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Comparative genotoxicity of the herbicides Roundup, Stomp and Reglone in plant and mammalian test systems

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The genotoxicities of the herbicides Roundup (glyphosate), Stomp (pendimethaline) and Reglone (diquat), were compared in plant (*Crepis capillaris L.*) and mouse bone marrow test systems using chromosomal aberrations and micronuclei. Roundup did not induce chromosomal aberrations or micronuclei in either test system. Reglone also did not induce chromosomal aberrations in either test system; however, it increased micronucleus frequency in both plant cells and mouse bone marrow polychromatic erythrocytes (PCEs). The responses of the two test systems to Stomp were quite different. Stomp did not induce chromosomal aberrations in the plant cells, but increased their incidence in mouse cells; Stomp increased the frequency of micronuclei in both test systems. The induction of micronuclei in plant cells may have been due to the spindle-destroying effect of the herbicide, since all concentrations of Stomp produced C-mitoses. The increased chromosomal aberration frequency in mouse bone marrow cells observed at later sampling times after administration of Stomp into animals suggests that the induction of aberrations may be due to biosynthesis of genotoxic metabolites. This conclusion was supported by the coincidence between the frequencies of chromosomal aberrations and of micronucleated PCEs in mouse cells. These data indicate that plant and animal assays are differentially responsive to some pesticides, and these differences may be due to metabolism and their responses to mitotic spindle disruption.

Introduction

Pesticides, including herbicides, insecticides and fungicides, are used extensively to improve crop yields and as a result, they accumulate in the environment. More than 2.5 million tons of pesticides are applied every year to agricultural crops worldwide (1). Pesticides tend to be very reactive compounds that can form covalent bonds with various nucleophilic centres of cellular biomolecules, including DNA (2). Because of their biological activity, the use of pesticides may cause undesired effects to human health. For instance, the induction of DNA damage can potentially lead to adverse reproductive outcomes, the induction of cancer and many other chronic diseases (3–6). Although studies on the biological effects of currently used pesticides have increased in recent years, there are often incomplete, and sometimes contradictory, data on their genotoxicity.

A great variety of tests and test systems based on microbes, plants and animals have been developed in order to assess the genotoxic effects of xenobiotic agents, including pesticides. Arguably, the most reliable genotoxicity evaluation for human health risk is conducted with mammals, whose enzyme systems and more specifically their monooxygenase enzyme complex, are responsible for the biotransformation of xenobiotic chemicals (7,8). Although plants have monooxygenase enzyme systems that are to a certain degree similar to the mammal monooxygenase enzyme complex, the plant enzyme complex possesses a number of distinguishing characteristics (9,10). Of particular importance are a few reports indicating that unlike animals, some chemicals, including pesticides, are metabolically activated by plant peroxidases and may express different responses compared to those of mammalian cytochromes P-450. Peroxidases are abundant and widely disseminated in plants and therefore they might play a major role in the plant activation of promutagens (11–14). These observations suggest the value of plant test systems for evaluating the genotoxicity of different chemicals used for agricultural purposes (15). Hence, additional information on the comparative responses of plant and mammalian test systems response to potentially genotoxic pesticides would be of special interest.

In the present investigation, we have evaluated the genotoxicity of three herbicides, Roundup, Stomp and Reglone, in plant (*Crepis capillaris*) and mammalian (mouse) test systems that measure the induction of structural chromosomal aberrations and micronuclei. Chromosomal aberrations qualitatively and quantitatively detect clastogenic activity, while the micronucleus assay detects both clastogenic effects and damage to the mitotic apparatus, some of which might have aneugenic consequences.

Materials and methods

Chemicals

The following three herbicides were obtained from Agria, Plovdiv, Bulgaria.

Roundup is a liquid water-soluble organophosphorus herbicide, containing glyphosate [*N*-(phosphonomethyl) glycine, $C_3H_8NO_5P$] as its active ingredient (a.i.) (CAS No. 1071-83-6; >90% purity). Roundup is used as a total, leaf herbicide with contact action and is applied at concentrations ranging from 0.26 to 1.152% a.i. (16).

Stomp 330 is a liquid emulsive herbicide of the dinitroaniline type, whose a.i. is pendimethaline [*N*-(1-ethylpropyl)-2,6-dinitro-3,4-xylidine, $C_{13}H_{19}N_3O_4$] (CAS No. 40487-42-1; 98.9% purity). Stomp is applied as a selective systematic soil herbicide at concentrations ranging from 0.264 to 0.6% a.i. (16).

Reglone is a liquid water-soluble bipyridylium herbicide, whose a.i. is diquat (1,1′-ethylene -2,2′-ipyridyl dibromide, $C_{12}H_{12}Br_2N_2$) (CAS No. 85-00-75; 98% purity). Reglone is applied in practice as a total, leaf herbicide with

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contact action and is used at concentrations ranging from 0.08 to 0.24% a.i. (16).

In addition, colchicine ($C_{22}H_{25}NO_6$; CAS No. 64-86-8; >98% purity) was obtained from Merck KGaA, Darmstadt, Germany; ethyleneimine (EI; C_2H_5N; CAS No. 151-56-4; >99% purity) was obtained from Serva Feinbiochemica GmbH, Heidelberg, Germany and cyclophosphamide (CP; $C_7H_{15}Cl_2N_2O_2P.H_20$; CAS No.6055-19-2; >98% purity) was obtained from Sigma Chemie GmbH, Deisenhofen, Germany.

Plant assays

The experiments with plant assay were conducted using *C. capillaris* root meristems. The plant originating from the collection of the Institute of Botanic, BAS, Sofia, Bulgaria was propagated for 30 years in the greenhouse of the Institute of Genetics, BAS, Sofia, Bulgaria.

