Bull. Environ. Contam. Toxicol. (1998) 60:387-394

Bulletin of Environmental Contamination and Toxicology

DNA Adduct Formation by the Pesticide Alachlor and Its Metabolite 2-Chloro-N-(2,6-diethylphenyl)acetamide (CDEPA)

G. B. Nelson, J. A. Ross

Biochemistry and Pathobiology Branch, National Health and Environmental Effects Research Laboratory, United States Environmental Protection Agency, MD-68, Research Triangle Park, North Carolina 27711, USA

Received: 8 September 1997/Accepted: 23 December 1997

Alachlor (2-chloro-N-methoxymethyl-N-(2,6-diethylphenyl)-acetamide) is a pre-emergent herbicide used primarily to control weeds in corn and soybeans. Over 70 million pounds of alachlor are used annually (EPA 1989). The extensive use of alachlor presents the potential for occupational and environmental exposure to humans. Alachlor and its metabolites have been detected in the urine of pesticide applicators (Cowell et al. 1987; Sanderson et al. 1995).

Alachlor is an *in vitro* clastogen in Chinese hamster ovary cells (Lin et al. 1987) and human blood lymphocytes (Georgian et al. 1983; Meisner et al. 1992; Ribas et al. 1996). *In vivo* studies with Wistar rats exhibited a dose-related increase in chromosomal aberrations in bone marrow following a single intraperitoneal injection of alachlor (Georgian et al. 1983). Several studies have tested the mutagenicity of alachlor in various systems with both positive and negative results. Negative results were observed in *Salmonella typhimurium* with and without S9 activation (Shirasu et al. 1982). Activation by maize 1S fraction produced positive results in *S. typhimurium* (Plewa et al. 1984). Alachlor also produced significant gene conversion in *Saccharomyces cerevisiae* after plant activation (maize 1S fraction) or mammalian S9 activation (Plewa et al. 1984). Alachlor was mutagenic in the *Drosophila melanogaster* wing-spot test (Torres et al. 1992). DNA single-strand breaks were observed *in vitro* in rat hepatocytes and *in vivo* in rat liver and brain tissue as shown by the alkaline elution assay (Bontanti et al. 1992; Bagchi et al. 1995). DNA single-strand breaks were also observed *in vitro* with human peripheral blood lymphocytes by using the single-cell gel electrophoresis assay (Ribas et al. 1995).

Alachlor induces nasal, thyroid, and stomach tumors in rats and lung tumors in mice after lifetime oral administration (EPA 1984; EPA 1987). Alachlor is metabolized through multiple metabolic pathways to 2-chloro-N-(2,6-

Correspondence to: G. B. Nelson

diethylphenyl)acetamide (CDEPA), diethylaniline (DEA), and other metabolites. Alachlor, CDEPA, and DEA bind to mouse liver DNA and hemoglobin protein (Brown et al. 1988). DNA binding could occur directly with alachlor or CDEPA and by further metabolic activation of DEA. Adducts of alachlor and CDEPA with 2'-deoxyguanosine, 2'-deoxytbymidine and their 3'-monophosphates have been synthesized and characterized (Nesnow et al. 1995). On the basis of the work by Brown et al. (1988) and Nesnow et al. (1995), we selected polydeoxyguanosine and polydeoxythymidine as targets for adduction by alachlor and CDEPA. Alachlor's genotoxic and carcinogenic effects in conjunction with alachlor's ability to bind to DNA led us to further investigate alachlor-DNA adducts. The objective of the present study was to examine the direct binding of alachlor and CDEPA to calf thymus DNA and polydeoxynucleotides.

MATERIALS AND METHODS

Alachlor was purchased from Chem Service (Westchester, PA). Poly-ethyleneimine(PEI)-cellulose thin-layer chromatography plates were purchased from Alltech Associates, Inc. (Deerfield, IL). Micrococcal nuclease (MN) and calf thymus DNA were purchased from Sigma Chemical Co. (St. Louis, MO). 1-butanol was obtained from Fisher Scientific (Pittsburgh, PA). Calf spleen phosphodiesterase (SPDE) and nuclease P1 were purchased from Calbiochem (La Jolla, CA). Polydeoxyguanosine (poly dG) and polydeoxythymidine (poly dT) were obtained from Pharmacia LKB Biotechnology (Piscataway, NJ). Polynucleotide kinase (3'-phosphatase free) was purchased from Boehringer Mannheim (Indianapolis, IN) and [γ-^{32}P]ATP from Amersham (Arlington Heights, IL). Synthetic alachlor adduct standards, synthetic CDEPA adduct standards, and CDEPA were a gift from Dr. Stephen Nesnow.

