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1,2-Dibromoethane as an Initiating Agent for Cell Transformation

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The two-stage transformation assay increases the sensitivity of cells to chemicals and permits detection of carcinogens acting as initiating agents. 1,2-Dibromoethane, a representative halogenated aliphatic, has been tested in the two-stage BALB/c 3T3 cells transformation test at dosages from 16 μM to 128 μM. This dose range is much lower than those previously found efficient in transforming BALB/c 3T3 cells. Apart from the lowest dose, which induced borderline effects, all the other assayed dosages appeared to induce heritable changes in the traget cells. The initiated cells were revealed as fully transformed foci both in the combination with a chronic promoting treatment and also by allowing cells to perform more rounds of cell replication. The results clearly show that 1,2-dibromoethane can act as an initiator of cell transformation.

Key words: 1,2-Dibromoethane — Cell transformation — Initiation — Carcinogenesis process

Halogenated compounds represent one of the most important classes of environmental pollutants. Among them, the smaller haloalkanes and haloalkenes are widely used for agricultural, pharmaceutical and also household purposes.[1,2] Most of them are capable of binding nucleic acids and proteins to an extent typical of weak or moderate initiating agents.[2] Some of them are mutagenic in bacteria and/or positive in short-term tests for DNA damage.[1] However, only a few of them have been adequately tested in long-term tests of carcinogenicity[1] and of those tested, all but 1,2-dichloroethane and 1,2-dibromoethane (1,2-DBE), which also induced forestomach and lung carcinomas,[1,3,4] were found to be carcinogenic only for the liver of B6C3F1 mice, a strain genetically susceptible to hepatocarcinogenesis.[1] Thus, results from these tests have been considered inconclusive. Chlorinated compounds have been suggested to induce hepatocarcinogenesis by a promoting mechanism. Several chlorinated aliphatics examined in a rat liver focus assay for tumor-initiating and promoting activities failed to exert any initiating effect but were positive in the promotion protocol.[5] However, we previously found that the most toxic chloroethane, 1,1,2,2-tetrachloroethane (1,1,2,2-TTCE) was capable of acting as an initiating agent in the two-stage cell transformation test[6] and of reacting covalently with DNA similarly to a moderate initiator.[7] 1,2-DBE which is capable of binding DNA to an extent similar to that of 1,1,2,2-TTCE,[8,9] also induces cell transformation at a much lower dosage and to a greater extent than 1,1,2,2-TTCE.[10] This compound is the only one among the smaller haloalkanes which has been adequately tested in rodent bioassays[1,3,4] and it has been classified as carcinogenic to animals.

To define better the role of 1,2-DBE in the carcinogenic process, we have assayed it in the two-stage BALB/c 3T3 cell system. Malignant cell transformation *in vitro* is regarded as a model for carcinogenesis *in vivo*,[11] and the two-stage assay is considered superior to the standard method to detect initiating activity of a wide range of chemicals.[12]

MATERIALS AND METHODS

Chemicals 1,2-DBE (98% pure) and 12-O-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Cells and cell culture The original stock of BALB/c 3T3 cells, clone A31, was obtained from American Type Culture Collection, Rockville, MD, USA. Working cultures were expanded from the original cryopreserved stock. Cells were grown in Dulbecco's modified Eagle's medium (D-MEM from GIBCO) supplemented with 10% newborn calf serum (GIBCO) and in the absence of antibiotics. Only subconfluent cells (60–70% confluent) were used in the experimental assay and the target cells were not maintained beyond the third passage after thawing.

Transformation assay The two-stage transformation test was carried out according to the protocol described previously.[6,11,13] Cells were seeded at a density of 10^4

