Mutation Research, 138 (1984) 93–98
Elsevier

MTR 0915

Detection of sister-chromatid exchanges in human peripheral lymphocytes induced by ethylene dibromide vapor

James D. Tucker *, Jing Xu, John Stewart and Tong-man Ong

Division of Respiratory Disease Studies, National Institute for Occupational Safety and Health, Morgantown, WV 26505 (U.S.A.)

(Received 2 April 1984)
(Revision received 4 June 1984)
(Accepted 12 June 1984)

Summary

A method using sister-chromatid exchanges (SCEs) for genotoxic testing of gaseous compounds is described. Human peripheral lymphocyte cultures previously stimulated with phytohemagglutinin were placed in sterile dialysis tubing and then put in an enclosed flask containing additional culture media. Air, with or without ethylene dibromide (EDB), was bubbled through the flask for up to 8 h. The cultures were harvested 75 h after culture initiation, and second-division cells were scored for induction of SCEs according to established procedures. The SCE frequency was approximately doubled in cultures treated with EDB. A similar experiment with air alone resulted in only slight increases in SCEs. The results indicate that this system is potentially useful for detecting genotoxicity of gases and vapors and may be useful for the detection of genotoxic agents in occupational settings.

In recent years, sister-chromatid exchange (SCE) assays have become an increasingly popular method of detecting genotoxic activity. Most investigators utilizing SCEs in vitro dissolve the test compound in a liquid solvent prior to addition to the cultures. Although this approach works well with many compounds, there are limitations. If one wishes to measure the genotoxic activity of vapors, gases or airborne dusts, then an alternative exposure method is needed.

Guerrero and Rounds (1982) developed two systems for exposing cells to gases. In the first, they exposed human diploid fibroblasts directly to ozone by use of a 'rocking table'. In a second procedure, enclosed roller tubes were used to expose Chinese hamster ovary (CHO) cells to CH_3Br gas. In each case, dose-response SCE frequencies were obtained. In another approach, Rasmussen and Crocker (1982) grew Chinese hamster lung fibroblasts on cellulose membrane filters and exposed the cells to ozone. No increase in the SCE frequency was found. White et al. (1979) exposed CHO cells to a variety of anesthetic gases by delivering the gases directly into the culture flask. An increase in the SCE frequency was observed with 3 of 11 gases. Zamora et al. (1983) grew CHO cells on hydrated collagen gels in glass bottles. Although the endpoint was gene mutation, this approach may also be used for SCEs.

This paper describes a method of exposing cells contained in dialysis tubing to gases or vapors for the purpose of genotoxicity testing. The purpose was to establish an assay system suitable for gassing human peripheral lymphocytes in occupational settings. With this approach, cells are treated un-

* Present address: Biomedical Sciences Division, L-452, Lawrence Livermore National Laboratory, P.O. Box 5507, Livermore, CA 94550 (U.S.A.)

0165-1218/84/$03.00 © 1984 Elsevier Science Publishers B.V.

der conditions that approximate those of typical culture conditions.

Materials and methods

Test compound

Ethylene dibromide (EDB, CAS 590-12-5) purchased from Sigma Chemical Company was employed in this study. Compressed breathable air was used to evaporate the EDB, and was also used as the control.