Poly dG (0.4 mg), poly dT(0.25 mg), and calf thymus DNA (3.9 mg) were dissolved in 1.0 ml TE buffer (10 mM Tris-HCl, 1 mM Na_2EDTA, pH=7.5). Alachlor and CDEPA were dissolved in DMSO (10 mg/ml). Alachlor or CDEPA was combined with calf thymus DNA or polydeoxynucleotides with final concentrations ranging from 0.75 - 1.0 mg/ml and the reactions proceeded at 37°C for 72 h. Nucleic acids were recovered by ethanol precipitation, rinsed with 70% ethanol, and then redissolved in 1.0 ml TE buffer.

DNA adducts were analyzed by ^{32}P-postlabeling assay using the 1-butanol extraction method (Gupta 1985) and nuclease P1 enrichment (Reddy and Randerath 1986) to isolate DNA adducts. DNA digestion by SPDE/MN was verified by labeling an aliquot of normal DNA nucleotides prior to adduct

enrichment. Adduct enriched calf thymus DNA (5 µg), polydeoxynucleotides-CDEPA (5 µg) and polydeoxynucleotides-alachlor (2.5 µg) samples were postlabeled. Radiolabeled adduct nucleotide biphosphates were then separated by thin layer chromatography on 10 x 10 cm PEI cellulose plates. The following solvent system was employed: D-1, 1.7 M sodium phosphate, pH=6.0, with overnight development onto a 10 cm Whatman grade 3MM Chr wick followed by a wash with H_2O; D-3, 2.2 M lithium formate, 5.25 M urea, pH=3.5, followed by a wash with water; D-4, 0.5 M lithium chloride, 0.22 M Tris-HCl, 4.25 M urea, pH=8.0, followed by a wash with water; D-5, 1.7 M sodium phosphate, pH=6.0, with development onto a 3 cm Whatman grade 3MM Chr wick, followed by a final wash with water. Separated adducts were visualized with a PhosphorImager system (Model 400E, Molecular Dynamics Inc., Sunnyvale, CA). Co-chromatography was completed by combining aliquots of calf thymus DNA and polydeoxynucleotide samples prior to SPDE/MN hydrolysis and carrying this mixture through the ^{32}P-postlabeling assay. Thin layer chromatography was completed on 20 x 20 cm PEI cellulose plates with the listed solvents to enhance adduct resolution.

RESULTS AND DISCUSSION

DNA adducts were observed in calf thymus DNA and polydeoxynucleotides reacted with alachlor (Fig. 1) or CDEPA (Fig. 2) following butanol extraction and ^{32}P-postlabeling analysis. Calf thymus DNA reacted *in vitro* with alachlor results in the formation of one major (spot 1) and one minor (spot 2) covalent DNA adduct (Fig. la). Total alachlor adduction in calf thymus DNA was 2898.6 ± 994.2 amol/µg DNA (mean ± SD of 4 replicates) with spot 1 levels at 2643.0 ± 875.4 amol/µg DNA and spot 2 levels at 255.6 ± 121.2 amol/µg DNA. No major DNA adducts were observed in calf thymus DNA alone (Fig. lb). One major and one minor adduct were observed in poly dG (spots 1 and 2) and one minor adduct (spot 3) was observed in poly dT (Fig. lc,d) following *in vitro* reaction with alachlor. Two covalent adducts (spots 1 and 2) were observed in calf thymus DNA following *in vitro* reaction with CDEPA (Fig. 2a). Total CDEPA adduction in calf thymus DNA was 118.0 ± 14.4 amol/µg DNA (mean ± SD of 4 replicates) with spot 1 levels at 84.4 ± 15.0 amol/µg DNA and spot 2 levels at 33.6 ± 0.5 amol/µg DNA. The poly dG-CDEPA reaction formed two adducts and the poly dT-CDEPA reaction resulted in one adduct (Fig. 2c,d). Calf thymus DNA, poly dG, and poly dT were also reacted with alachlor or CDEPA at 37° C for 24 h. DNA adduct patterns were identical at 24 and 72 h reaction times with lower adduct levels being observed at 24 h. This suggests that alachlor and CDEPA are stable at 37 °C, but react slowly with calf thymus DNA and polydeoxynucleotides. Alachlor and CDEPA adducts were not observed in calf thymus DNA

