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Environmental Contamination and Toxicology

Evaluation of Acute Immunotoxicity of Alachlor in Male F344/N Rats

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Alachlor (2-chloro-2',6'-diethyl-N-[methoxymethyl] acetanilide) is one of the most commonly used herbicides in the United States (see Figure 1), as it is the active ingredient in several trade name preparations that control pre-emergence of many broadleaf weeds and grasses. Alachlor acts as a herbicide by inhibiting protein synthesis in susceptible plants (Weed Science Society 1979). The Environmental Protection Agency (EPA) has classified alachlor, and pesticide products containing alachlor as an active ingredient, restricted to use by certified applicators or persons under their direct supervision (Federal Register 1987). These terms and conditions were instituted because pesticide products containing alachlor met or exceeded EPA's oncogenicity risk criteria. Specifically, EPA determined that exposure to alachlor resulted in increased incidence of tumors at multiple sites in two species of laboratory animals (Daly et al. 1977). However, epidemiological evidence for the carcinogenic potential of alachlor in humans is absent.

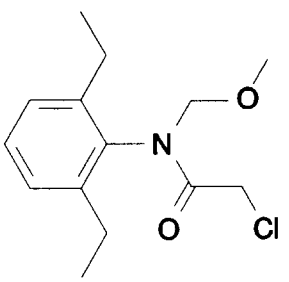

Figure 1. Structure of Alachlor

It has been well documented that certain environmental chemicals, at relatively low doses, adversely affect normal functions of the mammalian immune system (for reviews see Dean et al. 1982; Koller et al. 1979; Vos 1977). It has also been shown that chemical-induced immune dysfunction can be associated with increased incidence of infectious disease and cancer in laboratory animals (Exon et al. 1975; Exon et al. 1979; Dean et al. 1980; Ward et al. 1984). The purpose of the present study was to investigate the potential immunotoxicity of alachlor in rats using a general multiple immunoassay model (Exon et al. 1986).

MATERIALS AND METHODS

Male Fisher 344/N rats were obtained from the National Toxicology Program Animal

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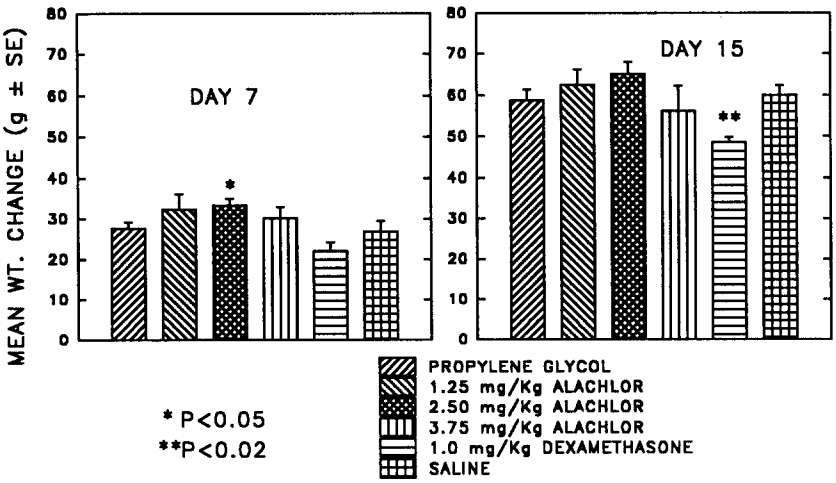

Figure 2. Effect of exposure to alachlor, dexamethasone and vehicles on body weight change at days 7 and 15.

Colony (Research Triangle Park, NC). The rats were 8 wks old at the start of the study, after being routinely quarantined for 2 wks upon arrival at our laboratory. Animals were randomly assigned to cages and to treatment groups and housed four to a cage in stainless-steel, hanging, wire enclosures with mesh bottoms. Animals were randomly assigned to six groups blocked on three separate days as follows: Group 1 (N=12), 10 mL/Kg propylene glycol vehicle; Groups 2-4 (N=12), Alachlor (solubilized in propylene glycol) at 1.25, 2.50 and 3.75 mg/Kg; Group 5 (N=9), Dexamethasone (positive control), 1 mg/Kg solubilized in physiological saline (PS); and Group 6, (N=9), PS control. All doses were administered as intraperitoneal (ip) injections at volumes of 10 mL/Kg using 18-20 ga needles. Doses and routes of exposure to alachlor were based on literature ports for acute toxicity studies in rats (Georgian et al. 1983). Three ip injections of the either alachlor, vehicle control, or positive control were administered on days -1, 6 and 13 of the 14-day study. The rats were given water and commercial rodent chow (Formulab Chow 5008, Purina Mills, St. Louis, MO) ad libitum. This study was conducted in facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

Alachlor (98.5%, lot No. 45-80A) was obtained from Chem Service, Inc. (West Chester, PA). Dexamethasone sodium phosphate was obtained from American Regent Laboratories (Shirley, NY).
All other chemicals used were reagent grade or of the highest grade commercially available. All water used for solutions was deionized following reverse osmosis pre-treatment.

