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In vitro Transformation of BALB/c 3T3 Cells by 1,1,2,2-Tetrachloroethane

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1,1,2,2-Tetrachloroethane (1,1,2,2-TTCE) was shown to be capable of inducing *in vitro* transformation of BALB/c 3T3 cells (clone A-31) either in the presence or in the absence of S9 activating system using an amplification-transformation (level-II) assay by reseeding confluent cells from each treatment and allowing additional rounds of cell replication. In the absence of metabolic activation, the highest assayed dose (1000 μg/ml), exerting the highest toxicity, was the only transforming dose. Lower doses of 1,1,2,2-TTCE were capable of transforming BALB/c cells in the presence of S9 activating system, the dose of 500 μg/ml exerting the highest transforming activity. The number and size of transformed foci recognized in the level-II plates were a function of the number of cells reseeded in the amplification assay. Foci obtained in the presence of S9 activating systems were larger in size, more deeply basophilic, and exhibited denser multilayering of constituent cells than foci recognized in the absence of exogenous metabolic activation.

Key words: Transformation — BALB/c 3T3 — 1,1,2,2-Tetrachloroethane

1,1,2,2-Tetrachloroethane (1,1,2,2-TTCE)[5] is a widely produced and used solvent and chemical intermediate. It is toxic to liver, kidney and lung and can be considered as the most toxic of the smaller chlorinated hydrocarbons.[1] It is mutagenic in *S. typhimurium*[2,3] and in *S. cerevisiae*[3] and inhibits the growth of DNA polymerase-deficient (pol A−) *E. coli*.[2,3]

Like other chlorinated ethanes, 1,1,2,2-TTCE is bioactivated by cytochrome P450-dependent enzymatic systems through oxidative metabolic pathways to reactive electrophilic metabolites which covalently react with nucleophilic sites of macromolecules.[4] On the basis of binding values to liver DNA, measured after ip injection into rats and mice, 1,1,2,2-TTCE can be classified as a moderate initiator.[5] The covalent binding index (CBI), calculated according to Lutz,[6] is compatible with the binding extent to exogenous DNA, measured after *in vitro* bioactivation of 1,1,2,2-TTCE by rat or mouse enzymatic fractions,[5] and with the oncogenic potency index (OPI) calculated according to Parodi *et al.*[7] from long-term carcinogenicity test data. This bioassay has demonstrated that 1,1,2,2-TTCE can give rise to hepatocellular carcinomas in mice, whereas only rare (not statistically significant) hepatocellular carcinomas and preneoplastic nodules were observed in rats.[8,9] The CBI of 1,1,2,2-TTCE is even higher than that of 1,2-dichloroethane (1,2-DCE),[10,11] a symmetric haloethane whose carcinogenicity and genotoxicity have been widely demonstrated. 1,1,2,2-TTCE is also capable of binding chromatin constituents, particularly the F_1 histone fraction (Colacci *et al.*, unpublished data). Moreover, 1,1,2,2-TTCE induced a significant increase in γ-glutamyl transpeptidase (GGT)(+) foci when administered in a promotion protocol with or without initiation with diethylnitrosamine (DEN).[12]

It has been recognized that the endpoint of *in vitro* cell transformation has direct relevance to carcinogenesis. The available data on chemicals show a good relationship between *in vitro* cell transformation and *in vivo* carcinogenesis (a 69 to 85% correlation has been obtained for the tested chemicals).[13,14] Thus, we have tested the *in vitro* transforming potential of 1,1,2,2-TTCE in an attempt to improve our knowledge of the molecular mechanisms by which this compound exerts carcinogenic effects.