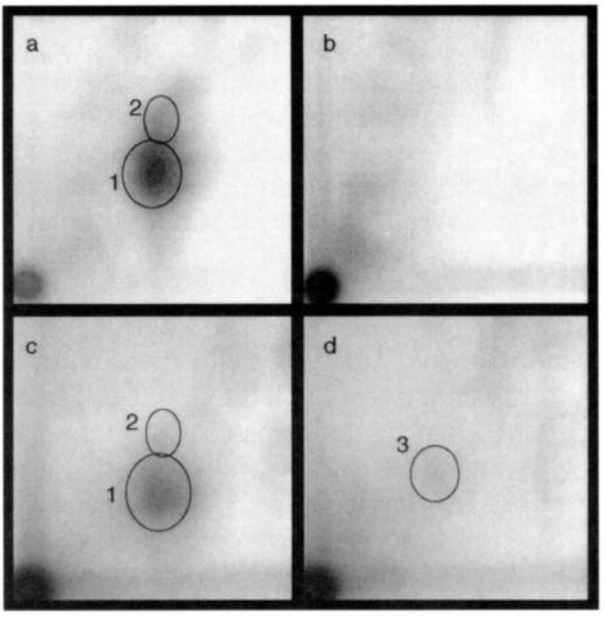

Figure 1. ^{32}P-postlabeling analysis of calf thymus DNA-alachlor adducts (a), calf thymus DNA alone (b), poly dG-alachlor adducts (c), and poly dT-alachlor adducts (d). The direction of elution for solvent D-1 was top to bottom; D-3 was bottom to top; D-4 and D-5 was left to right.

following nuclease P1 enrichment. The pesticides Guthion, Sencor, Lorox, Reglone, Daconil, and Admire have been studied for DNA adduct formation (Shah et al. 1997). Metabolites of the pesticides obtained enzymatically from arochlor induced rat liver S9 fraction formed *in vitro* calf thymus DNA adducts. DNA adducts were observed for all pesticides with nuclease P1 enrichment, but only in Sencor following butanol extraction. Alachlor and CDEPA adducts are sensitive to nuclease P1 as no adducts were observed by this enrichment method. Brown et al. (1988) reported that alachlor and CDEPA react directly with DNA and that this reactivity is enhanced by metabolic transformations. They used alachlor [^{14}C]-labeled in the phenyl and methoxy carbons to show that binding could occur directly from alachlor or CDEPA. They also showed that DNA adducts were formed by the metabolic activation of DEA or by formaldehyde released in the conversion of alachlor to CDEPA. The use of the ^{32}P-postlabeling assay in the present study confirms that alachlor and CDEPA bind directly to DNA.

DNA adducts from the calf thymus DNA and poly dG-alachlor reactions (spots 1 and 2) co-migrated on 20 x 20 cm TLC plates (data not shown). The poly dT-alachlor adduct (spot 3) did not co-migrate with either calf

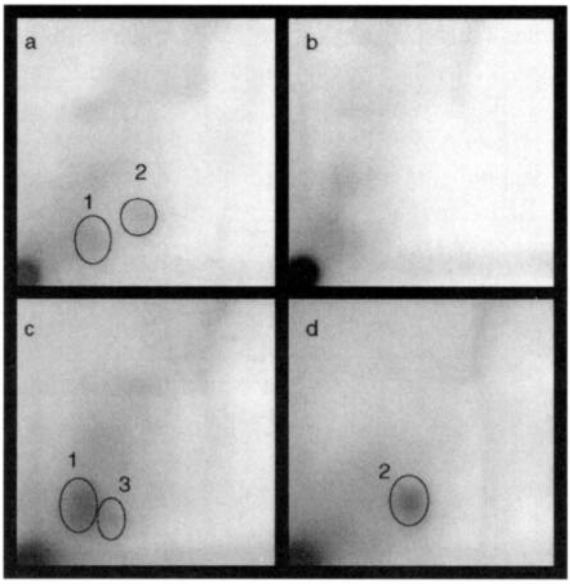

Figure 2. ^{32}P-postlabeling analysis of calf thymus DNA-CDEPA adducts (a), calf thymus DNA alone (b), poly dG-CDEPA adducts (c), and poly dT-CDEPA adducts (d). The direction of elution for solvent D-1 was top to bottom; D-3 was bottom to top; D-4 and D-5 was left to right.