The experiments with the three herbicides were carried out at concentrations bracketing those used in agricultural practice: 0.05, 0.1, 0.5 and 1.0% a.i. for Roundup; 0.005, 0.1, 0.2 and 0.4% a.i. for Stomp; and 0.005, 0.01, 0.05 and 0.1% a.i. for Reglone. Four plants were evaluated for each data point.

Primary root meristems of *C. capillaris,* 1.5–2 mm long, were treated with the three herbicides for 2 h. After treatment, the roots were washed with running tap water for 1 h, and then left for recovery in an incubator at 24°C. Two controls were also investigated; distilled water was used as negative control and the alkylating agent ethyleneimine at concentration 0.05% (9.72×10^{-3} M) as positive control.

The material was fixed in 3:1 alcohol : acetic acid after 4, 16 and 24 h recovery periods. Two hour before fixation, half of the material was pretreated with a 0.05% colchicine solution, which is necessary for metaphase analysis of the chromosomal aberrations. The other half was fixed directly without colchicine pretreatment for assessment of the micronucleus frequency. Squash preparations were made after hydrolysis of fixed material in 1 N HCl at 60°C for 8 min, and staining after Feulgen (17) in a mixture ofza Schiff's reagent and aceto-carmine (ratio 1:1). 400 cells, 50 metaphases per slide were analysed for chromosomal aberrations and 4000 cells, 1000 interphase cells per treatment were evaluated for micronuclei. The slides were coded and examined blind.

Animals assays

C57BL mice were from the vivarium of the Laboratory of Radiation Genetics, MA, Sofia, Bulgaria. Treatments were conducted with 12–14-week-old male mice, weighing 22–25 g. They were allowed free access to food and water in a room kept at 23 ± 1°C with a 12 h light/dark cycle. The starting solutions of Roundup, Stomp and Reglone, containing 9.80, 16.50 and 1.43% a.i., were diluted with distilled water and were administered orally at 0.2–0.5 ml per mouse for Roundup and from 0.05 to 0.1 ml per mouse for Stomp and Reglone. These doses correspond to the concentrations used in plant experiments.

In a preliminary study, the LD_{50} for the three herbicides was determined by the Kerber method using group of eight male mice:

$$LD_{50} = LD_{100} - \frac{\sum(zd)}{m}$$

where: *d,* an interval between every two studied concentrations; *z,* average number of animals, the studied effect after every consequent doses is included; *m,* number of animals in a group.

Chromosomal aberration analysis was conducted on mouse bone marrow cells after treatment with 1/2 LD_{50} doses of the three herbicides. Eight mice per group were analysed for each data point—6, 24, 48, 72, 96 and 120 h after treatment. Analysis of the micronuclei was conducted in polychromatic erythrocytes (PCEs). Eight mice per group were analysed for each data point—24, 48, 72, 96 and 120 h after treatment with 1/2 LD_{50} doses of the three herbicides, and 72 h after 1/4 and 1/8 LD_{50} doses of Stomp and Reglone. The 1/8 LD_{50} doses of these two herbicides were applied every 24 h for 5 consecutive. Two control groups were also investigated, negative (untreated) control administered 0.3 ml distilled water and a positive control given an oral dose of 100 mg/kg cyclophosphamide.

Bone marrow preparations for chromosomal aberrations analysis

The mice were given an intraperitoneal injection of colchicine solution, at a concentration of 4 mg/kg, 1–1.50 h before they were killed. The bone marrow was removed from the femurs and processed by the Ford and Woolam method (18). Chromosomal aberrations were analysed in preparations stained with basic fuchsine. Four hundred bone marrow metaphases cells per treatment group, 50 cells obtained from each of eight animals were scored for chromosomal aberrations. The slides were coded and examined blind.

Bone marrow preparations for micronucleus analysis

Cell preparations were made from mouse bone marrow derived from the femur. After the bone marrow and the serum were homogenised, the material was centrifuged for 10 min at 1000 *g*. The supernatant was discarded, leaving roughly 100 µl in which the pellet was carefully resuspended. One drop of this suspension was spread on a refrigerated glass slide. The slides were air dried for 24 h, then staining as follows: the slides were stained for 3 min in May-Grünwald solution, and then for 1 min in a 1:1 (v/v) solution of May-Grünwald: demineralised water. Afterwards, the slides were washed with demineralised water for 2 min, stained with Giemsa solution for 15 min, and washed again for 2 min. After air-drying for 24 h, the slides were analysed.

Four thousand PCEs per treatment group, 500 cells obtained from each of eight animals were scored for the presence of micronuclei. The ratio of PCEs to normochromatic erythrocytes (NCEs) was established after analysis of 200 erythrocytes per animal (19). The slides were coded and examined blind.

Statistical analysis

The data obtained from the experiments were analysed using the statistical functions of Sigma Plot 9 with Sigma Stat Integration, SYSTAT (Software Inc., Cincinnati, OH, 2004). The frequency of the chromosomal aberrations and micronuclei induced in plant and in mice cells was scored. The data obtained in four independently replicated experiments were expressed of mean percent for each recovery time within each dose of treatment and analysed for significance by one-way analysis of variance ANOVA comparing the treated groups with their untreated control. If a statistically significant F-value of $P \leq 0.05$ was obtained, a Holm–Sidak multiple comparison versus the untreated control was conducted. The power of the test statistic (β) was ≥ 0.8 at $\alpha = 0.05$.

Results

The pesticides were tested as complex commercial mixtures because this is the form in which they are applied in agriculture and introduced into the environment.