Delayed type hypersensitivity (DTH) reactions were evaluated as left minus right foot weights by a modification of the method of Henningsen, et al. (1984). Keyhole limpet hemocyanin (KLH, Calbiochem, LaJolla, CA) was injected subcutaneously (1.0 mg

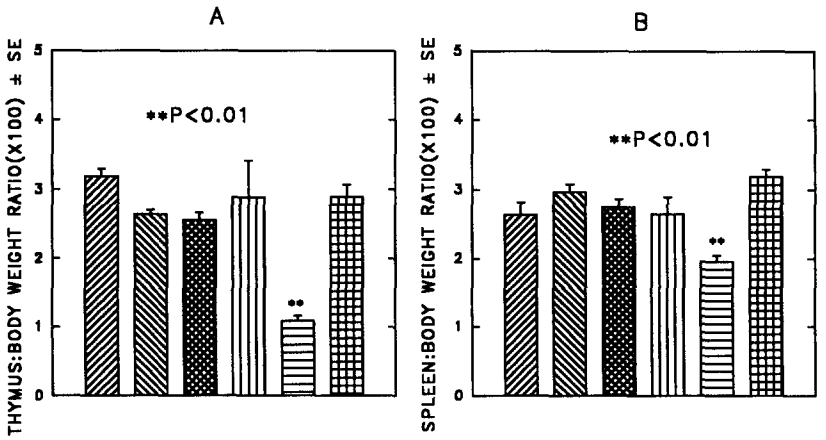

Figure 3. Effect of exposure to alachlor, dexamethasone and vehicles on thymus: (panel A) and spleen: body weight (panel B) ratios (mean ± SE; see legend on Figure 2).

KLH in 0.2 mL H_2O) at the base of each rat's tail on days 0 and 7. Two percent heat aggregated (HA) KLH was injected subcutaneously (sc) (0.1 mL) into the left footpad (producing a plantar bleb, using 25 ga x 1/2 in. needles) on day 13. A right footpad sc injection of 0.1 mL PS vehicle was also performed on day 13. On day 14, after sacrifice by CO_2 inhalation, the left and right feet of the rats were precisely severed at the tibio-tarsal joints and weighed. The difference in weight of the heavier, swollen left foot and the contralateral foot was recorded. Dorsal-ventral swelling of the feet was also measured and the difference in mms was recorded, but this method of measurement was less precise than the foot weight differences.

Body weights were measured on days 0, 7, and 14. Organ weights of the thymus, spleen, kidney and liver were recorded on day 14 following sacrifice. Blood was collected by posterior vena cava phlebotomy, and sera was harvested for the determination of humoral IgG antibodies to KLH and alachlor-bovine serum albumin conjugate (ALA-BSA). Observations concerning gross pathologic changes in major organ systems were also noted.

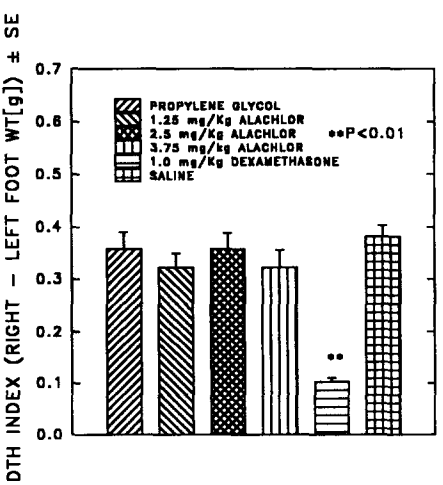

Figure 4. Effect of exposure to alachlor, dexamethasone and vehichles on delayed type hypersensitivity (DTH) index.

Sera samples were evaluated for anti-KLH IgG antibody by an indirect enzyme-linked immunosorbent assay as previously reported (Exon et al. 1986; Biagini et al. 1990). Optical density at 410 nm (490 nm as dual reference wavelength) was read on an automated ELISA plate reader (model MR700, Dynatech, Alexandria, VA). Data from a common sera dilution factor (1:1600), which was in the linear portion of the

titer curve for all animals (without exceeding the reader's absorbency maximum) was chosen in order to perform hypotheses tests. Anti-alachlor-bovine serum albumin (anti-ALA-BSA) IgG antibodies were measured by a procedure similar to the one outlined above, except the plates were coated with 100μl/well of a solution of 250μg/mL of ALA-BSA (obtained from Immunosystems, Inc., Scarsdale, NY) solubilized in PS. Sera were diluted 1:5 and analyzed in duplicate as outlined above.