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cells/60-mm dish (13 dishes for each treatment condition). At 24 h after seeding, cells were treated with 1,2-DBE (from 16 μM to 128 μM) dissolved in 0.5% dimethylsulfoxide (DMSO) and added to the culture medium. The exposure to the chemical was carried out for 72 h (initiating treatment). Cells were then washed with phosphate-buffered saline (PBS) and placed in fresh normal medium. After four days and thereafter each time the medium was changed (twice weekly), the cultures were treated with medium containing 100 ng/ml TPA (promotion treatment) until the end of the experiment (Fig. 1). When cells reached confluence (about 2 weeks after the start of treatment), the contents of three plates from each treatment group were pooled and reseeded at 2×105 cells/60-mm dish in 10 replicates (level-II transformation test). Then the cells were again exposed to the promotion treatment. Untreated cells and solvent vehicle-treated cells (e.g. 0.5% DMSO-treated cells) were used as negative controls. Five weeks after the treatment, level-I and level-II plates were fixed with methanol, stained with 10% aqueous Giemsa and scored for foci (Fig. 1). Only foci considered as positive,[11] deeply basophilic, showing a dense layer formation and a random orientation of cells at the edges of foci and not smaller than 1 mm were counted. Values were reported as number of positive plates (plates with foci)/plates scored, mean number of foci per plate, total number of foci per treatment and transformation frequency (TF) calculated on the cells at risk surviving after chemical treatment. Statistical evaluation of focus distribution was performed by means of the Mann-Whitney unpaired t test. The number of positive plates among the treated plates was statistically analyzed by use of the Fisher-Yates test of significance in 2×2 contingency tables.

Cytotoxicity test Colony-forming efficiency assay was performed in parallel with the transformation test by seeding 100 cells/60-mm plates in 5 replicates. Cells were treated with 1,2-DBE at the same doses as in the transformation test, and with 100 ng/ml TPA from 3 days after removal of 1,2-DBE. Ten days after seeding, the cultures were fixed and stained with 10% Giemsa and colonies of at least 50 cells were counted. Cytotoxicity was expressed as mean number of colonies obtained per dose level and as relative clonal efficiency with respect to solvent vehicle-treated cells.

RESULTS

Table I shows the results of the cytotoxicity tests performed in the absence and in the presence of TPA. Some cytotoxic effects were seen in cells treated with different dosages of 1,2-DBE in the absence of promoting treatment.

In Table II, the transforming effects of 1,2-DBE obtained in the absence or in the presence of a chronic TPA treatment in the level-I standard experiment are summarized. A slight but significant increment of the transformation frequency was observed. The increment did not appear to be related to the dose employed and it was only slightly affected by the treatment with TPA.

In the level-II transformation assays (Table III), the amplification process carried out by replating confluent cells yielded a much higher number of transformed foci