The results of chromosome analysis on the clastogenic potential of Roundup, Stomp and Reglone in plant and bone marrow cells are shown in Tables I and II. None of the three compounds produced a significant increase in the frequency of structural chromosomal aberrations in plant cells ($P > 0.05$) at the concentrations tested. Roundup and Reglone also were negative for the induction of chromosomal aberrations in mouse bone marrow. Stomp produced an increased frequency of chromosomal aberrations in mouse bone marrow, but the response was statistically significant only at 96 h after treatment of the animals with the highest test dose (1/2 LD_{50}, 489.0 mg/kg).

As can be seen in Table I, Stomp induced numerical aberrations in plant cells. This kind of damage includes both aneuploidy and polyploidy. The numerical aberration data are presented in Table I as hyperploid cells. They arose as a result of spindle disturbances that caused C-mitoses. Disturbances of the mitotic spindle resulting in C-mitosis were observed even with the lowest concentration of Stomp (0.005%), which is ~53-fold lower than the lowest effective concentration used in agricultural practice (0.264%) (16).

Tables III and IV show the results of micronucleus assays conducted in plants and mice. Roundup did not significantly increase micronucleus frequency in plant cells; a slight increase was observed with some treatments, e.g. 0.05% with the 24 h sample time, but these increases were not statistically significant ($P > 0.05$). Roundup was also negative for micronucleus induction in mouse PCEs. In contrast, Reglone was positive for micronucleus induction in both plant cells and mouse bone marrow cells. All test concentrations (0.005–0.1%) increased the frequencies of micronuclei in plant cells, with the increases most pronounced at the two highest concentrations (0.05 and 0.1%) (Table III). For mouse PCEs single treatments with Reglone produced a statistically significant increase in micronucleus frequency only at 24 h after treatment with the highest dose of the herbicide (1/2 LD_{50}, 489.0 mg/kg; Table IV). Single treatments with the other doses

Table I. Frequency of chromosomal aberrations and hyperploid cells in root meristems of *C. capillaris* after treatment with the herbicides Roundup, Stomp and Reglone

Herbicides	Concentration (%)	Recovery time (h)	No. of cells analysed	No. of cells with aberrations	Type of aberrations									Aberrations per 100 cells (without gaps) (Mean ± SEM)	Polyploid cells (%)	Aneuploid cells (%)
					Chromatid				Chromosome				Gaps			
					Breaks	Exchanges			Breaks	Exchanges						
						Intra-chromosomal	Interchromosomal			Intra-chromosomal	Interchromosomal					
							Asymmetric	Symmetric			Asymmetric	Symmetric				
Roundup	0.05	4	400	1	1	–	–	–	–	–	–	–	–	0.25 ± 0.10	–	–
		16	400	3	1	–	–	–	–	–	–	–	2	0.25 ± 0.18	–	–
		24	400	3	1	–	–	–	2	–	–	–	–	0.75 ± 0.27	–	–
	0.01	4	400	2	2	–	–	–	–	–	–	–	–	0.50 ± 0.14	–	–
		16	400	4	3	–	–	–	–	–	–	–	1	0.75 ± 0.27	–	–
		24	400	4	–	1	–	–	2	–	–	–	1	0.75 ± 0.27	–	–
	0.5	4	400	3	1	–	–	–	–	–	–	–	2	0.25 ± 0.10	–	–
		16	400	4	2	1	–	–	–	–	–	–	1	0.75 ± 0.27	–	–
		24	400	4	–	–	–	–	3	–	–	–	1	0.75 ± 0.27	–	–
	1.0[a]	4	–	–	–	–	–	–	–	–	–	–	–	–	–	–
		16	–	–	–	–	–	–	–	–	–	–	–	–	–	–
		24	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Control	–	24	400	1	–	–	–	–	1	–	–	–	–	0.25 ± 0.10	–	–
EI	0.05	24	400	88	46	9	13	7	1	–	–	–	12	19.00 ± 0.53***	–	–
Stomp	0.005	4	400	3	3	–	–	–	–	–	–	–	–	0.75 ± 0.23	–	–
		16	400	6	3	1	–	–	–	–	–	–	2	1.00 ± 0.23	6.55	–
		24	400	3	1	–	–	–	2	–	–	–	–	0.75 ± 0.10	10.75	1.75
	0.1	4	400	4	3	–	–	–	–	–	–	–	1	0.75 ± 0.18	–	–
		16	400	6	1	1	1	–	–	–	–	–	3	0.75 ± 0.18	9.75	2.25
		24	400	6	–	1	–	–	2	–	1	–	2	1.00 ± 0.31	17.75	5.75
	0.2	4	400	5	4	–	–	–	–	–	–	–	1	1.00 ± 0.31	–	–
		16	400	7	2	1	1	–	–	–	–	–	3	1.00 ± 0.31	20.89	3.91
		24	400	8	–	–	–	–	2	–	1	–	5	0.75 ± 0.18	38.81	9.14
	0.4	4	400	7	4	–	–	–	–	–	–	–	3	1.00 ± 0.31	–	–
		16	400	10	2	1	–	–	1	–	–	–	6	1.00 ± 0.31	48.25	9.50
		24	400	11	–	–	1	–	2	1	1	–	6	1.25 ± 0.27	71.84	23.97
Control	–	24	400	3	1	–	–	–	2	–	–	–	–	0.75 ± 0.18	–	–
EI	0.05	24	400	85	49	5	10	6	–	–	–	–	15	17.50 ± 0.64***	–	–
Reglone	0.005	4	400	2	2	–	–	–	–	–	–	–	–	0.50 ± 0.18	–	–
		16	400	3	3	–	–	–	–	–	–	–	–	0.75 ± 0.20	–	–
		24	400	4	–	–	–	–	3	1	–	–	–	1.00 ± 0.27	–	–
	0.01	4	400	4	3	–	1	–	–	–	–	–	–	1.00 ± 0.27	–	–
		16	400	6	3	1	1	–	–	–	–	–	1	1.25 ± 0.35	–	–
		24	400	7	–	–	–	–	3	–	1	–	3	1.00 ± 0.27	–	–
	0.05	4	400	5	4	–	–	–	–	–	–	–	1	1.00 ± 0.20	–	–
		16	400	7	–	1	2	–	–	–	–	–	4	0.75 ± 0.18	–	–
		24	400	6	–	–	–	–	4	1	–	–	1	1.25 ± 0.32	–	–
	0.1	4	400	5	3	–	–	–	–	–	–	–	2	0.75 ± 0.10	–	–
		16	400	5	1	1	–	–	2	–	–	–	1	1.00 ± 0.27	–	–
		24	400	7	–	–	–	–	4	–	1	–	2	1.25 ± 0.31	–	–
Control	–	24	400	2	2	–	–	–	–	–	–	–	–	0.50 ± 0.10	–	–
EI	0.05	24	400	102	54	12	14	4	–	1	1	–	16	21.50 ± 0.54***	–	–

