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Clastogenic and aneuploidogenic effects of cigarette smoke condensate, mitomycin C and vincristine sulfate

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Immunofluorescent staining of kinetochores in micronuclei (MN) of cytochalasin-B-blocked cells was used to distinguish between clastogenic and aneuploidogenic effects of cigarette smoke condensate (CSC), mitomycin C (MMC) and vincristine sulfate (VS) in Chinese hamster lung V79 cells by using an anti-kinetochore antibody. Within the base-line micronucleated cells (2%), 50 to 58% contained kinetochore-positive (KC+) MN. MMC induced a significantly higher number of MN compared to the controls ($P < 0.01$), and the majority of micronucleated cells (77% at 0.08 μg/ml) contained kinetochore-negative (KC−)MN. In contrast, VS induced a large number of MN ($P < 0.01$) but most of the micronucleated cells contained KC+ MN (93% at 0.08 μg/ml). Cigarette smoke condensate (CSC) induced both KC+ ($P < 0.01$) and KC− ($P < 0.05$) MN suggesting the presence of both clastogenic and aneuploidogenic agents. However, the higher frequency of KC+ than KC− micronucleated cells suggests a stronger aneuploidogenic effect of CSC.

Introduction

Many investigators have reported that cigarette smoke condensate (CSC) induces chromosomal aberrations (CA) and sister chromatid exchanges (SCE) (Hopkins and Evans, 1979; DeMarini, 1983; Sorsa and Lofroth, 1989). Some data from human studies, however, have failed to show the effects of cigarette smoke on induction of CA and SCE (Hollander *et al.*, 1978; Crossen and Morgan, 1980). Epidemiological studies on the relationship between smoking and teratogenesis have shown that the frequency of major congenital malformations of infants was increased with increased consumption of cigarettes by the parents (Mau and Netter, 1974). In addition, smoking has been associated with increased spontaneous abortions (Kline *et al.*, 1977; Himmelberger *et al.*, 1978). However, whether cigarette smoke possesses both clastogenic and aneuploidogenic activities and whether such activities are related to the disorders of reproduction are yet to be determined.

The incidence of aneuploidy in human populations can be monitored by cytogenetic studies. Some investigators have proposed using micronucleus (MN) induction as an alternative method for measuring chromosomal aberrations and for detecting aneuploidy (Oshimura and Barrett, 1986). A major limitation in this approach has been that MN may originate from either chromatid/chromosome fragments or lagging chromosomes, and are thus induced by agents damaging either the chromosomes directly or the spindle apparatus. It would be of great interest to be able to separate these two types of MN from each other since the inducing agents are often quite different. This would also enable us to identify the mechanism involved in the induction of MN in both *in vivo* and *in vitro* studies. Many studies to distinguish between chromosome- and fragment-containing MN have been attempted (Heddle and Carrano, 1977; Vig and Swearngin, 1986). However, clear evidence for the presence of centric fragments or whole chromosomes in micronuclei was obtained only when centromeres or related materials were identified (Moroi *et al.*, 1980).

Immunofluorescent staining of kinetochores (KC) in MN with CREST (Calcinosis, Raynaud phenomenon, Esophageal dysmobility, Sclerodactyly, Telangiectasia) anti-kinetochore antibodies and immunofluorescence was developed as an *in vitro* assay for aneuploidy-inducing agents (Thomson and Perry, 1988; Degrassi and Tanzarella, 1988; Eastmond and Tucker, 1989). In the present investigation, we employed immunofluorescent staining of KC in cytokinesis-blocked V79 cells fixed *in situ* followed by chemical treatment. By using this technique, the clastogenic and aneuploidogenic effects of CSC (a complex mixture) as a test compound and mitomycin C (a clastogen) and vincristine sulfate (an aneuploidogen) as model compounds were investigated.

Materials and methods

Cell culture

Cultures of the Chinese hamster lung V79 cell line were maintained in Minimum Essential Medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (HI—FBS), L-glutamine (2 mM), and penicillin/streptomycin (1%). Trypsinized cells were seeded directly onto sterile precleaned glass slides (0.5×10^5 cells in 1 ml of growth medium per slide) placed in a square petri dish (100 × 15 mm, Lab-Tek) and allowed to adhere for 2 h before 15 ml of growth medium was added. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Preparation of cigarette smoke condensate

Commercially available ultra-low-tar cigarettes were purchased from a retailer. The CSC was prepared by smoking the cigarettes on a smoking apparatus. The smoke from five cigarettes was trapped in a flask containing 40 ml cold acetone. The acetone solvent with the smoke condensate was transferred to a small glass vial and evaporated on a hotplate by passing N_2 gas. The residual condensate was dissolved in 5 ml dimethyl sulfoxide (DMSO) and stored at −20°C until use.