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[5] Abbreviations used in this paper: 1,1,2,2-TTCE, 1,1,2,2-tetrachloroethane; CBI, covalent binding index; OPI, oncogenic potency index; 1,2-DCE, 1,2-dichloroethane; GGT, γ-glutamyl transpeptidase; DEN, diethylnitrosamine; 3-MCA, 3-methylcholanthrene; B(a)P, benzo[*a*]pyrene; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; NADP, nicotinamide adenine dinucleotide phosphate; NADH, nicotinamide adenine dinucleotide, reduced form; PB, phenobarbital; D-MEM, Dulbecco's modified Eagle's minimal essential medium; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

MATERIALS AND METHODS

Radiochemicals and Chemicals [U-^{14}C]-1,1,2,2-Tetrachloroethane (1,1,2,2-TTCE; chemical structure: $CHCl_2CHCl_2$; specific activity: 29 mCi/mmol; purity >98%) was obtained from The Radiochemical Centre, Amersham. Unlabeled 1,1,2,2-tetrachloroethane (>98% pure) and 3-methylcholanthrene (3-MCA) were obtained from Fluka, Switzerland, and benzo[*a*]pyrene (B(a)P) from Sigma Chemical Co., St. Louis, Mo. Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), nicotinamide adenine dinucleotide phosphate (NADP), nicotinamide adenine dinucleotide, reduced form (NADH), *d*-glucose-6-phosphate, glucose-6-phosphate dehydrogenase grade II, RNAse, and proteinase K were obtained from Boehringer Biochemia Robin, Milan. Phenobarbital (PB) was from BDH, Milan. Sodium dodecyl sulfate (SDS) was purchased from Pharmacia-LKB, Milan.

Cells and cell culture The original stock of BALB/c 3T3 cells, clone A-31, was from the American Type Culture Collection, Md. Working cultures were expanded from the original cryopreserved stock. Cells were grown in Dulbecco's modified Eagle's minimal essential medium (D-MEM, GIBCO) supplemented with 10% newborn calf serum (GIBCO). The antibiotics streptomycin and penicillin were present at 50 μg/ml and 50 units/ml, respectively. Only subconfluent (60–70% confluent) cultures were used in the assays and the target cells were not maintained beyond the second passage after thawing.

Preparation of S9 fraction and metabolic activation Adult male inbred Wistar rats, 250 g (from Charles River, Calco, Milan) were pretreated with PB (1 g/liter added to the drinking water) during the six days before being killed. The animals were sacrificed and the livers were removed aseptically. The excised tissue was rinsed in cold sterile 0.15 *M* KCl and then homogenized. The supernatant fraction (S 9) was collected following centrifugation in the cold at 9000*g* for 20 min, and portioned into aliquots, which, after measurement of the protein content, were stored at −80°C until use.

The amount of each component of the activating system was lowered with respect to that reported in the recommended protocol[14, 15] in order to reduce toxic effects on target cells observed in preliminary experiments. Thus, the final concentrations of activating system mixture (S9-mix) components were: 50 μl/ml S9 fraction, 2 μl/ml glucose-6-phosphate dehydrogenase, 0.2 m*M* glucose-6-phosphate, 0.28 m*M* NADH, 0.25 m*M* NADP, and 0.27 m*M* NADPH.

Binding test to cell DNA For *in situ* treatment, cells were seeded in 75 cm^2 Falcon flasks (Falcon Plastics, Los Angeles, Calif.) and, when the flasks were approximately half-confluent, ^{14}C-1,1,2,2-TTCE (2.5 μCi/flask) was added to the growth medium with or without S9 activating system. For each treatment, 15 flasks were utilized. Incubation was carried out for 24 h in the absence of S9-mix or for 4 h in its presence, then the monolayers were rinsed three times with phosphate-buffered saline (PBS), and the cells were scraped off and counted. Cells from three flasks were collected and centrifuged, and pellets were resuspended in 10 m*M* Tris·HCl-100 m*M* NaCl-1 m*M* EDTA, pH 8.0 at a concentration of 2×10^7 cells/ml. The suspension was incubated overnight at 50°C in the presence of proteinase K (100 μg/ml) and 0.5% sodium dodecyl sulfate (SDS), then extracted three times with saturated phenol, twice with chloroform-isoamylic alcohol mixture, and twice with ether. The aqueous solution was incubated at 37°C for 1 h in the presence of predigested RNAse (100 μg/ml), then with 100 μg/ml pronase at 37°C for 2 h and finally reextracted twice with phenol-chloroform (1:1) mixture. DNA was precipitated from the aqueous solution by adding two volumes of cold ethanol and then resuspended in 1 ml of 1 m*M* EDTA, pH 8.0, DNA recovery, purity and labeling were assayed by ultraviolet absorption measurement and counting in a liquid scintillation spectrometer (Beckman LS 5000TD). For the treatment in suspension, exponentially growing cells were suspended in conical tubes at a density of approximately 22×10^6 in 5 ml of PBS containing the S9 reaction mixture and 2.5 μCi ^{14}C-1,1,2,2-TTCE. Treatment was carried out in triplicate at 37°C in air under gentle agitation for 4 h. The DNA extraction procedure was then performed as reported above.