[a]Data not obtained due to high toxicity of the herbicide.
***$P < 0.001$.

Table II. Frequency of chromosomal aberrations in mouse bone marrow cells after treatment with the herbicides Roundup, Stomp and Reglone

Herbicides	Dose (mg/kg)	Recovery time (h)	No. of cells analysed	No. of cells with aberrations	Type of aberrations									Aberrations per 100 cells (without gaps) (Mean ± SEM)
					Chromatid				Chromosome				Gaps	
					Breaks	Exchanges			Breaks	Exchanges				
						Intra-chromosomal	Interchromosomal			Intra-chromosomal	Interchromosomal			
							Asymmetric	Symmetric			Asymmetric	Symmetric		
Roundup	1 × 1080 (1/2 LD_{50})	6	400	1	1	–	–	–	–	–	–	–	–	0.25 ± 0.10
		24	400	4	1	–	–	–	1	–	–	–	2	0.50 ± 0.10
		48	400	2	–	–	–	–	1	–	–	–	1	0.25 ± 0.18
		72	400	4	1	–	–	–	1	1	–	–	1	0.75 ± 0.27
		96	400	6	–	1	1	–	1	–	1	–	2	1.00 ± 0.37
		120	400	7	1	–	–	–	2	–	–	–	4	0.75 ± 0.27
Control	–	24	400	1	1	–	–	–	–	–	–	–	–	0.25 ± 0.10
CP	100	24	400	37	10	3	8	6	1	–	–	–	9	7.00 ± 0.27***
Stomp	1 × 489.0 (1/2 LD_{50})	6	400	2	1	–	–	–	1	–	–	–	–	0.50 ± 0.10
		24	400	2	1	–	–	–	–	–	–	–	1	0.25 ± 0.10
		48	400	3	–	–	–	–	1	–	–	–	2	0.25 ± 0.10
		72	400	4	1	–	–	–	1	–	–	–	2	0.50 ± 0.18
		96	400	11	5	–	1	–	3	–	–	1	1	2.50 ± 0.77*
		120	400	7	2	–	–	–	2	–	–	–	3	1.00 ± 0.37
Control	–	24	400	1	1	–	–	–	–	–	–	–	–	0.25 ± 0.10
CP	100	24	400	44	13	5	10	4	–	–	1	–	11	8.25 ± 0.27***
Reglone	1 × 34.0 (1/2 LD_{50})	6	400	2	–	–	–	–	2	–	–	–	–	0.50 ± 0.10
		24	400	7	1	–	–	–	2	–	–	–	4	0.75 ± 0.10
		48	400	6	1	–	–	–	2	–	–	–	3	0.75 ± 0.10
		72	400	10	2	–	–	1	3	–	1	–	3	1.75 ± 0.51
		96	400	4	1	–	–	–	1	–	–	–	2	0.50 ± 0.10
		120	400	5	1	–	–	–	1	–	–	–	3	0.50 ± 0.10
Control	–	24	400	2	2	–	–	–	–	–	–	–	–	0.50 ± 0.10
CP	100	24	400	34	9	5	9	3	–	–	1	–	7	6.75 ± 0.44***

*$P < 0.05$; ***$P < 0.001$.

Table III. Frequency of micronuclei in root meristem cells of *C. capillaris* after treatment with the herbicides Roundup, Stomp and Reglone

Herbicides	Concentration (%)	Recovery time (h)	No. of cells analysed	Cells with micro nuclei	
				Number	%Mean ± SEM
Roundup	0.05	4	4000	4	0.10 ± 0.04
		16	4000	4	0.10 ± 0.01
		24	4000	12	0.30 ± 0.10
	0.1	4	4000	4	0.10 ± 0.02
		16	4000	9	0.22 ± 0.07
		24	4000	7	0.18 ± 0.02
	0.5	4	4000	10	0.25 ± 0.09
		16	4000	6	0.15 ± 0.02
		24	4000	10	0.25 ± 0.08
	1.0[a]	4	–	–	–
		16	–	–	–
		24	–	–	–
Control	–	24	4000	4	0.10 ± 0.03
EI	0.05	24	4000	550	13.75 ± 0.10***
Stomp	0.005	4	4000	92	2.30 ± 0.54**
		16	4000	148	3.70 ± 0.03***
		24	4000	224	5.60 ± 0.05***
	0.1	4	4000	88	2.20 ± 0.75*
		16	4000	158	3.95 ± 0.03***
		24	4000	230	5.75 ± 0.04***
	0.2	4	4000	96	2.40 ± 0.45**
		16	4000	172	4.30 ± 0.05***
		24	4000	270	6.75 ± 0.08***
	0.4	4	4000	196	4.90 ± 0.07***
		16	4000	184	4.60 ± 0.04***
		24	4000	298	7.45 ± 0.06***
Control	–	24	4000	5	0.12 ± 0.01
EI	0.05	24	4000	490	12.25 ± 0.06***
Reglone	0.005	4	4000	40	1.00 ± 0.20
		16	4000	44	1.10 ± 0.30
		24	4000	52	1.30 ± 0.23*
	0.01	4	4000	32	0.80 ± 0.22
		16	4000	50	1.25 ± 0.27*
		24	4000	44	1.10 ± 0.30*
	0.05	4	4000	70	1.75 ± 0.22**
		16	4000	60	1.50 ± 0.23**
		24	4000	56	1.40 ± 0.20*
	0.1	4	4000	56	1.40 ± 0.27*
		16	4000	70	1.75 ± 0.33**
		24	4000	60	1.50 ± 0.23**
Control	–	24	4000	20	0.50 ± 0.08
EI	0.05	24	4000	610	15.25 ± 0.19***

