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Results of *In Vitro* and *In Vivo* Genetic Toxicity Tests on Methyl Isocyanate

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Methyl isocyanate (MIC) was tested for genetic toxicity in a variety of *in vitro* and *in vivo* assays. Negative results were obtained in the Salmonella/mammalian microsome assay using five bacterial strains in a preincubation protocol. The Drosophila sex-linked recessive lethal test also gave negative results in studies that involved three routes of administration: inhalation, feeding, and injection. Positive results were obtained for three endpoints in cultured mammalian cells. Reproducible, dose-related increases in trifluorothymidine-resistant clones were induced in L5178Y mouse lymphoma cells, and the frequencies of both SCE and chromosomal aberrations increased in Chinese hamster ovary cells. These effects were independent of exogenous metabolism. In mice exposed to methyl isocyanate by inhalation, cytogenetic analyses were carried out on bone marrow, blood, and lung cells. A single, 2-hr exposure to concentrations of 0, 3, 10, and 30 ppm MIC produced no evidence of chromosomal effects in the bone marrow, although significant cell cycle delay was observed. In four experiments involving exposures on 4 consecutive days to 0, 1, 3, or 6 ppm, delays in bone marrow cell cycle were again observed. Increases in SCE and chromosomal aberrations were observed in bone marrow cells, and a dose-related increase in SCE occurred in lung cells but not in peripheral blood lymphocytes. A significant increase in micronucleated polychromatic erythrocytes in the peripheral blood was observed in male mice in one experiment.

From these results, it appears that methyl isocyanate has the capacity to affect chromosome structure but not to induce gene mutations. Furthermore, *in vitro* tests show that the induction of chromosomal effects is not dependent on an exogenous source of metabolism. Based on these results and on what is known about the binding of carbamoylating agents to cellular macromolecules, methyl isocyanate may exert its genetic toxicity by binding to nuclear proteins rather than by binding to DNA.

Introduction

Methyl isocyanate (MIC) was assayed in a number of *in vitro* and *in vivo* genetic toxicity tests to determine its ability to interact with DNA and/or to induce genetic damage. *In vitro* tests included the Salmonella/mammalian microsome test for bacterial gene mutations, the L5178Y mouse lymphoma cell test for thymidine kinase mutants, and tests for chromosomal aberrations and sister chromatid exchanges (SCE) in cultured Chinese hamster ovary (CHO) cells. *In vivo* studies were conducted using the sex-linked recessive lethal test in *Drosophila melanogaster*; tests for SCE in bone marrow, lung, and blood cells; and tests for chromosomal aberrations and micronuclei in bone marrow cells of mice exposed to MIC by inhalation. This paper presents summaries of test results presented elsewhere (*1–4*).

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Results

Salmonella

The Salmonella/mammalian microsome assay was conducted using a preincubation protocol employing strains TA1535, TA1537, TA97, TA98, and TA100 without metabolic activation and with S9 from Aroclor 1254-induced male Sprague-Dawley rats and similarly in-

Table 1. Methyl isocyanate: summary of genetic toxicity test results from *in vitro* and Drosophila assays.

Assay	Dose range	Level of effect[a]	Test result
Salmonella (−S9)	0.3–333 μg/plate	No effect	Negative
(+S9)	0.3–333 μg/plate	No effect	Negative
L5178Y TK$^{+/-}$ (−S9)	0.5–3.0 nL/mL	5× (trial 1)	Positive
		13× (trial 2)	Positive
Methylamine[b] (+S9)	50–400 nL/ML	2.4×	Positive
N,N'-dimethylurea[b] (−S9)	500–5000 μg/mL	No effect	Negative
(+S9)	500–5000 μ/mL	No effect	Negative
N-methylcarbamic acid, ethyl ester[b] (−S9)	500–5000 nL/mL	No effect	Negative
(+S9)	500–5000 nL/mL	2.2× (trial 1)	Positive
		2.5× (trial 2)	Positive
		1.8× (trial 3)	Positive
CHO–SCE (−S9)	0.3–3.1 μg/mL	1.9× (trial 1)	Positive
		1.4× (trial 2)	Positive
(+S9)	0.3–3.1 μg/mL	1.6×	Positive
CHO–CA (−S9)	10–25 μg/mL	22×	Positive
(+S9)	15–25 μg/mL	30×	Positive
Drosophila			
(inhalation)	14 ppm in air	No effect	Negative
(feeding)	600 μg/mL in food	No effect	Negative
(injection)	260 μg/mL (trial 1)	No effect	Negative
	350 μg/mL (trial 2)	No effect	Negative

[a] Greatest fold increase over the concurrent solvent control value.
[b] Hydrolysis products of MIC.

duced Syrian hamsters. Doses, at half log intervals, from 0.3 to 333 μg/plate failed to induce an increased frequency of revertant colonies under any of the test conditions employed. Toxicity, as evident from a thinning of the background lawn of bacteria, was noted at the higher doses (33, 100, or 333 μg/plate) in all tests, confirming that the bacteria were exposed to MIC or a breakdown product of MIC (*3*).