Cytotoxicity test Exponentially growing cells were seeded for each treatment at 500 cells/60-mm Falcon dish in five dishes per treatment. The plates were incubated at 37°C in a 5% CO_2 humidified atmosphere for 24 h. After this period, 1,1,2,2-TTCE dissolved in ethanol was added to the culture medium. In the presence of S9-mix activating system, incubation was carried out with serum-free medium. Six different concentrations were tested (10 μg/ml, 50 μg/ml, 100 μg/ml, 250 μg/ml, 500 μg/ml, 1000 μg/ml). Cells were exposed to the tested compound for 72 h in the absence of S9 activating system or for 4 h in the presence of S9 activating system. Negative control experiments (untreated cells and solvent vehicle-treated cells) were performed. The final solvent concentration (0.1%) was equal for all treatments. Following the exposure period, all treatment media were removed. Cells were washed with PBS and refed with 3 ml of complete growth medium. The medium was changed twice a week for the duration of the assay. Cells were maintained in culture for 7/10 days. At the end of this period, dishes were fixed with methanol, stained with 10% aqueous Giemsa and scored for colony formation. Only colonies larger than 50 cells were counted.

Transformation test Cell transformation assay was performed according to the recommended experimental protocol.[14, 15] Exponentially growing cells were seeded for each treatment at 1×10^4 cells/60-mm Falcon dish in 10 replicates per treatment. For each treatment a cytotoxicity test was also performed in two replicates. The plates were incubated at 37°C in a 5% CO_2 humidified atmosphere for 24 h. After this period, 1,1,2,2-TTCE dissolved in ethanol was added to the culture medium. The final solvent concentration (0.1%) was equal for all treatments. Four concentrations were tested (125 μg/ml, 250 μg/ml, 500 μg/ml, 1000 μg/ml), the most toxic dose being chosen as the highest assayed dose. Untreated cells and solvent vehicle-treated cells were used as negative controls. Positive control experiments were carried out utilizing 3-MCA or B(a)P as a transforming agent in the absence or in the presence of S9-mix activating system, respectively, at the concentration of 2.5 μg/ml.

In the absence of S9, cells were exposed to the test compound for 72 h. In the modified activation assay cells were incubated for 4 h in serum-free medium containing S9 activating system. At the end of the incubation period, all treatment media were removed, cells were washed with PBS and refed with 3 ml of complete growth medium. Plates from the cytotoxicity test were fixed, stained and scored for colony formation after 7/10 days. In the other plates the medium was changed twice a week until confluence was attained (about two weeks). At this time, a level-II transformation test was performed in order to obtain transformation amplification. Level-II transformation plates were generated by pooling the contents of five confluent plates (level-I transformation plates defined as the standard test) and reseeding cells at 1×10^5 or 2×10^5 cells/60 mm dish. Medium was changed twice a week. Level-I and level-II plates were fixed (methanol), stained (10% aqueous Giemsa) and scored for foci formation six weeks after the treatment.

Scoring of foci Only foci considered as positive (type III),[15, 16] showing deeply basophilic, dense multilayering of cells, random cell orientation at all parts of the focus edge, invasion into the surrounding contact-inhibited monolayer and domination of spindle-shaped cells, were counted. Foci of less than 2 mm in diameter were not scored. Transformation frequencies were estimated by calculating 1) the total number of foci per group of scored plates; 2) the average number of foci per plate; 3) the total number of foci per group divided by the number of cells at risk (cells surviving after treatment) estimated from the clonal efficiency observed in the cytotoxicity assay.