Chemical treatment

Chemical treatment was done after 24 h of initial incubation. Mitomycin C (MMC) was added directly to the cultures at concentrations of 0.01, 0.02, 0.04, and 0.08 μg/ml and remained in cultures until harvest (20 h). Prior to vincristine suflate (VS) treatment, culture medium was replaced with MEM without serum. VS was then added to the cultures at concentrations of 0.01, 0.02, 0.04, and 0.08 μg/ml and cultures were incubated for 4 h. Following treatment, cultures were washed once with Hanks Balanced Salt Solution (HBSS) containing 2% HI-FBS and incubated with fresh growth medium for an additional 24 h. CSC stock solution was thawed and diluted in DMSO (treated and control cultures received 60 μl DMSO/15 ml of MEM). Known concentrations of CSC (5,10, 20, and 40 μl/ml) and 15% freshly prepared S9 mix were added to the cultures containing serum-free MEM and incubated for 4 h. The chemical was removed after 4 h and cultures were washed once with HBSS and incubated with fresh growth medium. Cytochalasin-B (CYB) 4 μg/ml was added to the cultures after chemical treatment to block cytokinesis and to obtain bionucleated cells (Fenech and Morley, 1986; Channarayappa *et al.*, 1990). Cultures were incubated for an additional 20 h.

Replicate cultures were established for each experiment and all cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Preparation of slides and immunofluorescent staining

Cultures were terminated 20 h after CYB addition and washed once with phosphate-buffered saline (PBS), then treated with hypotonic solution (0.075 M KCl) for 15 min. Cells were fixed *in situ* with prechilled (−20°C) absolute methanol for 15 min at −20°C. Slides were washed twice with PBS (pH 7.4) and laid horizontally in petri dishes. Immunofluorescent staining of KC in binucleated cells with anti-kinetochore and FITC-conjugated antibodies was performed as described by Eastmond and Tucker (1989). Slides were counter-stained with propidium iodide (5 μg/ml) for 5 min and rinsed in distilled water. The slides were mounted with antifade solution, prepared by dissolving 50 mg of *p*-phenylenediamine in 10 ml of PBS (pH 8.0) and mixing with 45 ml glycerol, using 24 × 50 mm coverslips, and were stored at 4°C.

Scoring procedure for immunofluorescence-stained micronuclei

An Epi-fluorescence condenser IV F1 (Carl Zeiss, standard microscope) was used for fluorescence analysis. Slides from each treatment were randomized and coded prior to scoring. The number of MN at each dose level was determined by scoring 1000 binucleated cells at 1000 × magnification. Slides were stained with propidium iodide and the FITC-conjugated antibody, and KC were identified by using the same optical set-up. The criteria of Eastmond and Tucker (1989) for MN determination were followed.

Statistical analysis

The frequencies of micronucleated cells in treated and control cultures were analyzed by using contingency tables. An overall test was made to compare the doses with respect to the probability of finding MN. The 2 × 2 contingency tables were formed to compare each positive dose to control with respect to the probability