thymus DNA-alachlor adduct (data not shown). The co-migration of calf thymus DNA-alachlor adducts with poly dG-alachlor adducts suggests that both calf thymus DNA-alachlor adducts occur at guanosine. Co-chromatography of calf thymus DNA-CDEPA and poly dG-CDEPA revealed that calf thymus DNA adduct 1 co-migrates with the poly dG-CDEPA adduct 1 (data not shown). Co-chromatography of calf thymus DNA-CDEPA and poly dT-CDEPA demonstrates that calf thymus DNA-CDEPA adduct 2 co-migrates with the poly dT-CDEPA adduct (data not shown) thus, calf thymus DNA covalent adducts from reaction with CDEPA appear to form at both the guanosine and thymidine bases.

The ^{32}P-postlabeling assay has been used to identify potential structures of DNA adducts by co-chromatography with known standards. Adducts of alachlor and CDEPA with 2'-deoxyguanosine, 2'-deoxythymidine, 2'-deoxyguanosine-3'-monophosphate (3'-dGMP) and 2'-deoxythymidine-3'-monophosphate (3'-dTMP) have been synthesized and characterized (Nesnow et al. 1995). Alachlor and CDEPA form N-1 adducts with 2'-deoxyguanosine and N-3 adducts with 2'-deoxythymidine under basic conditions. In addition, alachlor and CDEPA form N-1 adducts with 3'-dGMP and N-3 adducts with

3'-dTMP. Co-chromatography was completed with calf thymus DNA-alachlor adducts and synthetic alachlor adducts: l-[[*N*-(methoxymethyl)- *N*-(2,6-diethyl-phenyl)carbamoyl]-methyl]-2'-deoxyguanosine-3'-monophosphate and 3-[[*N*-(methoxymethyl)- *N*-(2,6-diethylphenyl)carbamoyl]-methyl]thymidine-3'-monophosphate. Calfthymus DNA-alachlor adducts and synthetic adducts did not co-migrate (data not shown). Co-chromatography was completed with calf thymus DNA-CDEPA adducts and synthetic CDEPA adducts: 1-[[*N*-(2,6-diethylphenyl)-carbaoyl]-methyl]-2'-deoxyguanosine-3'-monophosphate and 3-[[*N*-(2,6-diethylphenyl)carbamoyl]-methyl]thymidine-3'-monophosphate. Calf thymus DNA-CDEPA adducts and synthetic adducts did not co-migrate (data not shown). The chemical nature of the covalent calf thymus DNA-alachlor and CDEPA adducts from direct binding is currently unknown.

Studies examining cancer incidence in alachlor manufacturing workers exhibit uncertainty in possible elevated rates of cancer. However, alachlor does produce nasal, thyroid, and stomach tumors in rats and lung tumors in mice (EPA 1984; EPA 1987) following chronic feeding at high doses. Non-genotoxic mechanisms have been proposed to explain the increase in thyroid tumors (Wilson et al. 1996). However, numerous studies have demonstrated the genotoxicity of alachlor. Alachlor produces DNA single-strand breaks (Bontanti et al. 1992; Bagchi et al. 1995; Ribas et al. 1995), cytogenetic effects (Lin et al. 1987; Georgian et al. 1983; Meisner et al. 1992; Ribas et al. 1996), and has been shown to be mutagenic (Plewa et al. 1984; Tort-es et al. 1992). The formation of DNA adducts by genotoxic chemicals is a critical step in the initiation of carcinogenesis. This study demonstrates that alachlor and CDEPA form adducts *in vitro* with calf thymus DNA. The ability of alachlor and its metabolite CDEPA to form DNA adducts demonstrates a possible mechanism for alachlor genotoxic and carcinogenic potential.