L5178Y Mouse Lymphoma Cells

Cultured L5178Y mouse lymphoma cells were used to test MIC for the induction of thymidine kinase mutants selected for resistance to trifluorothymidine. In the absence of exogenous metabolic activation, MIC was tested at six doses from 0.5 to 3.0 nL/mL in the medium. Two trials yielded reproducible, dose-related increases in the fraction of trifluorothymidine-resistant mutants. In both trials, the mutant fraction at the highest dose was more than five times that for the solvent control, indicating a clear mutagenic effect for MIC in this assay. In both trials, mutants that recovered from the high dose treatment were subjected to colony sizing analysis. Both trials produced a preponderance of small colony mutants, suggesting that the primary type of damage induced may have been chromosomal aberrations.

Some of the hydrolysis products of MIC were also tested in this assay. *N,N'*-dimethylurea gave negative results in the presence and absence of rat liver S9. Methylamine was mutagenic in two trials in the absence of S9, but effects were seen only at 200 to 300 nL/mL, whereas much higher mutant frequencies were seen with MIC at 3 nL/mL. The ethyl ester of *N*-methylcarbamic acid was shown to be weakly mutagenic in the presence of rat liver S9, giving no more than a doubling of the mutant fraction at doses up to 5000 nL/mL (*1*).

CHO Cytogenetics

The induction of chromosomal aberrations and SCE by MIC was assessed in cultured CHO cells in the presence and absence of Aroclor-1254 induced rat liver S9. Tests for SCE induction were reproducibly positive with and without S9 over a dose range from 0.3 to 3 μg/mL. SCE induction occurred at doses of MIC that resulted in extended cell cycles. Chromosomal aberration tests were conducted at doses ranging from approximately 10 to 25 μg/mL MIC. Test results were positive both in the absence and presence of S9. Again, cell cycle delay occurred, and cells were harvested at 20 hr rather than the standard 12.5-hr harvest time (*3*).

Drosophila

Sex-linked recessive lethal tests were conducted in Drosophila using three different routes of administration to expose males to MIC. MIC was tested up to toxic doses by all three routes (feeding, inhalation, and injection). MIC gave negative results under all conditions used in these studies (*3*) (Table 1).

Table 2. Summary of results from cytogenetic assays on mice exposed to MIC by inhalation.

A. Bone marrow analyses: single 2-hr exposure to 0, 3, 10, or 30 ppm.

Sex	Expt.#	CA	SCE	AGT	MN-PCE
M	I	0	0[a]	+	0
M	II	0	0[a]	+	0

B. Bone marrow, blood, and lung analyses: 4-day (6 hr/day) exposure regimen.

			Bone marrow			Peripheral blood		Lung Cells
Sex	Expt.#	MIC (ppm)	CA	SCE	AGT	MN-PCE	SCE-PBL	SCE
M	I	0,1,3	0	+	+	0	—	—
	II	0,1,3	0	0	+	0	—	—
	III	0,1,3	NS	0[b]	+	0	0	—
	IV	0,1,3,6	+	0[c]	+	+	0	—
F	I	0,1,3	0	0	0	0	—	—
	II	0,1,3	+	0	0	0	—	—
	III	0,1,3	NS	+	0	0	—	+
	IV	0,1,3,6	+	+	+	0	—	+

CA = chromosomal aberration; SCE = sister chromatid exchange; AGT = average generation time; MN = micronucleus; PCE = polychromatic erythrocyte; PBL = peripheral blood lymphocyte. NS = nonscorable; 0 = no significant difference; — = no data obtained; + = significant difference at $p \leq 0.05$.

[a] Highest concentration tested was 10 ppm.

[b] Highest concentration evaluated was 1 ppm.

[c] Highest concentration evaluated was 3 ppm.

Mouse Bone Marrow Cytogenetics: Chromosomal Aberrations (CA), SCE, and Micronuclei (MN)

A series of experiments was conducted to assess the ability of MIC to induce chromosomal effects in the bone marrow cells of B6C3F1 mice exposed via inhalation (*4*). Two exposure regimens were used. In the first regimen, two groups of male mice were exposed to 3, 10, and 30 ppm MIC for a single 2-hr period. In the second regimen, male and female mice were exposed to MIC for 6 hr a day on 4 consecutive days. Four experiments were conducted using this regimen. The first three experiments included test groups exposed to 1 and 3 ppm; a 6 ppm exposure group was included in the final 4-day exposure experiment. Test results are presented in Table 2. For details on exposure, see Adkins et al. (*5*).

The 2-hour exposure of male mice at 3, 10, and 30 ppm did not significantly affect frequencies of CA, SCE, or MN in the bone marrow. The only significant bone marrow effect in these two experiments was a marked increase in the cell cycle time. In the 30 ppm groups, cell cycle times were estimated to be more than twice the normal 11 to 13 hr-cycle.

In the four, 4-day exposure experiments, bone marrow cell cycle delay was again evident in treated males. Only in the final experiment, which included a 6 ppm exposure group, was this effect seen in females.