Tumorigenicity testing Transformed foci obtained by 1,1,2,2-TTCE treatment as well as those obtained in the positive controls were isolated. The cells were allowed to multiply in minimal essential medium. Groups of five nude mice (Charles River) per treatment were sc injected with 5×10^6 cells of each clone in a volume of 200 μl of PBS. Transformed cells from plates treated with 1000 μg/ml in the absence of S9-mix or with 500 μg/ml in the presence of metabolic activation were assayed for tumorigenicity.

RESULTS

^{14}C-1,1,2,2-TTCE covalently bound to DNA of BALB/c 3T3 cells both in the absence and in the presence of metabolizing fractions (Table I). In the absence of S9 activating system, binding to DNA was higher than in its presence.

In Table II, the results of cytotoxicity test are reported. In the absence of S9-mix activating system the highest assayed dose (1000 μg/ml) was a toxic dose leading to a reduction of cloning efficiency (about 30%) with respect to that of the solvent control. In the presence of S9-mix activating system, the doses of 500 μg/ml and 1000 μg/ml exerted toxic effects. Thus, the high dose employed in the transformation test was selected to give the highest toxicity.

No transformation foci were obtained in the standard transformation assay (level-I) in the absence of metabolic activation and only a few foci, too small or not fully corresponding to the morphologic characteristics of "mature" type III foci, were recognized in the level-I standard assay performed in the presence of S9-mix (Table III). No or very few foci were obtained in the untreated BALB/c 3T3 cells cultured under the same experimental conditions as the treated cells (data not shown).

Tables IV and V summarize the results from level-II amplification cell transformation experiments. The transformation frequency in solvent control groups both in the absence and in the presence of metabolic activation was

Table I. Binding of ^{14}C-1,1,2,2-Tetrachloroethane (1,1,2,2-TTCE) to DNA and Protein of BALB/c 3T3 Cells in the Presence or in the Absence of Metabolic Activation[a)]

Treatment	Specific activity (pmol/mg)	
	DNA	Protein
In situ (−)S9 activating system	54.95±4.68	2279.60±191.38
In situ (+)S9 activating system	7.50±1.43	331.20±36.17
In suspension (+)S9 activating system	20.53±2.17	80.61±18.20

a) Data are reported as mean values of three replicates±SE, each from five treated flasks of cells.

Table II. Cytotoxic Effects of 1,1,2,2-Tetrachloroethane (1,1,2,2-TTCE) on BALB/c 3T3 Cells in the Absence or in the Presence of the Exogenous Metabolic Activation System[a)]

Treatment	In the absence of activating system		In the presence of activating system	
	Mean no. colonies/ plate scored	Relative clonal efficiency (%)[b)]	Mean no. colonies/ plate scored	Relative clonal efficiency (%)[b)]
Untreated cells	69.4±5.5	112	28.7±5.3	80
Ethanol-treated cells	61.6±7.6	100	35.7±7.6	100
10 μg/ml 1,1,2,2-TTCE	49.7±4.8	80.7	25.5±5.6	71
50 μg/ml 1,2,2,2-TTCE	61.2±2.4	99.3	21.9±3.2	61
100 μg/ml 1,1,2,2-TTCE	61.3±5.4	99.5	27.6±4.8	75
125 μg/ml 1,1,2,2-TTCE	59.2±4.7	96.1	31.7±6.5	89
250 μg/ml 1,1,2,2-TTCE	60.2±7.2	97.7	32.7±4.9	92
500 μg/ml 1,1,2,2-TTCE	45.1±6.0	73.2	23.3±4.1	65
1000 μg/ml 1,1,2,2-TTCE	35.7±7.5	58.0	13.3±4.1	37

a) Data are reported as mean values of five replicates±SE from two different experiments.
b) Values are expressed as total number of colonies per treatment relative to the negative control.