In conclusion, the results of this study show that alachlor and CDEPA bind directly to calf thymus DNA. In addition, the calf thymus DNA adducts observed from direct binding do not co-migrate with previously described alachlor and CDEPA synthetic adducts. Alachlor and CDEPA adducts may provide the initial step for the genotoxic and carcinogenic potential of alachlor.

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

REFERENCES

Bagchi D, Bagchi M, Hassoun EA, Stohs SJ (1995) In vitro and in vivo generation of reactive oxygen species, DNA damage and lactate dehydrogenase leakage by selected pesticides. Toxicology 104: 129-140

Bontanti M, Taverna P, Chiapetta L, Villa P, D'Incalci M, Bagnati R, Fanelli R (1992) DNA damaage induced by alachlor after *in vitro* activation by rat hepatocytes. Toxicology 72:207-219

Brown MA, Kimmel EC, Casida JE (1988) DNA adduct formation by alachlor metabolites. Life Sci 43:2087-2094

Cowell JE, Danhaus RG, Kuntsman JL, Hackett AG, Oppenhuizen ME, Steinmetz JE (1987) Operator exposure from closed system loading and application of alachlor herbicide. Arch Environ Contam Toxicol 16:327-332

Environmental Protection Agency (1984) Alachlor. Special Review Position Document. Office of Pesticides and Toxic Substances, Washington, DC

Environmental Protection Agency (1987) Alachlor: Notice of Intent to Cancel Registration: Conclusion of Special Review.Office of Pesticides and Toxic Substances, Washington, DC

Environmental Protection Agency (1989) Pesticide Industry Sales and Usage: 1988 Market Estimates. Office of Pesticide Programs, Washington, DC

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

Gupta RC (1985) Enhanced sensitivity of ^{32}P-postlabeling analysis of aromatic carcinogen: DNA adducts. Cancer Res 45:5656-5662

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

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

Nesnow S, Agarwal SC, Padgett WT, Lambert GR, Boone P, Richard AM (1995) Synthesis and characterization of adducts of alachlor and 2-chloro-N-(2,6-diethylphenyl)acetamide with 2'-deoxyguanosine, thymidine, and their 3'-monophosphates. Chem Res Toxicol 8:209-217

Plewa MJ, Wagner ED, Gentile GJ, Gentile JM (1984) An evaluation of the genotoxic properties of herbicides following plant and animal activation. Mutat Res 136:233-245

Reddy MV, Randerath K (1986) Nuclease P1-mediated enhancement of sensitivity of ^{32}P-postlabeling test for structurally diverse DNA adducts. Carcinogenesis 7: 1543-1551

Ribas G, Frenzilli G, Barale R Marcos R (1995) Herbicide-induced DNA damage in human lymphocytes evaluated by the single-cell gel electrophoresis (SCGE) assay. Mutat Res 344:41-54

Ribas G, Surralles J, Carbonell E, Xamena N, Creus A, Marcos R (1996) Genotoxicity of the herbicides alachlor and maleic hydrazide in cultured human lymphocytes. Mutagenesis 11:221-227

Sanderson WT, Biagini R, Tolos W, Henningsen G, MacKenzie B (1995) Biological monitoring of commercial pesticide applicators for urine metabolites of the herbicide alachlor. Am Ind Hyg Assoc J 56:883-889

Shah RG, Lagueux J, Kapur S, Levallois P, Ayotte P, Tremblay M, Zee J, Poirier GG (1997) Determination of genotoxicity of the metabolites of the pesticides Guthion, Sencor, Lorox, Reglone, Daconil and Admire by ^{32}P-postlabeling. Mole Cell Biochem 169: 177-184

Shirasu Y, Moriya M, Tezuka H, Teramoto S, Ohta T, Inoue T (1982) Mutagenicity screening studies on pesticides. Environmental Mutagens and Carcinogens. Alan R. Liss, NY 331-335.

Torres C, Ribas G, Xamena N, Creus A, Marcos R (1992) Genotoxicity of four herbicides in the Drosophila wing spot test. Mutat Res 280:291-295

Wilson AGE, Thake DC, Heydens WE, Brewster DW, Hotz KJ (1996) Mode of action of thyroid tumor formation in the male Long-Evans rat administered high doses of alachlor. Fund App Toxicol 33 : 16-23