Bull. Environ. Contam. Toxicol. (1994) 52:498–504

Bulletin of Environmental Contamination and Toxicology

Genotoxic Effects of the Herbicides Alachlor, Atrazine, Pendimethaline, and Simazine in Mammalian Cells

H. Dunkelberg,[1] J. Fuchs,[2] J. G. Hengstler,[2] E. Klein,[1] F. Oesch,[2] K. Strüder[1]

[1]Hygiene-Institut, Johannes Gutenberg-Universität, 55131 Mainz, Germany
[2]Institut für Toxikologie, Johannes Gutenberg-Universität, 55131 Mainz, Germany

Received: 1 December 1992/Accepted: 10 August 1993

The extensive application of pesticides in modern agriculture requires an intensive investigation of the impact of these chemicals on the environment and the public health. With the dispersal of hundreds of millions of kilograms each year, these agents must be analysed for their mutagenic properties.
In the present study we tested the genotoxic activity of the herbicides alachlor, atrazine, pendimethaline and simazine using the in vitro SCE test with human lymphocytes and the alkaline elution assay with rat-hepatocytes, V79 cells and human lymphocytes.

MATERIALS AND METHODS

Herbicides tested were obtained from Promochem (Wesel, FRG; purity of pesticides: alachlor 98,3%; atrazine 98,7%; pendimethaline 99,0%; simazine 99,9%).
For the human lymphocyte in vitro sister-chromatid exchange (SCE)-test cultures were set up from fresh blood collected aseptically from healthy donors. Each culture consisted of 0.3 mL blood in 5.0 mL chromosome-medium (Chromosomenmedium B PHA-M from Biochrom, Berlin, FRG). 5-bromo-2`deoxyuridine (Serva, Heidelberg, FRG) at a final concentration of 6 µg/mL was added to each culture.
Cultures were treated with various concentrations of each test compound for 24 hrs and 48 hrs in the absence of a metabolic activating system. In a third assay lymphocytes were also treated in the presence of rat-liver S9-Mix (rats were aroclor 1254-pretreated) for 2 hrs (100 µL/mL). The test compounds were added 24 hrs after the start of culture for the exposure times of 48 and 2 hrs and 48 hrs after the start of culture for the exposure time of 24 hrs. Herbicides were tested in concentrations up to a cytotoxic response in the culture determined by an absence of dividing cells. All pesticides tested were dissolved in 100 µL DMSO (Merck, Darmstadt, FRG), aliquots of DMSO were also added to control cultures.
In total, lymphocytes were cultivated for 72 hrs at 37°C. Two hours before termination colcemid (Serva, Heidelberg, FRG) (0.4 µg/mL) was added.

Correspondence to: H. Dunkelberg

As positive control we used cyclophosphamide at a concentration of 10^{-5} mol/L with and without S9-Mix.
The cells were harvested and slides were prepared according to the method of Perry and Wolff (1974) for counting sister-chromatid exchanges. One hundred metaphases were scored to determine the cell-proliferation (Lamberti et al., 1983); furthermore thirty metaphases were examined for sister-chromatid exchanges for each concentration of the test-compounds and the controls.
To evaluate the statistical significance of our results we applied the t-test at multiple level $\alpha = 0.01$ using Bonferroni correction.
For the alkaline elution test, V79 cells, a Chinese hamster lung fibroblast cell line, were cultured as described previously (Hengstler et al., 1992). Stock solutions of alachlor were freshly prepared in DMSO, the concentration of DMSO in the culture medium was 0.5%. Mononuclear blood cells were isolated by ficoll-metrizoate centrifugation according to the method of Boyum (1964). Adult, male Sprague-Dawley rats were used for the preparation of isolated rat liver cells as described previously (Utesch et al., 1987). The alkaline elution method and the trypan blue exclusion test were performed as described (Hengstler et al., 1992).