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

Treatment	Plates with foci/plates scored	Mean no. of transformed foci/plate	TF[b)] ($\times 10^{-4}$)
Untreated cells	0/5	0.00±0.00	0.00
Ethanol-treated cells	1/5	0.20±0.20	3.51
B(a)P (2.5 μg/ml)[c)]	3/4	0.75±0.25	4.84
1,1,2,2-TTCE (125 μg/ml)	1/2	0.50±0.50	7.94
1,1,2,2-TTCE (250 μg/ml)	0/4	0.00±0.00	0.00
1,1,2,2-TTCE (500 μg/ml)	1/4	0.25±0.25	5.30
1,1,2,2-TTCE (1000 μg/ml)	1/4	0.25±0.25	9.26

a) Only positive foci (type III) not smaller than 2 mm have been considered. In the absence of S9-mix activation no foci having these characteristics were scored.
b) Transformation frequency expressed as the number of transformation foci per number of cells at risk after chemical treatment.
c) Dissolved in dimethylsulfoxide (DMSO) which, at the final concentration employed (0.25%), did not exert any transforming activity.

very low and did not exceed the maximum allowed by the recommended protocol. Also, positive controls induced a significant number of transformed foci relative to the negative control. In the absence of exogenous metabolic activation, 1,1,2,2-TTCE was capable of inducing foci at the highest assayed dose, especially when cells from the level-I transformation test (from which no foci were obtained) were reseeded at 1×10^5 cells/plate (Table IV). In the presence of S9-mix (Table V) marked transforming activity by 1,1,2,2-TTCE and positive dose-response correlations were observed up to the dose of 500 μg/ml, exerting the highest transforming activity. The number and the size of transformed foci were more evident when cells were reseeded at a number of 1×10^5 per plate.

Transformed cells from 1,1,2,2-TTCE-treated plates at the dosage of 500 μg/ml or 1000 μg/ml as well as from positive controls induced tumors within two months in all injected mice.

DISCUSSION

1,1,2,2-TTCE is considered the most toxic and carcinogenic chloroethane. It is also the most effective among the chloroethanes we studied[5,17] in interacting with DNA, reaching an *in vivo* CBI value typical of moderate initiators, according to Lutz's classification,[6] and even higher than that detected for 1,2-DCE, which is carcinogenic in rodents as found in long-term tests and

Table IV. *In vitro* BALB/c 3T3 Cell Transformation (Level-II) (Amplification) by 1,1,2,2-Tetrachloroethane in the Absence of Exogenous Metabolic Activation[a)]

Treatment	Cells replated at 2×10^5/plate			Cells replated at 1×10^5/plate		
	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)] ($\times10^{-4}$)
Experiment I						
Untreated cells	0/5	0.00±0.00	0.00	0/5	0.00±0.00	0.00
Ethanol-treated cells	1/4	0.50±0.50	6.36	1/3	0.33±0.33	4.20
3-MCA (2.5 μg/ml)[c)]	5/5	3.00±0.63	19.35	4/4	4.25±0.48	27.42
1,1,2,2-TTCE (125 μg/ml)	2/4	0.75±0.48	8.06	3/5	0.60±0.25	6.45
1,1,2,2-TTCE (250 μg/ml)	0/7	0.00±0.00	0.00	0/4	0.00±0.00	0.00
1,1,2,2-TTCE (500 μg/ml)	0/5	0.00±0.00	0.00	0/5	0.00±0.00	0.00
1,1,2,2-TTCE (1000 μg/ml)	5/5	5.20±0.86	173.33	3/3	7.33±0.67	244.33
Experiment II						
Untreated cells	2/10	0.20±0.13	1.84	0/10	0.00±0.00	0.00
Ethanol-treated cells	1/8	0.12±0.12	1.53	2/7	0.33±0.33	4.20
Acetone-treated cells[d)]	1/10	0.10±0.10	ND	7/7	3.71±0.47	ND
3-MCA (2.5 μg/ml)[e)]	ND	ND	ND	11/11	8.45±0.91	54.52
1,1,2,2-TTCE (125 μg/ml)	ND	ND	ND	ND	ND	ND
1,1,2,2-TTCE (250 μg/ml)	ND	ND	ND	ND	ND	ND
1,1,2,2-TTCE (500 μg/ml)	ND	ND	ND	ND	ND	ND
1,1,2,2-TTCE (1000 μg/ml)	9/10	1.90±0.41	63.33	9/9	4.44±0.67	148.00

a) Results from two independent cell transformation experiments are reported. Confluent cells from level-I (standard) transformation were reseeded at 2×10^5 cells/plate or 1×10^5 cells/plate.
b) Transformation frequency expressed as the number of transformation foci per number of cells at risk after chemical treatment.
c) Dissolved in dimethylsulfoxide (DMSO) which, at the final concentration employed (0.25%), did not exert any transforming activity.
d) Solvent-treated cells used as the negative control for 3-MCA-treated cells.
e) Dissolved in acetone.
ND=not determined.