Sister-chromatid exchange assay and cell treatment protocol

Heparinized human peripheral lymphocytes from healthy unrelated non-smokers were used in this study. Whole blood (0.6 ml/10 ml media) was cultured in the dark at 37 °C in RPMI-1640 culture medium supplemented with 15% fetal bovine serum (FBS), L-glutamine (final concentration 2 mM), 1% penicillin–streptomycin (Gibco), 0.1 ml phytohemagglutinin (PHA, Gibco), and 25 μM bromodeoxyuridine (BrdU). The equipment is illustrated in Fig. 1. The set-up is similar to that described by Whong et al. (1984) for in situ microbial mutagenesis assay system. The cultures were treated under subdued red light beginning at 22–26 h after culture initiation by placing the entire culture contents into about 10 in. of sterilized dialysis tubing (Arthur H. Thomas Company) with a molecular weight cutoff of 12000. The dialysis tubing was then placed in a 100-ml pear-shaped, foil-wrapped flask with a fritted bubbler, containing 20 ml of RPMI-1640 culture medium supplemented with L-glutamine, BrdU, and PHA in the same concentrations as the cultures. Because the CO_2 buffer in RPMI-1640 is rapidly removed by the bubbling of air, 0.4 ml of 7.5 M phosphate buffer was also added to each flask and the pH adjusted to 7.4 as necessary with 1 N HCl or NaOH. Periodic minor readjustments of the pH were necessary, especially in the first hour. Water loss due to evaporation was controlled by careful addition of distilled water as needed. FBS could not be added to the exposure flask because of frothing. In the experiments with EDB, a small evaporation chamber containing the test compound (Fig. 1) was inserted in the air line. The amount of EDB evaporated in the course of the experiment was determined, and the parts per million (ppm) calculated. The air flow rate was maintained at 1.2 l/min per culture by Dwyer type RMB Mini-Master air flow gauges.

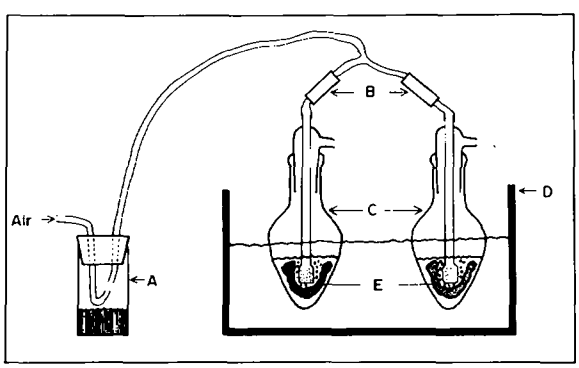

Fig. 1. Apparatus set-up used in these experiments. A, EDB evaporation chamber; B, gas flow meters, to equilize the flow rate to each of two flasks; C, pear-shaped flasks containing dialysis tubing; D, 37 °C water bath; E, cells contained inside dialysis tubing.

Upon removal of the dialysis tubing from the flasks, the cells were washed twice in Hanks' balanced salt solution. They were then resuspended in media supplemented as described in the initial culture set-up, and grown for the remainder of the 75-h culture period. Colcemid (0.1 μg/ml) was added for the final 3 h of incubation. The cells were swollen with 0.075 M KCl, washed 3 times in fresh fixative (methanol : acetic acid, 3 : 1), dropped onto slides and air dried. Differential staining was achieved by a modified fluorescent-plus-Giemsa technique (Perry and Wolff, 1974; Goto et al., 1978). Slides were stained in Hoechst 33258 (5 μg/ml) for 15 min in 0.15 M phosphate buffer (pH 6.8), and exposed at a distance of 1 cm and 55–60 °C to UV light at approximately 365 nm for 7 min. The slides were then stained with 5% Giemsa and coded prior to scoring. 25 cells were scored per person per dose.

Statistical analysis

A one-tailed Student *t* test was used to compare the SCE frequencies in the treated cultures with those of the controls. The SCE counts were first subjected to the square root transformation because the data were skewed towards the higher frequencies. A Chi-squared test was used to compare the mitotic indices.

Results and discussion

The results of the first experiment with EDB are presented in Table 1. Clear dose responses were seen in both individuals tested. The longest treatment time (8 h) was lethal in the first trial of the experiment, consequently, this dose was not attempted again. In the second experiment with EDB (Table 2), blood from two other donors was treated simultaneously. Cultures were also exposed to air alone as controls. Clear dose responses were again observed, which were statistically significant when compared to both the negative and the air controls. Cell-cycle delay was apparent in each experiment (data not shown). It should be noted that the concentration of EDB in the second experiment was much higher than the first, however, the increase in SCE frequencies was not proportional to the EDB concentration. It is possible that the concentration of EDB used in the second experiment was higher than the solubility of EDB in culture media.