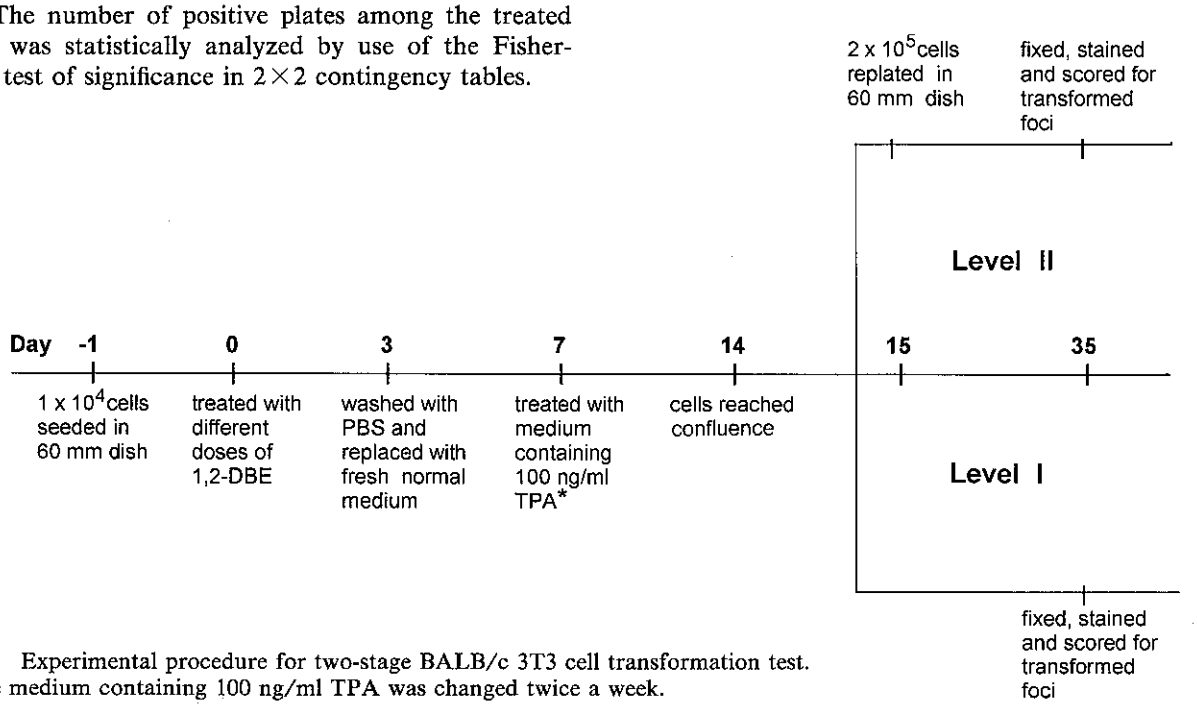

Fig. 1. Experimental procedure for two-stage BALB/c 3T3 cell transformation test.
* The medium containing 100 ng/ml TPA was changed twice a week.

Table I. Cytotoxic Effects of 1,2-DBE on BALB/c 3T3 Cells in the Absence or in the Presence of TPA[a)]

Treatment	In the absence of TPA			In the presence of TPA		
	Mean no. colonies/plate scored	Relative clonal efficiency (%)[b)]	Clonal efficiency (CE)[c)]	Mean no. colonies/plate scored	Relative clonal efficiency (%)[b)]	Clonal efficiency (CE)[c)]
UC[d)]	18.80±2.03	106	0.19	14.80±1.43	117	0.15
0.5% DMSO	17.75±2.06	100	0.18	12.60±1.08	100	0.13
2.5 μg 3-MCA[e)]	14.80±1.62	83	0.15	13.20±1.66	105	0.13
16 μM DBE	13.50±0.64	76	0.13	15.20±0.86	121	0.15
32 μM DBE	15.40±0.98	87	0.15	13.00±0.89	103	0.13
64 μM DBE	14.00±1.15	79	0.14	12.60±0.80	100	0.13
128 μM DBE	16.40±1.21	92	0.16	11.80±0.49	94	0.12

a) Data are reported as mean of five replicates±SE from two different experiments. The number of colonies scored in the treated plates as well as the clonal efficiency are not significantly different from those detected in the solvent-treated plates.
b) Values are expressed as total number of colonies per treatment relative to the negative control.
c) Number of colonies/1×10^2 seeded cells per plate.
d) UC=Untreated cells, i.e., negative controls performed with BALB/c 3T3 not exposed and cultured like treated cells.
e) MCA=3-methylcholanthrene.

Table II. *In vitro* BALB/c 3T3 Cells Transformation (Level-I) by 1,2-DBE in the Presence of Exogenous Metabolic Activation[a)]

Treatment	Transformation activity in the absence of TPA			Transformation activity in the presence of TPA		
	Plates with foci/plates scored	Mean no. of transformed foci/plate	TF[b)] ($\times10^{-4}$)	Plates with foci/plates scored	Mean no. of transformed foci/plate	TF[b)] ($\times10^{-4}$)
UC[c)]	1/10 (1)	0.10±0.10	0.53	1/10 (1)	0.10±0.10	0.67
0.5% DMSO	1/10 (1)	0.10±0.10	0.56	1/10 (1)	0.10±0.10	0.77
2.5 μg 3-MCA[d)]	2/10 (2)	0.20±0.13	1.33	4/10[e)] (5)	0.50±0.22	3.85
16 μM DBE	5/10[f)] (7)	0.70±0.26	5.38	5/10[f)] (7)	0.70±0.26	4.67
32 μM DBE	2/10 (3)	0.30±0.21	2.00	6/10[g)] (9)	0.90±0.28[h)]	6.92
64 μM DBE	7/10[i)] (13)	1.30±0.42[h)]	9.29	5/10[f)] (7)	0.80±0.33	6.15
128 μM DBE	6/10[g)] (9)	0.90±0.28[h)]	5.63	7/10[i)] (10)	1.00±0.26[h)]	8.33

