polycyclic aromatic hydrocarbons, amides and nitrosamines; i.e., the major groups of environmental mutagens and carcinogens. Therefore, it may be expected that the genotoxic effects, if any, of chemicals from these groups will be detected

using the in vitro micronucleus assay in BALB/c-3T3 cells. Further studies are needed to explore the metabolising capacity of 3T3 cells for other chemicals and/or complex mixtures.

Nitrosated coal-dust extracts and 2-nitrofluorene have exhibited their mutagenic and clastogenic effects without metabolic activation (Whong et al., 1983; Tucker et al., 1984; Tucker and Ong, 1985; Nachtman and Wolff, 1982). However, an exogenous activation system enhanced the induction of sister chromatid exchanges by 2-nitrofluorene (Nachtman and Wolff, 1982). Coal-dust extracts may contain certain indirect-acting substances which are responsible for their transformation activity (Wu et al., 1990). The use of BALB/c-3T3 cells instead of V79 cells may result in an enhanced induction of MN.

From the methodological aspect, the MN frequencies of solvent control groups in BALB/c-3T3 cells were higher than in V79 cells. The ranges were from 16 to 30.5 per 1000 cells scored against 5–10 for V79 cells. Perhaps this is due to different numbers of chromosomes in the two types of cells or to the differences in chromosome stability. The average number of chromosomes of BALB/c-3T3 cells used in the present experiments is about 3 times that of V79 cells. Nevertheless, the induced MN frequencies may be correspondingly increased, and therefore a high background of MN frequency would not affect the capacity of detecting the genotoxic effects. For each dose 2000 cells were scored in in vitro micronucleus assay with Syrian hamster embryo fibroblasts (Schmuck et al., 1988). In our experiments, scoring 2000 cells per treatment appears to be adequate to detect the genotoxic effects of tested chemicals. The variability seen in the background micronucleus frequency among experiments might be due to cells from different passages, different sampling time and high chromosome instability. Recently, elevated frequencies of MN in cultured fibroblasts after freezing and thawing were observed (Schmidt-Preuss et al., 1990). Therefore, the proper control should be settled for each experiment.

Recently, Fitzgerald et al. (1989) have employed BALB/c-3T3 cells in a simultaneous cell transformation and mutation assay protocol to see whether both genotoxic and non-genotoxic carcinogens can be identified. The in vitro MN assay in BALB/c-3T3 cells may also be used for this purpose. Since they are metabolically active and can be used for the different toxicological endpoint studies, MN assay in BALB/c-3T3 cells may become a useful in vitro short-term assay system for the detection of genotoxic agents and potential carcinogens.

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