[a]Data not obtained due to high toxicity of the herbicide.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

(1/4 LD_{50} and 1/8 LD_{50}) were uniformly negative. Administration of the 1/8 LD_{50} dose of the compound on five consecutive days resulted in 90% animal lethality. Table III show that Stomp produced the greatest increase in plant micronucleus frequency. It was positive at all concentrations (0.005–0.4%) and at all sample times. The highest test concentration of Stomp (0.4%) enhanced the micronucleus frequency in *C. capillaris* by between 38- and 62-fold that of the control. Stomp also increased the frequency of micronuclei in mouse PCEs. Single treatments were effective only at the 489.0 mg/kg dose, corresponding to 1/2 the LD_{50}. In addition, five consecutive treatments with the 1/8 LD_{50} dose of Stomp were very effective in inducing micronuclei.

The positive control compounds ethyleneimine in plant cells and cyclophosphamide mice cells caused a high incidence of chromosomal aberrations and micronuclei in all experiments.

Ranking the chemicals according to values of PCE:NCE ratio, Stomp was the least toxic and Roundup was the most toxic compound (Table IV).

Discussion

This study investigated genotoxicity of the pesticides Roundup, Stomp and Reglone in two phylogenetically distant test systems. The three herbicides are widely used against a range of annual and perennial weeds. Bearing in mind that most pesticides are capable of inducing mutations in at least one test system (20), it is worthwhile to test the genotoxicity of such compounds in an animal system for its relevance to assessing human risk and in a plant system because the agents are used on plants and plants may produce unique genotoxic metabolites.

The genotoxic potential of Roundup has been studied extensively and inconsistent results have been reported using the same assay as well as using different assays. Usually either the a.i., glyphosate or its commercial formulation, Roundup, has been tested; more rarely both compounds were investigated. Thus, glyphosate did not induce gene mutations in a variety of *in vitro* bacterial assays including the *Salmonella typhimurium* reversion assay, with and without metabolic activation (21–23) and in *Escherichia coli* WP-2 (22,23). It was also negative in the *Chinese hamster* ovary cell *HGTRT* gene mutation assay, in the primary hepatocyte DNA repair assay (23). The technical formulation, Roundup, was negative in the *S. typhimurium* reversion assay (24,25) and in the sex-linked recessive lethal assay with *Drosophila melanogaster* (26).

There are limited data published on the cytogenetic damage induced by Roundup (27). It was negative for *in vivo* micronucleus induction in mouse bone-marrow (19, 25). The results of our study agree with these negative results for both mouse bone marrow and plants cells. However, induction of chromosomal aberrations was observed in *Allium cepa* root meristem cells (19). Roundup also causes an increase in reverse mutations in *S. typhimurium* TA 98 and TA 100 (in the presence of S9 fraction). It was reported to induce a high frequency of lethal in larval spermatocytes and spermatogonia of *D. melanogaster* (28). Roundup induced DNA damage in *Rana catesbeiana* tadpoles (29).

On balance, the available data indicate that the technical formulation Roundup is at best weakly genotoxic in short-term assays. Differences in the response of test organisms to the a.i., glyphosate and the commercial formulation, Roundup, might be due to the toxicity of different coformulants and surfactants contained in commercial product. Several studies with parallel testing of glyphosate and Roundup showed that only the commercial formulation was genotoxic (19,30–32). What chemicals are used as coformulants and surface-acting agents is difficult to define because of patent protections. For this reason we have no information on the coformulants involved in the production of the Roundup that was used in our study, complicating comparisons between our results and those of others.

Data on Stomp genotoxicity are scarce. In this respect only two investigations are known. Stomp was negative in mouse bone marrow micronucleus induction in either male or female animals (31). It was also negative for human lymphocytes sister chromatide exchange (SCE) induction (33). The genotoxic responses of the plant and mouse bone marrow assays to the herbicide Stomp in our experiments were quite different. No clastogenicity was observed in plant cells, but the highest dose (489 mg/kg) of Stomp resulted in an increase in chromosomal aberrations in mouse bone marrow. This increase, although weak, was statistically significant.