The total number of foci per treatment is shown in parentheses.
a) Experiment was performed according to the standard method.
b) Transformation frequency expressed as the number of transformation foci per number of cells at risk after chemical treatment.
c) UC=Untreated cells, i.e., negative controls performed with BALB/c 3T3 not exposed and cultured like treated cells.
d) MCA=3-methylcholanthrene.
e) Significantly different from control (solvent-treated plates) in the Fisher-Yates test of significance in 2×2 contingency tables ($P<0.05$).
f) Significantly different from control (solvent-treated plates) in the Fisher-Yates test of significance in 2×2 contingency tables ($P<0.01$).
g) Significantly different from control (solvent-treated plates) in the Fisher-Yates test of significance in 2×2 contingency tables ($P<0.005$).
h) $P<0.05$ using the distribution-free Mann-Whitney unpaired *t* test between control (solvent-treated plates) and 1,2-DBE-treated plates.
i) Significantly different from control (solvent-treated plates) in the Fisher-Yates test of significance in 2×2 contingency tables ($P<0.001$).

even in the absence of the TPA treatment. The focus distribution and the number of positive plates became statistically significant, leading to TF values about 6 times higher as compared to the solvent-treated cultures. The treatment with TPA produced an even higher number of transformed foci, which increased with increasing concentration of 1,2-DBE. TF values increased correspondingly up to 12 times with respect to DMSO-treated cells. The lowest assayed dosage (16 μM) seemed to induce borderline effects in that the proportion of

Table III. *In vitro* BALB/c 3T3 Cells Transformation (Level II-Amplification) by 1,2-DBE in the Presence of Exogenous Metabolic Activation[a)]

Treatment	Transformation activity in the absence of TPA			Transformation activity in the presence of TPA		
	Plates with foci/plates scored	Mean no. of transformed foci/plate	TF[b)] ($\times 10^{-4}$)	Plates with foci/plates scored	Mean no. of transformed foci/plate	TF[b)] ($\times 10^{-4}$)
UC[c)]	3/10 (3)	0.30±0.15	1.58	3/10 (4)	0.40±0.22	2.67
0.5% DMSO	3/10 (4)	0.40±0.22	2.22	5/10 (7)	0.70±0.26	5.38
2.5 μg 3-MCA[d)]	10/10[e)] (28)	3.00±0.51[f)]	20.00	9/10[g)] (9)	2.70±0.47[h)]	20.77
16 μM DBE	5/10[i)] (16)	0.90±0.31	6.92	5/10 (27)	0.90±0.23	6.00
32 μM DBE	10/10[e)] (26)	2.60±0.40[j)]	17.33	10/10[k)] (41)	4.10±0.64[f)]	31.54
64 μM DBE	10/10[e)] (23)	2.30±0.55[j)]	16.43	10/10[k)] (48)	4.80±0.84[f)]	36.92
128 μM DBE	9/10[i)] (20)	2.00±0.30[j)]	12.50	10/10[k)] (64)	6.40±0.67[f)]	53.33

The total number of foci per treatment is shown in parentheses.
a) Experiment was performed according to the standard method.
b) Transformation frequency expressed as the number of transformation foci per number of cells at risk after chemical treatment.
c) UC=Untreated cells, i.e., negative controls performed with BALB/c 3T3 not exposed and cultured like treated cells.
d) MCA=3-methylcholanthrene.
e) Significantly different from control (solvent-treated plates) in the Fisher-Yates test of significance in 2×2 contingency tables ($P<0.005$).
f) $P<0.001$ using distribution-free Mann-Whitney unpaired *t* test between control (solvent-treated plates) and 1,2-DBE-treated plates.
g) Significantly different from control (solvent-treated plates) in the Fisher-Yates test of significance in 2×2 contingency tables ($P<0.01$).
h) $P<0.01$ using distribution-free Mann-Whitney unpaired *t* test between control (solvent-treated plates) and 1,2-DBE-treated plates.
i) Significantly different from control (solvent-treated plates) in the Fisher-Yates test of significance in 2×2 contingency tables ($P<0.001$).
j) $P<0.005$ using distribution-free Mann-Whitney unpaired *t* test between control (solvent-treated plates) and 1,2-DBE-treated plates.
k) Significantly different from control (solvent-treated plates) in the Fisher-Yates test of significance in 2×2 contingency tables ($P<0.05$).

dishes with transformed foci and the mean number of foci, although increased, were not statistically significantly different from the negative control values.