To determine whether air by itself had any effect on SCE frequencies, blood from 4 donors was treated for up to 8 h (Table 3). The SCE frequencies of donors A and C peaked at 48 and 160 min, respectively, and decreased at the longer exposure times. The SCE frequency in the culture of donor J never reached statistical significance, while the response of donor B was significant only at the longest exposure. When the results of all 4 donors are combined, a slight but consistent increase in the SCE frequency was observed with increasing exposure time. Even though the SCE responses reach statistical significance, the actual increases are not large. The second EDB experiment yielded an SCE increase of 179% at 48 min (Table 2, mean of both donors) compared to 17% at 48 min in the air experiment (Table 3, mean of all 4 donors). The slight increase in SCE with air, therefore, will have little or no effect upon the detection of SCEs induced by genotoxic agents. Care must be taken, therefore, to compare the SCE frequency of treated cultures with the air control, rather than the negative control if the treatment duration is more than 5 min.

A surprising find in the air experiments was that of cell-cycle delay. Because an early trial with air indicated that this might be a problem, the culture duration in these experiments was extended from the usual 72 to 75 h. The data in Table 3 suggest that a further extension would be warranted if the test chemical permitted survival at 8 h. The reason(s) for this cell-cycle delay are not clear. The medium inside the flasks, but outside the dialysis tubing, is as similar to actual culture

TABLE 1

SISTER-CHROMATID EXCHANGES INDUCED BY ETHYLENE DIBROMIDE GAS [a]

Treatment time (min)	Donor	Number of SCEs	SCEs/ chromosome	SCEs/cell ± S.E.
0 (control	D	230	0.201	9.20 ± 0.56
	B	235	0.205	9.40 ± 0.73
5	D	265	0.232	10.60 ± 0.81
	B	261	0.228	10.44 ± 0.60
16	D	310	0.271	12.40 ± 1.11 [b]
	B	272	0.237	10.88 ± 0.88
48	D	379	0.333	15.16 ± 1.00 [c]
	B	368	0.320	14.72 ± 0.79 [c]
160	D	449	0.391	17.96 ± 1.08 [c]
	B	407	0.356	16.28 ± 1.04 [c]

[a] Cells from the two donors were gassed on separate occasions. The concentration of EDB was 245 and 418 ppm, for donors D and B, respectively.

[b] $P < 0.01$.

[c] $P < 0.0001$.

TABLE 2

SISTER-CHROMATID EXCHANGES INDUCED BY ETHYLENE DIBROMIDE GAS WITH SIMULTANEOUS AIR CONTROLS [a]

Treatment time (min)	Donor	Cell kinetics index [b]	Number of SCEs	SCEs/ chromosome	SCEs/cell ± S.E.	Significance level compared to controls	
						Negative	Air
0 (negative control)	A	1.68	237	0.206	9.48 ± 0.69	-	0.40
	C	1.79	206	0.180	8.24 ± 0.72	-	N.S. [c]
160 (air control)	A	1.32	289	0.255	11.56 ± 0.90	0.40	-
	C	1.19	235	0.206	9.40 ± 0.52	N.S.	-
1.66	A	1.67	292	0.255	11.68 ± 0.89	0.25	N.S.
	C	1.40	233	0.203	9.32 ± 0.74	N.S.	N.S.
5	A	1.58	370	0.322	14.80 ± 1.26	0.0001	0.016
	C	1.44	261	0.228	10.44 ± 0.83	0.025	N.S.
16	A	1.45	446	0.390	17.84 ± 1.16	< 0.0001	< 0.0001
	C	1.34	379	0.332	15.16 ± 1.04	< 0.0001	< 0.0001
48	A	1.31	671	0.588	26.84 ± 1.40	< 0.0001	< 0.0001
	C	1.25	565	0.493	22.60 ± 1.04	< 0.0001	< 0.0001
160	A	1.18	550	0.482	22.00 ± 1.14	< 0.0001	< 0.0001
	C [d]	-	-	-	-	-	-