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

Treatment	Cells replated at 2×10^5/plate			Cells replated at 1×10^5/plate		
	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)] ($\times10^{-4}$)
Untreated cells	3/10	0.30±0.15	5.26	2/7	0.29±0.18	5.09
Ethanol-treated cells	3/9	0.33±0.17	4.62	3/10	0.40±0.22	5.60
B(a)P (2.5 μg/ml)[c)]	4/10	0.50±0.22	3.22	10/10	6.10±0.48	39.35
1,1,2,2-TTCE (125 μg/ml)	6/9	0.78±0.22	12.38	10/10	3.40±0.72	53.97
1,1,2,2-TTCE (250 μg/ml)	4/6	0.67±0.21	10.30	8/8	2.75±0.37	58.51
1,1,2,2-TTCE (500 μg/ml)	9/9	3.33±0.67	70.85	3/3	19.33±1.45	411.27
1,1,2,2-TTCE (1000 μg/ml)	10/10	4.10±0.60	151.85	3/3	5.20±1.39	192.59

a) Confluent cells from level-I (standard) transformation were reseeded at 2×10^5 cells/plate or 1×10^5 cells/plate.
b) Transformation frequency expressed as the number of transformation foci per number of cells at risk after chemical treatment.
c) Dissolved in dimethylsulfoxide (DMSO) which, at the final concentration employed (0.25%), did not exert any transforming activity.

also genotoxic as demontrated by positive results in many different short-term tests. 1,1,2,2-TTCE is capable of inducing a significant excess of liver tumors in the mouse (data from carcinogenicity bioassay are too limited to draw any conclusion as to its carcinogenicity in the rat). The transforming activity of 1,1,2,2-TTCE was analyzed in a previous study[18] using a standard transformation assay, in the absence of exogenous activating systems and employing doses up to 250 μg/ml. No significant increment of transformation foci with respect to controls were observed under these experimental conditions.[18] The reported data show that this compound is capable of inducing *in vitro* transformation in BALB/c 3T3 cells in a cell amplification-transformation assay. This replating process allows additional rounds of cell replication which may be required for complete expression of the transformed phenotype as judged by the numerous level-II transformed foci exhibiting the characteristic morphological aberrations recognized in the amplification assay. Indeed, in the standard transformation test performed in the absence of replating, only a few foci were observed which were too small and did not exhibit the morphological characteristics of a fully "matured" transformed focus. The amplification of the transformation is even more evident when cells were replated at a number of 1×10^5 cell/plate, thus allowing even more additional rounds.

Moreover, 1,1,2,2-TTCE requires metabolic activation in order to become biologically reactive.[5] Thus, the limited inherent metabolizing activity associated with BALB/c 3T3 cells has necessitated supplementing the endogenous metabolic capacity with exogenous enzymatic fractions. Indeed, in the absence of S9-mix no transformation foci were detected at doses lower than 1000 μg/ml, a four times higher dose than that assayed previously[18] which is also the only one exerting toxic effects among the assayed doses. It has been reported that cytotoxic chemical products formed through metabolism of compounds requiring bioactivation are similar to those which transform cells, either qualitatively or quantitatively and even 80–90% toxicity is often required to help ensure sufficient metabolism in the production of transforming products.[13] Conversely, lower non-toxic doses of 1,1,2,2-TTCE are capable of transforming BALB/c cells in the presence of the activating system, although the greatest number of transformation foci was obtained when the doses also eliciting toxic activity were employed, 500 μg/ml showing the highest transforming ability. Foci obtained at this dosage are larger in size, and more deeply basophilic, exhibiting denser multilayering of constituent cells. In contrast, no particular differences seem to exist between foci recognized at the highest assayed dose (1000 μg/ml) in the presence or in absence of exogenous metabolic activation with regard to the transformation frequency, size and morphologic characteristics.