RESULTS AND DISCUSSION

Table 1. Induction of sister-chromatid exchanges by alachlor in cultivated human lymphocytes

S9-Mix	Time [h] of Incubation	alachlor [mol/L] 0	$5 \cdot 10^{-6}$	10^{-5}	$5 \cdot 10^{-5}$	10^{-4}
no	24	11.4*±4.7** ±0.9*** 2.1****	12.9 ±4.2 ±0.8 2.0	13.3 ±6.0 ±1.1 1.9	15.3 ±5.8 ±1.1 1.8	18.7#±5.5 ±1.0 1.6
no	48	10.4 ±4.7 ±0.9 1.9	9.1 ±4.1 ±0.8 2.2	13.8#±4.4 ±0.8 2.0	19.9#±8.6 ±1.6 1.6	
yes	2	11.9 ±5.0 ±0.9 1.7	12.0 ±3.7 ±0.7 1.8	11.8 ±4.6 ±0.8 2.1	13.0 ±5.8 ±1.1 1.9	11.8 ±5.2 ±0.9 2.0

* SCE-frequency (average of 30 metaphases), ** standard deviation,
*** standard error, **** proliferation index (average of 100 metaphases)
\# SCE frequency is significantly higher (at level $\alpha = 0.01$, Bonferroni adjusted) than the value of the control incubation

Alachlor induced a dose-dependent increase of the SCE-frequency in human lymphocytes in vitro. For example in one assay with 24 hrs incubation the base rate

of 11.4 SCE/cell was elevated up to 18.7 SCE/cell for the highest concentration of the test-compound (10^{-4} mol/L). These effects were only ascertained in cultures without the metabolically activating system, whereas in the presence of rat-liver S9-Mix, no genotoxic response could be detected (table 1.). Therefore in the SCE test-system alachlor could be identified as directly acting mutagen.
All other herbicides tested (atrazine, pendimethaline, simazine) did not increase the number of SCEs compared to non-treated lymphocytes either in presence or in absence of the external metabolically activating system (table 2.).
The SCE frequencies induced by cyclophosphamide which served as positive control were in the range between 70 and 85 SCEs in the presence and between 8,9 and 10,5 in the absence of S9-Mix for these four experiments.

Table 2. Induction of sister-chromatid exchanges by pendimethaline, atrazine and simazine in cultivated lymphocytes

S9-Mix	Time [h] of Incubation	pendimethaline [mol/L] 0	10^{-5}	$5 \cdot 10^{-5}$	10^{-4}
no	24	9.7*±3.0**	10.1 ±4.9		8.8 ±3.3
no	48	10.4 ±4.2	9.7 ±3.3	9.3 ±3.5	12.0 ±4.9
yes	2	9.4 ±3.9	10.8 ±4.5	9.9 ±3.5	9.0 ±2.7
		atrazine [mol/L] 0	$5 \cdot 10^{-6}$	$5 \cdot 10^{-5}$	
no	24	9.8 ±3.5	7.6 ±3.0	10.6 ±4.5	
no	48	10.1 ±5.6	9.9 ±3.3	9.5 ±3.4	
yes	2	9.4 ±3.9	8.5 ±3.6	9.6 ±3.6	
		simazine [mol/L] 0	$5 \cdot 10^{-6}$	$5 \cdot 10^{-5}$	
no	24	9.7 ±3.0	9.6 ±3.5	8.9 ±3.7	
no	48	10.2 ±4.1	11.0 ±4.7	8.5 ±3.4	
yes	2	8.9 ±3.5	7.0 ±2.4	8.7±3.0	

* SCE-frequency (average of 30 metaphases)
** standard deviation

By alkaline elution a dose dependent increase in elution rates was observed in V79 cells between 0.1 mM and 0.2 mM alachlor after 24 hrs incubation, indicating an increase in DNA single strand breaks or alkali labile lesions of the DNA. Using 0.1 mM alachlor the elution rate was 0.0086/hr ± 0.0013/hr (control cells: 0.0076/hr ± 0.0018/hr). With 0.2 mM alachlor an elution rate of 0.190/hr ± 0.024/hr was

detected. In the same dose range, however, toxic effects of alachlor were apparent because the cells lost their ability to exclude trypan blue. With 0.1 mM alachlor 83% of the V79 cells excluded trypan blue (control cells: 92%). Using 0.2 mM alachlor only 7% of the cells excluded trypan blue. 50% of trypan blue exclusion was estimated to be achieved with 0.12 mM alachlor.
Using a 72 hrs culture of mononuclear blood cells stimulated by phytohaemagglutinin, an acceleration in elution rates was observed between 0.03 mM and 0.1 mM of alachlor, accompanied by a loss of trypan blue exclusion.
Elution rates were 0.0074/hr ± 0.0010/hr with 0.03 mM (control cells: 0.0062/hr ± 0.0013/hr) and 0.072/hr ±0.020/hr with 0.1 mM alachlor. Using 0.03 mM alachlor 75% of the lymphocytes excluded trypan blue (control cells: 79%).
With 0.1 mM the percentage of trypan blue exclusion decreased to 14%. No DNA-protein cross links could be detected in V79 cells and lymphocytes by adding proteinase K (0.5 mg/mL) to the lysing solution.
In primary rat hepatocytes no DNA strand breaks and no damage of the cell membrane were observed up to 0.2 mM alachlor after 24 hrs incubation.
In summary, induction of DNA strand breaks measured by alkaline elution was accompanied by a loss of cell membrane integrity. Therefore single strand breaks caused by alachlor are more likely due to a secondary effect caused by the release of DNAses after damage of lysosomal membranes (Williams et al., 1974) than to a primary attack on the DNA.