[a] Cells from the two donors were gassed simultaneously. The concentration of EDB was 1996 ppm.
[b] 100 cells scored per dose; cell kinetics index = (first-division cells plus 2 × second-division cells plus 3 × third-division cells)/100.
[c] Not significant.
[d] Insufficient dividing cells for analysis.

conditions as possible, but some differences do exist. Serum was not included because uncontrollable frothing results. However, serum is present inside the dialysis tubing. Most of the serum proteins are too large to pass through the tubing, thus the lymphocytes have access to normal concentrations of most of the serum components. Molecules with a molecular weight less than 12000 could pass through the tubing, but would remain inside the flask. The maximum dilution effect would be one third of the original concentration, since there is twice as much medium outside the dialysis tubing as inside. This dilution would not seem to be sufficient enough to cause cell-cycle delay of the magnitude seen after 8 h, particularly because the cells are washed twice following exposure with replacement of medium (plus 15% serum). The additional phosphate buffer employed (0.10 M final concentration, total flask contents considered) was the minimum found to be necessary to maintain the pH.

Separate experiments were conducted to determine whether the presence of phosphate and the absence of serum induced SCEs or caused cell-cycle delay. The results indicate that slight, but non-significant, increases in the SCE frequency resulted when phosphate was added and serum removed simultaneously for 8 h beginning 23 h after culture initiation (data not shown). However, compared to control cultures, marked cell-cycle delay was apparent. The mitotic index decreased from 1.96 to 1.22 ($P < 0.001$, mean of 100 cells for each of two donors).

The dialysis tubing may also be responsible for SCE induction or cell-cycle delay. The tubing used in these experiments is pure cellulose containing 25% glycerol by weight. It is expected that some of this glycerol is washed off by the water used to prepare the tubing for these experiments. However, the amount of glycerol lost is unknown. Experiments were conducted to determine whether glycerol in concentrations similar to those antic-

TABLE 3

SISTER-CHROMATID EXCHANGES INDUCED BY AIR [a]

Treatment time (min)	Donor	Cell kinetics index [b]	Number of SCEs [c]	SCEs/ chromosome	SCEs/cell ± S.E.
0 (control)	A	1.78	208	0.182	8.32 ± 0.56
	B	1.56	231	0.201	9.24 ± 0.44
	C	1.61	197	0.172	7.88 ± 0.67
	J	1.60	237	0.208	9.48 ± 0.86
	Pooled data	1.64	218	0.191	8.37 ± 0.33
5	A	1.51 [e]	232	0.203	9.28 ± 0.75
	B	1.45	195	0.171	7.80 ± 0.66
	C	1.55	218	0.190	8.72 ± 0.56
	J	1.63	258	0.225	10.32 ± 0.74
	Pooled data	1.54	226	0.197	9.03 ± 0.35
16	A	1.50 [d]	280	0.247	11.20 ± 0.75 [e]
	B	1.42	204	0.178	8.16 ± 0.66
	C	1.63	234	0.204	9.36 ± 0.62
	J	1.53	246	0.215	9.84 ± 0.64
	Pooled data	1.52 [d]	241	0.211	9.64 ± 0.35 [d]
48	A	1.39 [f]	274	0.240	10.96 ± 0.87 [e]
	B	1.48	228	0.199	9.12 ± 0.77
	C	1.51	217	0.190	8.68 ± 0.73
	J	1.53	261	0.228	10.44 ± 0.57
	Pooled data	1.48 [e]	245	0.214	9.80 ± 0.38 [d]
160	A	1.41 [f]	258	0.226	10.32 ± 0.75 [d]
	B	1.34 [d]	236	0.207	9.44 ± 0.76
	C	1.54	279	0.245	11.16 ± 0.58 [f]
	J	1.42 [e]	262	0.230	10.48 ± 0.59
	Pooled data	1.43 [g]	259	0.227	10.35 ± 0.34 [f]
480	A	1.29 [g]	249	0.219	9.96 ± 0.76 [d]
	B	1.14 [g]	312	0.277	12.48 ± 0.77 [f]
	C	1.28 [f]	245	0.214	9.80 ± 0.63 [d]
	J	1.34 [e]	258	0.225	10.32 ± 0.70
	Pooled data	1.26 [g]	266	0.234	10.64 ± 0.37 [f]