Table IV. Frequency of micronuclei in PCEs after treatment with the herbicides Roundup, Stomp and Reglone

Herbicides	Dose (mg/kg)	Recovery (h)	No. of PCEs analysed	Cells with micronuclei		Ratio of PCE:NCE
				Number	% Mean ± SEM	
Roundup	1 × 1080 (1/2 LD_{50})	24	4000	20	0.50 ± 0.07	0.78
		48	4000	20	0.50 ± 0.08	0.62
		72	4000	24	0.60 ± 0.14	0.60
		96	4000	24	0.60 ± 0.15	0.72
		120	4000	20	0.50 ± 0.08	0.65
Control	–	24	4000	20	0.50 ± 0.12	1.40
CP	100	24	4000	152	3.80 ± 0.36***	0.60
Stomp	1 × 489.0 (1/2 LD_{50})	24	4000	30	0.75 ± 0.15	1.10
		48	4000	50	1.25 ± 0.28	0.95
		72	4000	40	1.00 ± 0.18	0.90
		96	4000	96	2.40 ± 0.46**	1.07
		120	4000	46	1.15 ± 0.26	1.00
	1 × 244.5 (1/4 LD_{50})	72	4000	36	0.90 ± 0.25	0.90
	1 × 122.2 (1/8 LD_{50})	72	4000	32	0.80 ± 0.18	1.03
	5 × 122.2 (1/8 LD_{50})	120	4000	94	2.35 ± 0.39**	0.87
Control	–	24	4000	30	0.75 ± 0.20	1.43
CP	100	24	4000	116	2.90 ± 0.04***	0.63
Reglone	1 × 34.0 (1/2 LD_{50})	24	4000	97	2.42 ± 0.29*	1.10
		48	4000	36	0.90 ± 0.32	0.82
		72	4000	44	1.10 ± 0.19	0.73
		96	4000	44	1.10 ± 0.29	1.00
		120	4000	33	0.82 ± 0.14	1.29
	1 × 17.0 (1/4 LD_{50})	72	4000	36	0.90 ± 0.17	1.07
	1 × 8.5 (1/8 LD_{50})	72	4000	24	0.60 ± 0.25	0.90
	5 × 8.5 (1/8 LD_{50})	120	4000	–	90% lethality	0.18
Control	–	24	4000	32	0.80 ± 0.19	1.45
CP	100	24	4000	116	2.90 ± 0.14***	0.63

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

The appearance of aberrations at a relatively long time after administration of the herbicide suggests that the clastogenic effect may be due to metabolism of the herbicide by the animal. Metabolites with genotoxic properties may be responsible for the increase in chromosomal aberrations that was observed.

Stomp induced significant increases in micronucleus frequency in both plant cells and mouse PCEs. The magnitude of micronucleus response in plant cells was greater and the origin of micronuclei in the two assays appears to be rather different. The presence of aneuploid and polyploid cells in the plant chromosome aberration assay is an indication of antimitotic activity resulting from the destruction of mitotic spindle microtubules (34). The complete destruction of spindle microtubules results in C-mitoses and in our assays, typical C-mitoses were observed even after treatment with the lowest concentration of Stomp (0.005%). The a.i. in Stomp is pendimethaline, which belongs to the nitroaniline class of herbicides, some of which (e.g. oryzalin) have well-documented antimicrotubule effects (35). These agents block mitosis at metaphase and depolymerise spindle microtubules and cortical cytoplasmic microtubules with the subsequent disruption of the orientation of the newly deposited wall cellulose microfibrils. Thus, they produce colchicine-like effects (34). These effects have been observed in a number of plant species and protozoa, but nitroanilines do not act on fungal or vertebrate microtubules (34,36). So the micronuclei produced by Stomp in mouse bone marrow cells likely resulted from a clastogenic effect. The frequencies of chromosomal aberrations and micronuclei in this case were similar.

The herbicide Reglone contains diquat as its a.i. Most investigations on the genotoxicity of this herbicide involve the assessment of the a.i. and only one report (37) evaluated the genotoxic potential of the commercial formulation, Reglone. Diquat produced a small increase in gene conversions in *Sacharomyces cerevisiae* (38), and it induced DNA damage in cultured SV-40-transformed human cells and 8-azaguanine resistance in *S. typhimurium* (39,40). Benigni *et al*. (39) also reported that diquat induced gene mutations in *Aspergillus nidulans* and increased unscheduled DNA synthesis in human epithelial-like cells (37). However, Benigni *et al*. (39) and Levin *et al*. (41) reported negative results in the *S. typhimurium* reversion assay with and without metabolic activation. Diquat was also negative in dominant lethal assays in mice (42,43) and for chromosomal aberrations in mouse bone marrow (44). Our results on the clastogenicity of Reglone are consistent with these results. We did not observed any clastogenic activity of the herbicide in either the plant or the mouse bone marrow assays. These data indicate that Reglone does not produce DNA damage that can lead to chromosomal aberration. At the same time, Reglone was positive for micronucleus induction in both plant cells and mouse PCEs. Because Reglone did not have any clastogenic activity in mouse bone marrow or plants, the micronuclei may originate from partial damages to the mitotic apparatus leading to the loss of whole chromosomes. Similar effects have been observed for different classes of herbicides, not only in plants, but also in animals (45,46). Mechanisms for the deletion of one or more chromosomes were discussed by Natarajan (47).

In conclusion, the results from the present study indicate both similarities and differences for the genotoxicity of the herbicides Roundup, Stomp and Reglone in plant cells and in mouse bone marrow. The genotoxicity of Roundup and Reglone are quite similar in the two systems. Differences may arise when metabolic activation of the chemicals is

involved in the response as may be the case with Stomp in mouse bone marrow cells. Our results also indicate that the plant test system may be more susceptible to spindle poison effects (i.e., aneugenic effects) of these agents. The parallel use of the two test systems may provide a more complete evaluation of both clastogenic and aneugenic potential of the test agents.