The results of cytogenetic studies on alachlor indicate, that this agent induces mutagenic effects in procaryotes and lower eucaryotic cells only after plant metabolic activation (Gentile et al., 1977; Plewa et al., 1984). Negative results are reported from UDS-tests with rat-hepatocytes (Probst et al., 1981). On the other hand, alachlor was found to be clastogenic in CHO-cells with and without metabolic activation in a chromosome aberration induction-test (Lin et al., 1987) and in human lymphocytes using no activating-system (Georgian et al., 1983; Meisner et al., 1992).

These data and our results suggest that this acetamide herbicide may have a genotoxic potential in higher eucaryotic test systems also without and, following our results, only without metabolic activation.

Analysis of the mutagenicity of the s-triazine herbicides atrazine and simazine using microbial assays showed contradictory results. Clastogenic effects were not observed either with or without mammalian metabolic activation. In the light of information from activation protocols using plant systems (Plewa et Gentile, 1982) a pattern emerges from seemingly incongruous data. Clearly s-triazine herbicides are not activated by mammalian microsomal suspensions. The biotransformation of atrazine and simazine requires plant activation in microbial genetic indicator organisms. Only a few papers exist in which the induction of chromosome damage in cytogenetic test systems by s-triazine herbicides have been reported. Murnik and Nash (1977) showed that atrazine and simazine induced an increased frequency in

the loss of x- or y-chromosomes in Drosophila melanogaster. Both herbicides also elevated the rate of apparent dominant lethal mutations in the same test-organism. However, these effects might be caused by toxic effects rather than by mutation (Murnik and Nash, 1977).
Atrazine did not induce chromosome aberrations or sister chromatid exchanges in chinese hamster ovary cells (Plewa et al., 1984). However, at high in vivo doses (1500- and 2000 mg/kg) the herbicide induced a significant increase in the frequency of chromosome aberrations in bone marrow cells of treated mice compared to control mice (Loprieno et al., 1980).
Simazine induced forward mutations in mouse lymphoma cells (Waters et al., 1982). On the other hand, the substance caused no increase in SCE-frequency in CHO cells (Waters et al., 1982) and was also found to be non-genotoxic in the UDS- and chromosome abberation-test in human fibroblasts (Waters et al., 1982; Simmons et al., 1979; Ishidate, 1988).
Seiler (1977) also reported negative results, using an assay that measured the inhibition of mouse testicular DNA synthesis as an index of genotoxicity, up to 1000 mg/kg of simazine administered orally to male mice.

Only few data are available concerning the mutagenicity of the herbicide pendimethaline. Eisenbeis et al. (1981) reported the absence of reverse mutation in a Salmonella typhimurium spot test with and without mammalian activation system alone or in combination with other pesticides. The U.S. EPA (1984) has evaluated three tests, one dominant lethal study, one Ames assay and one host mediated assay with mice. The agency reports no mutagenic activity of pendimethaline in these test systems. Although the pesticide may contain a nitrosamine contaminant, the U.S. EPA (1984) has concluded, that this contaminant is not present at levels high enough to pose unacceptable level of risk to man. In summary, the limited information given by literature precludes any conclusion on the mutagenicity of pendimethaline.

Alachlor exhibits a very complex profile of metabolic products in vivo as well as in vitro (Feng and Patanella, 1989; Sharp, 1988). Glutathione conjugation, O-demethylation, benzylic hydroxylation, and N-desalkylation have been reported as major pathways of metabolism. Which pathway leads to ultimate genotoxic agents has not yet been totally elucidated. After activation with plant microsomes as well as in human lymphocytes without external metabolic activation, alachlor exhibits mutagenic or clastogenic properties. In the presence of mammalian liver microsomes and in rat hepatocytes alachlor seemed to be detoxified efficiently. To evaluate the hazard to human health by this herbicide it is therefore necessary to investigate different potentially exposed human cell systems as well as the impact on the total human organism.

Acknowledgment. This work has been partly supported by BMFT, FRG.