The molecular mechanisms of 1,1,2,2-TTCE induction of transformation remain to be identified. Both initiating and promoting activity by this compound could be involved. The genotoxic properties of 1,1,2,2-TTCE have been described.[3, 5] It is metabolized through oxidative steps which involve the microsomal mixed function oxidase system and microsomal and cytosolic GSH-transferase(s), all dependent on PB-inducible forms of cytochrome P450.[5] Reactive products are electrophilic enough to react covalently with nucleophilic sites of biological macromolecules.[5]

Like other haloalkanes, except for 1,2-dibromoethane, whose major DNA-adduct has been identified as S-[2-(N^7-guanyl)ethyl]glutathione,[19] the specific DNA-adduct(s) formed by 1,1,2,2-TTCE reactive metabolite(s) is unknown as yet. Nevertheless, more than one product of 1,1,2,2-TTCE metabolism may be capable of interaction with biological macromolecules. The main metabolic product of 1,1,2,2-TTCE, identified as dichloroacetyl chloride, can bind covalently to macromolecules or be hydrolyzed to dichloroacetic acid. Moreover, products formed by GSH-conjugation as well as free radicals can cooperate in the genotoxic process involving 1,1,2,2-TTCE.[5] 1,1,2,2-TTCE is also capable of binding chromatin constituents, particularly the lysine-rich histone defined as F_1, leading to some modification which may cause a change within the nucleosome, possibly affecting transcription of the immediate genome (Colacci *et al.*, unpublished data). The genotoxic activity of 1,1,2,2-TTCE is confirmed by its ability to bind covalently DNA of BALB/c 3T3 cells both in the presence and in the absence of metabolic activation. The binding extent to BALB/c DNA in the absence of S9-mix is similar to that obtained in an *in vitro* cell-free system utilizing calf thymus DNA as the binding acceptor.[5] In the presence of the exogenous activating system binding to DNA is lower probably because of competitive reactions between endogenous and exogenous metabolizing activities and/or to detoxifying mechanisms triggered by the supplemented enzymes. Also, the S9 itself may serve as a nucleophilic receptor for reactive electrophilic metabolites. Moreover, S9 poorly catalyzes, if at all, the GSH-conjugation whose products may be responsible for DNA binding to a greater extent than other reactive metabolites. Nevertheless, the lower binding to DNA in the presence of S9-mix does not affect the transformation response, which is probably the result of both initiating and promoting effects exerted by 1,1,2,2-TTCE. Indeed, promoting activity of this compound has been postulated.[12] Generally, promoting agents give mixed results with regard to the relationship between *in vitro* transformation and *in vivo* carcinogenesis. We are performing

further studies in order to clarify the role of 1,1,2,2-TTCE in the multistep carcinogenesis.