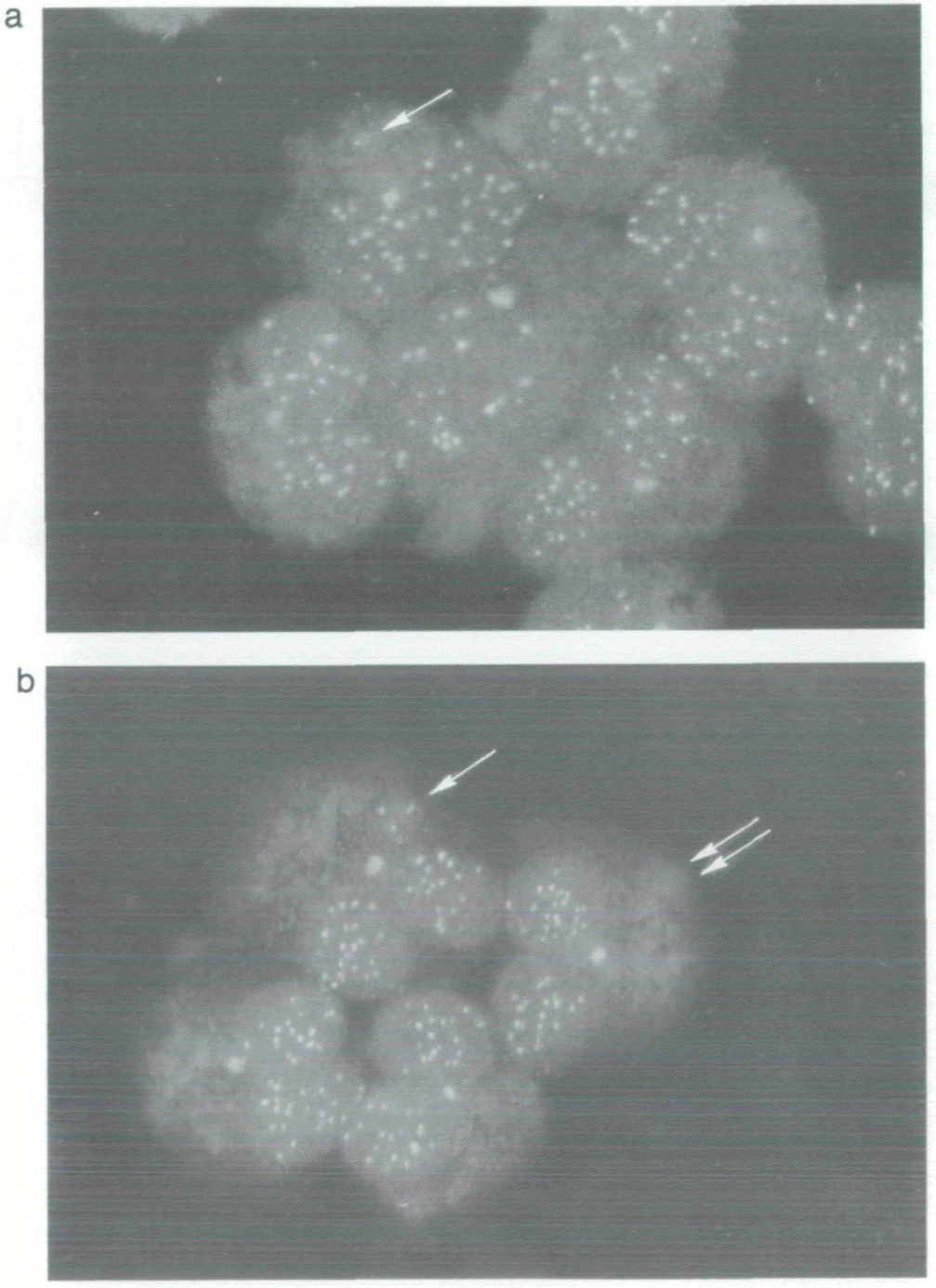

Fig. 1. Photomicrographs of cytokinesis-blocked V79 cells stained with anti-kinetochore antibody followed by FITC-conjugated anti-human IgG and counter-stained with propidium iodide, (a) binucleated cell with kinetochore-positive micronucleus (arrow), and (b) with kinetochore-negative micronucleus (double arrows).

of micronucleated cells. For comparison of kinetochore-positive (KC+) to kinetochore-negative (KC−) micronucleated cells for each treatment, data were pooled over duplicate samples and the occurrence of micronucleated cells was tested as H_o:$p(t) = p(-)$ or equivalently H_o: $p(t) = 0.5$. The sample sizes were large enough to do a Chi-square test (multinomial test).

Results and discussion

Many studies have reported that MN induced by clastogens are likely to originate from chromosome/chromatid fragments and those induced by aneuploidogens from lagging of whole chromosomes (Viaggi *et al.*, 1987; Eastmond and Tucker, 1989; Nusse *et al.*, 1989). However, it has also been reported that alkylating agents, although principally clastogenic, may also exhibit some degree of aneuploidogenic properties (Bonatti *et al.*, 1986). In contrast, many aneuploidogens, besides causing major damage to the spindle apparatus, may induce chromosomal breaks (Thomson and Perry, 1988). Thus the proportion of KC+ to KC− micronucleated cells (Figure 1) may be used to differentiate or quantify the clastogenic and aneuploidogenic activity of various genotoxic agents including complex mixtures such as CSC.

The results obtained in this study show that untreated cultures of V79 cells contained 2−3% micronucleated binucleate cells, and within the total micronucleated cells the KC+ micronucleated cells are 50−58% indicating thier origin from both acentric and centric chromosome/chromatid fragment(s) and whole chromosome(s). These data are comparable to the MN frequencies from immunofluorescent stained hamster cell line C1-1C (Degrassi and Tanzarella, 1988) and CHO cells (Eastmond and Tucker, 1989).