The treatment with TPA seemed not to affect the transformation rate in the level-II plates treated with 3-MCA (Table III). This was probably because the parental (level-I) plates, randomly chosen to be replated in the amplification test, contained a lower number of incipient transformed clones capable of yielding positive foci under promoting treatment.

DISCUSSION

The cell transformation test has been considered as indicating the inducibility in cultured cells of phenotypic alterations strongly related to neoplasia[14)] and is regarded as a model system for carcinogenesis *in vivo*. As in the mouse skin model *in vivo*, there are at least two different steps, initiation and promotion, in the malignant transformation of established cells such as C3H10T1/2 and BALB/c 3T3 cells. The BALB/c 3T3 cell transformation test has been employed for studying cellular and molecular mechanisms of carcinogenesis.[15)] Results from studies on BALB/c 3T3 cells, as well as on other established cell lines,[16)] have given evidence for a close correlation between molecular events observed in transformed cells as a consequence of chemical exposure and those detected in chemically induced rodent epithelial tumors.[17, 18)] The two-stage transformation test, performed by treating cells first with a suspect carcinogen and subsequently with a known tumor promoter, increases the sensitivity of target cells to the transforming potential of chemicals.

1,2-DBE has previously been demonstrated to induce cell transformation over a wide range of dosages in the absence or in the presence of an exogenous metabolic system.[10)] In the absence of exogenous bioactivation, 128 μM DBE was the minimal transforming dose. At that dosage, when rat liver S9 fraction and an NADPH-regenerating system (S9-mix) were added to improve BALB/c 3T3 cell metabolism, 1,2-DBE failed to transform cells, probably because the efficiency of detoxifying metabolic steps was enhanced.[10)] Results reported here

show that, in the absence of S9-mix, 1,2-DBE succeeds in transforming cells at even lower dosages. A modest but significant increment in cell transformation was already apparent in the standard assay (level-I) cultures, becoming more evident, at all assayed dosages, in the combination with TPA treatment, and being fully revealed in the amplification level-II test both in the absence and in the presence of TPA treatment. That means that 1,2-DBE can work as an initiator in a two-stage cell transformation system at very low concentrations, inducing a heritable alteration in the target cells which can be revealed under appropriate environmental conditions (i.e. by allowing cells to proliferate further or by treating them with a promoting substance). The result also shows that the BALB/c 3T3 cells used in this study retain metabolic enzymes in small but sufficient amounts to give low levels of active metabolites capable of inducing changes which can be revealed as visible foci of transformed cells by TPA treatment. This metabolite(s) remains to be identified. Although 1,2-DBE has been shown to induce tumors directly at the contact site,[1] it requires metabolic activation to form biologically reactive products. Although products from a mixed function oxidases (MFO)-mediated pathway seem to be efficient in binding nucleic acids,[9] GSH transferases have been suggested to play the major role in inducing covalent reaction and damage to DNA.[8, 9, 19, 20] The main DNA adduct has been proposed to be a product of glutathione conjugation,[21] whereas the oxidative pathway is thought to lead to the formation of metabolites, such as bromoacetaldehyde, that, although highly reactive, are rapidly detoxified through glutathione conjugation.[21] Thus, the decrement of BALB/c 3T3 cell transformation by 1,2-DBE in the presence of S9-mix could be related to improvement of this detoxifying pathway since S9-fraction seems to affect the metabolic steps involving MFO enzymes rather than glutathione-mediated pathways.

It is noteworthy that 1,2-DBE is one of the most widespread environmental pollutants. It was widely used in the past as a pesticide and as a lead scavenger in gasoline. Although its commercial use has recently been restricted, it is still widely employed and there is also some evidence that it has been used illegally in agriculture. For all these reasons, this compound constitutes a potential hazard to human health. Two epidemiological studies on industrial workers exposed to 1,2-DBE were inconclusive, but the deaths of two humans were associated with acute exposure to 1,2-dibromoethane.[22] Despite a large number of mutagenicity studies, the precise mode of action of 1,2-DBE is still poorly understood. Other reports give evidence for genotoxic and clastogenic activity of this compound[23] and recent findings suggest a possible action of 1,2-DBE as a progressor agent.[24] It seems important to quantify the minimal active dose of 1,2-DBE and to attempt to understand its role in carcinogenesis by assessing its promoting potential.

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