References

1. Van der Werf,H.M.G. (1996) Assessing the impact of pesticides on the environment. *Agric., Ecosyst. Environ.*, **60**, 81–96.
2. Crosby,D.G. (1982) Pesticides as environmental mutagens. In Fleck,R.A. and Hollander,A. (eds), *Genetic Toxicology: An agricultural Perspective*. Plenum Press, New York, London, pp. 201–218.
3. Ribas,G., Surrales,J., Carbonell,E., Xamena,N., Creus,A. and Marcos,R. (1996) Genotoxicity of the herbicides alachlor and maleic hydrazide in cultured human lymphocytes. *Mutagenesis*, **11**, 221–227.
4. Lander,B.F., Knudsen,L.E., Gamborg,M.O., Jarventaus,H. and Vorppa,H. (2000) Chromosome aberrations in pesticide-exposed greenhouse workers. *Scand J Work Environ Health*, **26**, 436–442.
5. Meinert,R., Schuz,J., Kaatsch,P. and Michaelis,J. (2000) Leukemia and non-Hodgkin's lymphoma in childhood and exposure to pesticides: results of a register-based cite- control study in Germany. *Am. J. Epidemiol.*, **151**, 639–646.
6. Ji,B.T., Silverman,D.T. and Stewart,P.A. *et al.* (2001) Occupational exposure to pesticides and pancreatic cancer. *Am. J. Ind. Med.*, **39**, 92–99.
7. Malling,H.V. (1966) Mutagenicity of two potent carcinogens, dimethilnitrosamine and diethylnitrosamine, in *Neurospora crassa*. *Mutat. Res.*, **3**, 537–540.
8. Ames,B.N., McCan,J. and Yamasak,E. (1975) Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. *Mutat. Res.*, **3**, 347–364.
9. Plewa,M.J. and Gentile,J.M. (1982) The activation of chemical mutagens by Green plants. In Hollander,A. and de Serres,F.J. (eds), *Chemical Mutagens, Principles and Methods for Their Detection.* Plenum Vol. VII, New York, pp. 401–420.
10. Higashi,K. (1988) Metabolic activation of environmental chemicals by microsomal enzymes of higher plants. *Mutat. Res.*, **197**, 273–288.
11. Plewa,M.J. and Wagner,E.D. (1993) Activation of promutagens by green plants. *Annu. Rev. Genet.*, **27**, 93–113.
12. Plewa,M.J., Wagner,E.D., Gentile,G.J. and Gentile,J.M. (1984) An evaluation of the genotoxic properties of herbicides following plant and animal activation. *Mutat. Res.*, **146**, 233–245.
13. Gentile,J.M. and Plewa,M.J. (1988) The use of cell free systems in plant activation studies. *Mutat. Res.*, **197**, 173–182.
14. Plewa,M.J., Seo,K.Y., Ju,Y.-H., Smith,S.R. and Wagner,E.D. (1997) Plant activation of environmental agents: the utility of the plant cell/microbe coincubation assay. In Wang,W., Gorsuch,J.W. and Hughes,J.S. (eds), *Plant Environmental Studies*. CRC Press LLC, Lewis Publishers, Boca Raton, New York, pp. 105–126.
15. Grant,W.F. (1999) Higher plant assays for the detection of chromosomal aberrations and gene mutations-a brief historical background on their use for screening and monitoring environmental chemicals. *Mutat. Res.*, **426**, 107–112.
16. Fetvadjieva,N., Straka,F., Michailova,P., Balinov,I., Lubenov,I., Balinova,A., Pelov,V., Karsova,V. and Tsvetkov,D. (1994) In Fetvadjieva,N. (ed.), *Handbook of Pesticides*. 2nd revised edn. Zemizdat Inc., Sofia, pp. 330.
17. Darlington,C.D. and La Cour,L.E. (1976) *The Handling of Chromosomes*. George Allen and Unwind Ltd, London, pp. 201.
18. Ford,H.E.R. and Woolam,H.M. (1963) A study of the mitotic chromosomes of mice of strong a line. *Exp. Cell Res.*, **32**, 320–326.
19. Rank,J., Jensen,A.G., Skov,B., Pedersen,L.H. and Jensen,K. (1993) Genotoxicity testing of Roundup and its active ingredient glyphosate isopropylamine using the mouse bone marrow micronucleus test, *Salmonella* mutagenicity test and *Allium* anaphase-telophase test. *Mutat. Res.*, **300**, 29–36.
20. Rodrigues,G.S., Pimentel,D. and Weinstein,L.H. (1998) In situ assessment of pesticide genototoxicity in an integrated pest management program: I. *Tradescantia* micronucleus assay. *Mutat. Res.*, **412**, 235–244.
21. Wildeman,A.G. and Nazar,R.N. (1982) Significance of plant metabolism in the mutagenicity and toxicity of pesticides. *Can. J. Genet. Cytol.*, **24**, 437–449.
22. Moriya,M., Ohta,T., Watanabe,K., Miyasawa,T., Kato,K. and Shirasu,Y. (1983) Further mutagenicity studies on pesticides in bacterial reversion assay systems. *Mutat. Res.*, **116**, 185–216.
23. Li,A.P. and Long,T.J. (1988) An evaluation of genotoxic potential of glyphosate. *Fundam. Appl. Toxicol.*, **10**, 537–546.
24. Njagi,G.D.E. and Gopalan,H.N. (1980) Mutagenicity testing of some selected food preservatives herbicides and insecticides: II. Ames test. *Bangladesh J. Bot.*, **9**, 141–146.
25. Kier,L.D., Stegeman,S.D., Dudek,S., McAdams,J.G., Flowers,F.J., Huffman,M.B. and Heydens,W.F. (1997) Genotoxicity studies of glyphosate, alachlor and butachlor formulations. *Fundam. Appl. Toxicol.*, **36**, (N1, Part 2), p. 305.