Both MMC and VS caused a dose-dependent increase in the frequency of micronucleated V79 cells (Table I). Based on the nuclear division index (NDI), both compounds, at high concentrations, appear to be slightly toxic to the cells (Table I). The immunofluorescence-labelled MN data show that most of the micronucleated cells (73−78%) induced by MMC lack KC fluorescence. This is expected since the MMC-induced MN originate predominantly from acentric chromatid fragments. The frequency of KC− micronucleated cells is almost parallel to the total micronucleated cells. However, at the highest concentration (0.08 μg/ml), MMC caused a slight increase of KC+ micronucleated cells ($P < 0.05$) indicating its low-level aneuploidogenic activity. These results are similar to those reported by Thomson and Perry (1988). On the other hand, VS, as expected, induced primarily KC+ micronucleated cells. At the highest concentration (0.08 μg/ml) tested, 93% of micronucleated cells contained at least one KC+ MN. The increase of KC+ micronucleated cells is nearly parallel to the total micronucleated cells. In contrast, the KC− micronucleated cells are almost equal in number to the baseline frequency indicating that VS is a purely aneuploidogenic agent. These results agree with those of Eastmond and Tucker (1989).

This study demonstrates that CSC, at high concentrations, is toxic to V79 cells. It is effective in the induction of MN formation in this cell line (Table I). Data from immunofluorescent staining of MN show that CSC induces both KC+ and KC− micronucleated cells in a dose-dependent fashion ($P < 0.01$) indicating that CSC contains both clastogenic and aneuploidogenic factors. However, the higher incidence of KC+ micronucleated cells compared to KC− micronucleated cells within the total micronucleated cells suggests a significant aneuploidy-inducing effect of CSC. The significant increase in the number of both chromatid and chromosome aberrations in blood lymphocytes of smokers (Vijayalaxmi and Evans, 1982) and the significant increase of both hypodiploid and hyperdiploid cells in pulmonary alveolar macrophages of exposed rats (Rithidech *et al.*, 1989) also suggest the aneuploidy-inducing potential of CSC. Further,

Table I. Kinetochore-positive (KC+) and kinetochore-negative (KC−) micronucleated cells in cytokinesis-blocked V79 cells following treatment with mitomycin C, vincristine sulfate and cigarette smoke condensate

Concentration	Total cells scored	Total micronucleated cells		KC+ micronucleated cells		KC− micronucleated cells		% micronucleated cells		NDI[c]
		Number	% cells	Number	% cells	Number	% cells	KC+	KC−	
Mitomycin C treatment for 20 h										
0[a]	3246	88	2.71	48	1.48	40	1.23	54.5	45.5	1.95
0.01	4604	289**	6.28	78	1.69	211**	4.58	26.9	73.0	1.93
0.02	4826	395**	8.18	97	2.01	298**	6.18	24.6	75.4	1.92
0.04	3348	273**	8.15	63	1.88	210**	6.27	23.1	76.9	1.79
0.08	4028	477**	11.84	107*	2.66	370**	9.19	22.4	77.6	1.73
Vincristine sulfate treatment for 4 h										
0[a]	2750	52	1.89	28	1.02	24	0.87	53.8	46.2	1.68
0.01	3277	90*	2.75	60*	1.83	30	0.92	66.7	33.3	1.60
0.02	3693	126**	3.41	85**	2.30	41	1.11	67.5	32.5	1.58
0.04	2935	135**	4.56	116**	3.95	19	0.65	85.9	14.1	1.56
0.08	2554	255**	9.98	238**	9.32	17	0.68	93.3	6.7	1.56
Cigarette smoke condensate with S9 mix treatment for 4 h										
0[b]	3220	92	2.86	55	1.71	37	1.15	59.8	40.2	2.59
5	3867	199**	5.15	158**	4.09	41	1.06	79.4	20.6	2.35
10	2913	172**	5.90	145**	4.98	27	0.93	84.3	15.7	1.96
20	3842	288**	7.50	202**	5.26	86**	2.24	70.1	29.9	1.41
40	3158	265**	8.39	184**	5.83	81**	2.56	69.4	30.6	1.39

[a] μg/ml in HBSS *$P < 0.05$; **$P < 0.01$.

[b] μl/ml of stock solution (stock solution was one cigarette smoke condensate per ml of DMSO)

[c] NDI = $[(1 \times M_1) + (2 \times M_2) + (3 \times M_3) + (4 \times M_4)]/N$, where M_1, M_2, M_3 and M4 are cells with 1,2 and 3 or more nuclei, respectively and N = total number of cells.

the induction of KC+ micronuclei in cytokinesis-blocked V79 cells exposed to CSC may be indicative of spindle-fiber disruptive activity of CSC. Since over 3800 chemicals including at least 40 known carcinogens have been identified in tobacco smoke (IARC, 1986) it will be difficult to determine what chemical or chemical classes are responsible for clastogenic and aneuploidogenic activities.

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