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REFERENCES

1) Torkelson, T. R. and Rowe, V. K. Halogenated haliphatic hydrocarbons. *In* "Patty's Industrial Hygiene and Toxicology," ed. G. D. Clayton, Vol. 2, part B, pp. 3513–3516 (1981). John Wiley & Sons, Inc., New York.
2) IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans, Vol. 20. "Some Halogenated Hydrocarbons" (1979). International Agency for Research on Cancer, Lyon.
3) IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans, Suppl. 6. "Genetic and Related Effects: an Updating of Selected IARC Monographs from Volumes 1 to 42," (1987). International Agency for Research on Cancer, Lyon.
4) Macdonald, T. L. Chemical mechanisms of halocarbon metabolism. *CRC Crit. Rev. Toxicol.*, **11**, 85–120 (1983).
5) Colacci, A., Grilli, S., Lattanzi, G., Prodi, G., Turina, M. P. and Mazzullo, M. The covalent binding of 1,1,2,2-tetrachloroethane to macromolecules of rat and mouse organs. *Teratog. Carcinog. Mutag.*, **7**, 465–474 (1987).
6) Lutz, W. K. *In vivo* covalent binding of organic chemicals to DNA as a quantitative indicator in the process of chemical carcinogenesis. *Mutat. Res.*, **65**, 289–356 (1979).
7) Parodi, S., Taningher, M., Zunino, A., Ottaggio, L., De Ferrari, M. and Santi, L. Quantitative predictivity of carcinogenicity for sister chromatid exchanges *in vivo*. *In* "Sister Chromatid Exchanges," ed. R. R. Tice and A. Hollaender, pp. 409–429 (1984). Plenum Publishing Corporation, New York.
8) National Cancer Institute Technical Report Series. Bioassay of 1,1,2,2-tetrachloroethane in F344/N rats and B6C3F1 mice (gavage studies). DHEW Publication (NIOH) 78-827. Cas no 79-34-5 (1978).
9) National Toxicology Program. Carcinogenic studies of 1,1,2,2-tetrachloroethane in F344/N rats and B6C3F1 mice (gavage studies). NTP Technical Report Series: No. 237, p. 146 (1983).
10) Arfellini, G., Bartoli, S., Colacci, A., Mazzullo, M., Galli, M. C., Prodi, G. and Grilli, S. *In vivo* and *in vitro* binding of 1,2-dibromoethane and 1,2-dichloroethane to macromolecules in rat and mouse organs. *J. Cancer Res. Clin. Oncol.*, **108**, 204–213 (1984).
11) Colacci, A., Arfellini, G., Mazzullo, M., Prodi, G. and Grilli, S. *In vitro* microsome- and cytosol-mediated binding of 1,2-dichloroethane and 1,2-dibromoethane with DNA. *Cell. Biol. Toxicol.*, **1**, 45–55 (1985).
12) Milman, H. A., Story, D. L., Riccio, E. S., Sivak, A., Tu, A. S., Williams, G. M., Tong, C. and Tyson, C. A. Rat liver foci and *in vitro* assays to detect initiating and promoting effects of chlorinated ethanes and ethylenes. *Ann. N.Y. Acad. Sci.*, **534**, 521–530 (1988).
13) Heidelberger, C., Freeman, A. E., Pienta, R. J., Sivak, A., Bertram, J. S., Casto, B. C., Dunkel, V. C., Francis, M. W., Kakunaga, T., Little, J. B. and Schechtman, L. M. Cell transformation by chemical agents — a review and analysis of the literature. A report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutat. Res.*, **114**, 283–385 (1983).
14) IARC/NCI/EPA Working Group. Cellular and molecular mechanisms of cell transformation and standardization of transformation assays of established cell lines for the prediction of carcinogenic chamicals: overview and recommended protocols. *Cancer Res.*, **45**, 2395–2399 (1985).
15) Schechtman, L. M. BALB/c 3T3 cell transformation: protocols, problems and improvements. *In* "Transformation Assay of Established Cell Lines: Mechanisms and Application," ed. T. Kakunaga and H. Yamasaki, IARC Sci. Publ. 67, pp. 165–184 (1985). International Agency for Cancer Research on Cancer, Lyon.
16) IARC/NCI/EPA Working Group. Recommendations for experimental protocols and for scoring transformed foci in BALB/c 3T3 and C3H10T1/2 cell transformation. *In* "Transformation Assay of Established Cell Lines: Mechanisms and Application," ed. T. Kakunaga and H. Yamasaki, IARC Sci. Publ. 67, pp. 207–223 (1985). International Agency for Cancer Research on Cancer, Lyon.
17) Prodi, G., Colacci, A., Grilli, S., Lattanzi, G., Mazzullo, M. and Turina, P. Comparison of the covalent binding of various chloroethanes with nucleic acids. *In* "Chemical Carcinogenesis," ed. F. Feo, P. Pani, A. Columbano and R. Garcea, pp. 93–102 (1988). Plenum Publishing Corporation, New York.
18) Tu, A., Murray, T. A., Hatch, K. M., Sivak, A. and Milman, H. A. *In vitro* transformation of BALB/c 3T3 cells by chlorinated ethanes and ethylenes. *Cancer Lett.*, **28**, 85–92 (1985).
19) Inskeep, P. B., Koga, N., Charik, S. J. and Guengerich, F. P. Covalent binding of 1,2-dihaloalkanes to DNA and stability of the major DNA adduct, S-[2(N^7-guanyl)ethyl]-glutathione. *Cancer Res.*, **46**, 2839–2844 (1986).