Significantly elevated frequencies of CA occurred in both sexes in the 4-day exposure experiment that included the 6 ppm group, and in females an elevated chromosomal aberration frequency was seen in one earlier 4-day exposure experiment at 3 ppm. SCE frequencies were significantly elevated in females in two experiments, including the experiment with the 6 ppm group. An increase in SCE frequency was also found in males in the first 4-day exposure experiment, but subsequent experiments were negative. These negative results may be attributable to the fact that, due to cell cycle delays, the high dose groups of males could not be scored for SCE in the third and fourth experiments because there were no (or inadequate) second division cells to score.

The frequency of MN polychromatic erythrocytes (PCE) and normochromatic erythrocytes in the peripheral blood was determined in both sexes in all 4-day experiments. The only significant elevation in incidence of micronucleated cells was in the MN-PCE of males in the 6 ppm group of the last experiment. Table 2 presents a summary of cytogenetic results.

SCE in Mouse Lung Cells and Peripheral Blood Lymphocytes (PBL)

Groups of mice were included in two experiments to conduct SCE studies on PBL and primary lung cells obtained by perfusion with trypsin, EDTA, and collagenase (Table 2). Because of technical difficulties, final results were limited to lung cells from females and PBL from males. In both experiments with cultured lung cells, a dose-related significant elevation in SCE was observed. SCE results from PBL determinations were uniformly negative when compared to the concurrent controls. It was noted that in both experiments, leukocyte counts were significantly reduced in the high dose groups (*2*).

Discussion

As a carbamoylating agent (R–N = C = O), MIC might be expected to react with nucleophilic sites on cellular macromolecules, including proteins, RNA, and DNA. Isocyanates have been studied to a very limited extent in biological systems, because of their extreme volatility and toxicity. One report was found on the direct interaction between nucleic acid bases and isocyanates (*6*), where carbamoylation of both endocyclic and exocyclic nitrogens were reported. Most of the literature on carbamoylation of nucleic acids and proteins has come from studies of dialkylnitrosoureas that have been proposed or used as cancer chemotherapeutic agents. These compounds are known to decompose under physiological conditions to yield alkylating and carbamoylating moieties, the latter being an isocyanate (*7,8*).

The carbamoylation of proteins by alkylnitrosoureas

is well established (*9–11*). Carbamoylation by dialkylnitrosoureas has also been reported for DNA and RNA bases (*12*), DNA and polynucleotides (*12–14*), and RNA (*15*). With respect to the possible mutagenic effects of carbamoylation, a key set of experiments was reported on the binding of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) to nucleic acids and proteins (*10*). Using CCNU labeled in either the ethylene or cyclohexyl group, Cheng et al. determined the relative binding of these two groups to isolated nucleic acids and proteins, to nucleic acids and proteins in cultured L1210 leukemia cells, and to proteins and nucleic acids in L1210 leukemia-bearing mice. With all three experimental conditions, the labeled ethyl group was found to bind extensively to both nucleic acids and proteins, while the labeled cyclohexyl moiety, presumably present as a cyclohexylcarbamoyl group, was bound almost exclusively to protein.

A case can be made that the genetic toxicity of MIC reported in this paper might be due in whole or in part to MIC disruption of the integrity of eukaryotic chromosomes through interaction with nuclear proteins. Although speculative, this possibility is supported by reports that alkylnitrosoureas lead to carbamoylation of the chromatin (*16,17*) and the nuclear matrix (*18*).

The negative results obtained with MIC in the Salmonella and Drosophila assays suggest that MIC may not induce base pair changes in DNA. The positive results in the L5178Y mouse lymphoma cell assay, an assay originally designed to detect gene mutations, may reflect the capacity of this assay to detect trifluorothymidine-resistant mutants that have lost thymidine kinase activity as the result of chromosomal rearrangements; a mechanism for which evidence has been published (*19*). The other assays, including the *in vitro* cytogenetics test in CHO cells and the *in vivo* studies on inhalation-exposed mice, all rely on chromosomal endpoints—aberrations, micronuclei, and sister-chromatid exchanges.

In conclusion, MIC gave negative results in the Salmonella/mammalian microsome assay and the Drosophila sex-linked recessive lethal test, but was found to increase the frequency of aberrations and SCE in cultured CHO cells and trifluorothymidine-resistant clones in cultured L5178Y cells. In mice exposed to MIC by inhalation for 4 days, there were increases in chromosomal aberrations, SCE, and micronuclei in bone marrow cells, and increased SCE frequencies in lung cells but not in peripheral blood lymphocytes. It is noteworthy that no cytogenetic effects were observed in the bone marrow of mice exposed for 2 hr to near lethal doses of MIC. However, MIC was found to reproducibly delay bone marrow cell cycle time in male mice subjected to single or multiple exposures.

These results, from both *in vitro* and *in vivo* tests, provide convincing evidence that MIC is capable of inducing chromosomal damage, and that this genetic toxicity is not strongly expressed *in vivo*, perhaps because of the selective reactivity of MIC with proteins. Although carbamoylation of DNA has been implicated in the induction of mutations (*14*), based on what is known about the binding of isocyanates to cellular macromolecules, the interesting possibility that the chromosomal effects of MIC reported here may result from the binding of MIC to DNA-associated proteins rather than directly to DNA cannot be ruled out.

The research described in this article has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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