REFERENCES

Boyum A (1964) Separation of white blood cells. Nature 204:793

Eisenbeis SJ, Lynch DL, Hampel AE (1981) The ames mutagen assay tested against herbicides and herbicide combinations. Soil Sc 131:44-47

Environmental Protection Agency (1984) Health and environmental effects profile for pendimethaline. Environmental Criteria and Assessment Office, Office of Research and Development U.S. Environmental Protection Agency, Cincinnati

Feng PCC, Pantanella IE (1989) In vitro oxidation of alachlor by liver microsomal enzymes from rats, mice, and monkeys. Pestic Biochem Physiol 33:16-25

Gentile J M, Wagner ED, Plewa MJ (1977) The detection of weak recombinogenic activities in the herbicides alachlor and propachlor using a plant-activation bioassay. Mutat Res 48:113-116

Georgian L, Moraru I, Draghicescu T, Dinu I, Ghizelea G (1983) Cytogenetic effects of alachlor and mancoceb. Mutat Res 116:341-348

Hengstler JG, Loeffler S, Schaefer M, Glatt H, Fuchs J, Flesch P, Oesch F (1992) DNA strand break induction, mutagenicity, and cytotoxicity of the mycotoxins 11- ß-hydroxy-7-deoxy-rosenonolactone, rosenonolactone, and trichothecin. Mycotox Res 8:77-83

Ishidate M (1988) Data book of chromosomal aberration test in vitro. Elsevier, Amsterdam:p34

Lamberti L, Brigatti Ponzetto P, Ardito G (1983) Cell kinetics and sister chromatid exchange frequency in human lymphocytes. Mutat Res 102:193

Lin MF, Wu CL, Wang TC (1987) Pesticide clastogenicity in chinese hamster ovary cells. Mutat Res 188:241-250

Loprieno N, Barale R, Mariani L, Presciuttini S, Rossi AM, Sbrana I, Zaccaro L, Abbondandolo A, Bonatti S (1980) Results of mutagenicity tests on the herbicide atrazine. Mutat Res 74:250

Meisner LF, Belluck DA, Roloff BD (1992) Cytogenetic effects of alachlor and/or atrazine in vivo and in vitro. Environ Molec Mutagen 19:77-82

Murnik MR, Nash CL (1977) Mutagenicity of the triazine herbicides atrazine, cyanacine, and simazine in drosophila melanogaster. J Tox Environ Health 3:691-697

Perry P, Wolff S (1974) New giemsa method for the differential straining of sister chromatids. Nature 251:156

Plewa MJ, Gentile JM (1982) The activation of chemical mutagens by green plants, in: A. Hollaender and F. J. de Serres (Eds.): Chemical mutagens, principles and methods for their detection. Vol. 7, Plenum Press, New York

Plewa MJ, Wagner ED, Gentile GJ, Gentile JM (1984) An evaluation of the genotoxic properties of herbicides following plant and animal activation. Mutat Res 1366:233-245

Probst GS, Mc Mahon RE, Hill LE, Thompson CZ, Epp JK, Neal SB (1981) Chemically induced unscheduled DNA synthesis in primary rat hepatocyte cultures: A comparion with bacterial mutagenicity using 218 compounds. Environ Mutagen 3:11-32

Seiler JP (1977) Inhibition of testicular DNA synthesis by chemical mutagens and carcinogens. Preliminary results in the validation of a novel short term test. Mutat Res 46:305-310

Sharp DB (1988) Alachlor, in: Kearney PC, Kaufman DD (Eds) HerbicidesChemistry, degradation and mode of action, Marcel Dekker Inc. New York, Vol 3:301-333

Simmons VF, Poole DC, Riccio ES, Robinson DE, Mitchell AD, Waters MD (1979) In vitro mutagenicity and genotoxicity assay of 38 pesticides. Environ Mutagen 1:142-143

Utesch D, Glatt H, Oesch F (1987) Rat hepatocyte-mediated bacterial mutagenicity in relation to the carcinogenic potency of benz(a)anthracene, benzo(a)pyrene, and twenty-five methylated derivatives. Cancer Res 47:1509-1515

Waters MD, Sandhu SS, Simmon VF, Mortelmans KE, Mitchell AD, Jorgenson TA, Jones DCL, Valencia R, Garrett NE (1982) Study of pesticide genotoxicity. Basic Life Sci 21:275-326

Williams JR, Little JB, Shipley WU (1974) Association of mammalian cell death with a specific endonucleolytic degradation of DNA. Nature 252:754-755