26. Gopalan,H.N.B. and Njagi,G.D.E. (1981) Mutagenicity testing of pesticides: III. *Drosophila*: recessive sex-linked lethals. *Genetics*, **97** (Suppl), S44.
27. Williams,G.M., Kroes,R. and Munro,I.C. (2000) Safety evaluation and risk assessment of the herbicide roundup and its active ingredient, glyphosate, for humans. *Regul. Toxicol. Pharmacol.*, **31**, 117–165.
28. Kale,P.G., Petty,B.T., Walker,S., Ford,J.B., Denkordi,N., Tarasia,S., Tasie,B.O., Kale,R. and Sohni,Y.R. (1995) Mutagenicity testing of nine herbicides and pesticides currently used in agriculture. *Environ. Mol. Mutagen.*, **25**, 148–153.
29. Clements,C., Ralph,S. and Petras,M. (1997) Genotoxicity of select herbicides in *Rana catesbeiana* tadpoles using the alkaline single-cell gel DNA electrophoresis (comet) assay. *Environ. Mol. Mutagen.*, **29**, 277–288.
30. Bolognesi,C., Bonatti,S., Degan,P., Callerani,E., Peluso,M., Rabboni,R., Roggeri,P. and Abbondandolo,A. (1997) Genotoxic activity of glyphosate and its technical formulation Roundup. *J. Agric. Food Chem.*, **45**, 1957–1962.
31. Gebel,T., Kevekordes,S., Pav,K., Edenharder,R. and Dunkelberg,H. (1997) *In vivo* genotoxicity of selected herbicides in the mouse bone-marrow micronucleus test. *Arch. Toxicol.*, **71**, 193–197.
32. Grisolia,C.K. (2002) A comparison between mouse and fish micronucleus test using cyclophosphamide, mitomycin C and various pesticides. *Mutat. Res.*, **518**, 145–150.
33. Dunkelberg,H., Fuchs,J., Hengstler,J.G., Klein,E., Oesch,F. and Strüder,K. (1994) Genotoxic effects of the herbicides alachlor, atrazine, pendimethaline and simazine in mammalian cells. *Bull Environ. Contam. Toxicol.*, **52**, 498–504.
34. Morejohn,L.C. and Fosket,D.E. (1986) Tubulins from plants fungi and protests; a review. In Shay,J.W. (ed.), *Cell and Molecular Biology of the Cytoskeleton*. Plenum Publishing Corp, New York, pp. 257–329.
35. Morejohn,L.C., Bureau,T.E., Molè-Bajer,J., Bajer,A.S. and Fosket,D.E. (1987) Oryzalin, a dinitroaniline herbicide, binds to plant tubulin and inhibits microtubule polymerization in vitro. *Planta*, **172**, 252–264.
36. Morissette,N.S., Mitra,A., Sept,D. and Sibley,D. (2004) Dinitroanilines bind α-tubulin to disrupt microtubules. *Mol. Biol. Cell*, **15**, 1960–1968.
37. Shah,R.G., Laguex,J., Kapur,S., Levallois,P., Avotte,P., Tremblav,M., Lee,J. and Poirer,G.G. (1977) Determination of genotoxicity of the metabolites of the pesticides Guthion, Sencor, Lorox, Reglone, Daconil and Admire by 32 p-postlabeling. *Mol. Cell. Biochem.*, **169**, 177–184.
38. Siebert,D. and Lemperle,E. (1974) Genetic effects of herbicides: induction of mitotic gene conversation in *Saccharomyces cerevisiae*. *Mutat. Res.*, **22**, 111–120.
39. Benigni,R., Bighnami,A., Carera,A., Conti,G., Conti,R., Crebelli,E., Dogliotti,E., Gualandi,G., Novelleto,A. and Ortali,V.A. (1979) Mutational studies with diquat and paraquat in vitro. *Mutat. Res.*, **68**, 183–193.
40. Bignami,M. and Crebelli,R. (1979) A simplified method for the induction of *s*-azaguanine resistance in *S. typhimurium*. *Toxicol. Lett.*, **3**, 169–175.
41. Levin,D.E., Hollstein,M., Christman,M.F., Schwiers,E.A. and Ames,B.N. (1982) A new *Salmonella* tester strain (TA102) with AT base pairs at the site of mutation detects oxidative mutagens. *Proc. Natl Acad. Sci. USA*, **79**, 7445–7449.
42. Pasi,A., Embree,J.W., Eisenlord,G.A. and Hine,G.H. (1974) Assessment of the mutagenic properties of diquat and paraquat in the murine dominant lethal test. *Mutat. Res.*, **26**, 171–175.
43. Anderson,D., McGregor,D.B. and Purchase,I.F.H. (1976) Dominant lethal studies with diquat and paraquat in male CD-I mice. *Mutat. Res.*, **40**, 349–358.
44. Selypes,A., Nagymajtenyl,L. and Berenesi,G. (1980) Mutagenic and embryologic effects of paraquat and diquat. *Bull Environ. Contam. Toxicol.*, **25**, 513–517.
45. Robinson,D.G. and Herzog,W. (1977) Structure synthesis and orientation of microfibrils: III. A survey of the action of microtubule inhibitors on microtubules and microfibril orientation in *Oocystis solitaria*. *Cytobiologie*, **15**, 463.

46. Magistrini,M. and Szollasi,D. (1980) Effects cold and of isopropyl – *N* – phenylcarbamate of the second meiotic spindle of mouse oocytes. *Eur. J. Cell. Biol*., **22**, 699.
47. Natarajan,A.T. (1993) An overview of the results of testing of known or suspected aneugens using mammalian cells in vitro. *Mutat. Res*., **287**, 113–118.

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