[a] Cells from the 4 donors were gassed on separate occasions.
[b] See footnote b, Table 2.
[c] Totals for 25 cells per donor.
[d] $P < 0.05$.
[e] $P < 0.01$.
[f] $P < 0.001$.
[g] $P < 0.0001$.

ipated in these experiments can induce SCEs or cell-cycle delay. The results showed that glycerol does not cause cell-cycle delay, but can induce small increases in the SCE frequency (data not shown). The other remaining alternative to the problem of SCE induction by air alone is that one or more medium components interact with the air itself to form a compound or compounds that react with the lymphocytes.

EDB was selected for this study because it is a volatile compound. It has been estimated that more than 108 000 American workers are potentially exposed to EDB in manufacturing, formulation and agricultural application. This compound

is known to induce SCEs and chromosome aberrations in cultured mammalian cells (Tezuka et al., 1980). The present report indicates that EDB induces high frequencies of SCEs in cultured human lymphocytes. These results and those from others, therefore, suggest that EDB may pose potential genetic, carcinogenic and reproductive hazards to the exposed workers. However, in vivo mutagenicity studies in mice by Shafik and Legator (1984) did not demonstrate genotoxicity.

The procedure described in this paper may be useful for testing gaseous or volatile compounds under controlled laboratory conditions, or for in situ monitoring of genotoxic agents in occupational settings. Although SCEs in human peripheral lymphocytes were employed here, alternative cell systems and biological endpoints may also be used.

References

Goto, K., S. Maeda, Y. Kano and T. Sugiyama (1978) Factors involved in differential Giemsa-staining of sister chromatids, Chromosoma, 66, 351–359.

Guerrero, R.R., and D.E. Rounds (1982) In vitro analysis of mammalian cells exposed in vitro and in vivo to airborne agents, in: R.R. Tice, D.L. Costa and K.M. Shaich (Eds.), Genotoxic Effects of Airborne Agents, Plenum, New York, pp. 51–73.

Perry, R., and S. Wolff (1974) New Giemsa method for the differential staining of sister chromatids, Nature (London), 251, 156–158.

Rasmussen, R.E., and T.T. Crocker (1982) Lung cells grown on cellulose membrane filters as an in vitro model of the respiratory epithelium, in: R.R. Tice, D.L. Costa and K.M. Shaich (Eds.), Genotoxic Effects of Airborne Agents, Plenum, New York, pp. 105–120.

Shafik, H.M., and M.S. Legator (1984) In vivo mutagenicity studies in mice following ethylene dibromide exposure, Environ. Mutagen., in press.

Tezuka, H., N. Ando, R. Suzuki, M. Terahata, M. Moriya and Y. Shirasu (1980) Sister-chromatid exchanges and chromosome aberrations in cultured Chinese hamster cells treated with pesticides positive in microbial reversion assays, Mutation Res., 78, 177–191.

White, A.E., S. Takehisa, E.I. Eger, S. Wolff and W.C. Stevens (1979) Sister chromatid exchanges induced by inhaled anesthetics, Anesthesiology, 50, 426–430.

Whong, W.-Z., J. Stewart and T. Ong. (1984) Development of an in situ microbial mutagenicity test system for airborne workplace mutagens: Laboratory evaluation, Mutation Res., 130, 45–51.

Zamora, P.O., J.M. Benson, A.P. Li and A.L. Brooks (1983) Evaluation of an exposure system using cells grown on collagen gels for detecting highly volatile mutagens in the CHO/HGPRT mutation assay, Environ. Mutagen., 5, 795–801.