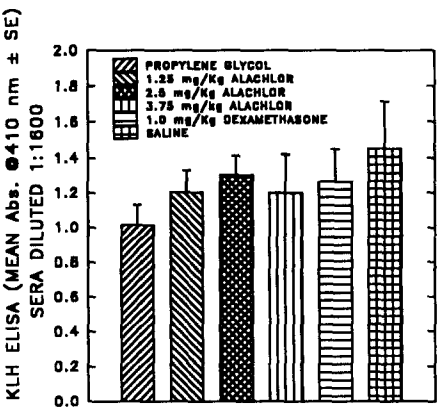

Figure 5. Effect of exposure to alachlor, dexamethasone and vehichles on anti-KLH-IgG levels.

Non-adherent splenic cell suspensions were used as the source of natural killer (NK) cells for the NK assay (Exon et al. 1986), The cells were resuspended at 1 x 10^7 cells/mL. Cell viability determinations and counts were performed by trypan blue exclusion and optical microscopy with hemocytometers, respectively.

Briefly, 100 μl aliquots of the NK-containing cell suspensions were dispensed into appropriate wells of 96-well round-bottomed tissue culture plates (with covers) to yield 1 x 10^6, 5 x 10^5 and 2.5 x 10^5 cells/well in individual wells (cellular additions were performed in triplicate for all dilutions). This yielded 100:1, 50:1 and 25:1 effector:target cell ratios as explained further below. The target cells (YAC-1 and EL-1, obtained from ATCC, Rockville, MD) were grown as stationary suspension cultures.. They were labelled with 250-400 μCi of ^{51}Cr (as sodium chromate, NEN Research Products, Boston, MA) per 10^7 cells. The cells were labelled during their exponential growth phase (24-28 hr) after transfer. The labelled cells were dispensed at 100μl (10^4 cells) into appropriate wells of 96-well culture plates containing NK cells, from above, and incubated for 4 hours at 37°C. After the incubation, cell free supernatants were collected from each well using a filter press (Skatron SCS Filter Press, Lier, Norway) and the filters were counted in a gamma counter (COBRA, Packard Instruments Co., Downers Grove, IL). The percentage of maximal ^{51}Cr release was calculated by the following formula:

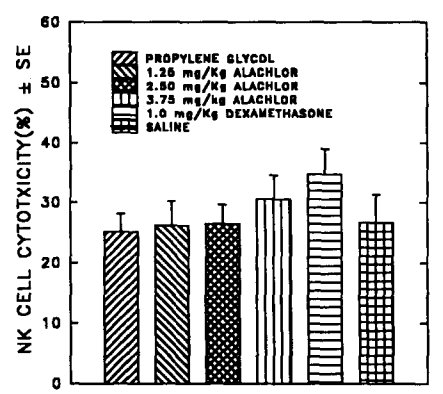

Figure 6. Effect of exposure to alachlor, dexamethasone and vehicle on NK cell cytolytic activity.

$$\frac{\text{cpm experimental release} - \text{cpm spontaneous release}}{\text{cpm maximal release (2\% SDS)} - \text{cpm spontaneous release}} \times 100$$

NK-resistant EL-4 cells were labeled in exactly the same way and plated for total release (3 wells), spontaneous release (3 wells), and experimental release (only 1 sample was run in triplicate for each animal at the 100:1 target:effector ratio).

Hematocrit (HCT), total white blood cell counts (WBC), and the number and percentages of lymphocytes\monocytes and granulocytes were counted using a centrifugal hematology system (QBC, Becton Dickinson, Franklin Lakes, NJ).

All null hypothesis tests were performed using non-parametric methods, as the results of tests for population distribution indicated, the data were not normally dispersed. Kruskal-Wallis's ANOVA (Number Cruncher Statistical System, Dr. Jerry Hintze, Kaysville, UT.) followed by Wilcoxon signed rank tests were used to investigate group differences. Gross pathological observations were investigated for significance using 2X6 Chi Square analyses. Body weights were taken at days 0, 7 and 14. Treatment effects on body weight were investigated by the ANOVA methods outlined above with contrasts between vehicle control and exposure groups (i.e., propylene glycol vehicle vs. alachlor; saline vs. dexamethasone). A Type 1 error level of $P \leq 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

The animals in this study experienced no major clinical signs from the experimental regimens. Body weight gains over the course of the experimental procedures were generally unaffected by treatment, except for a statistically significant increase in weight gain for the rats treated with 2.5 mg/Kg alachlor at seven days, which disappeared by day 15. Dexamethasone treatment significantly ($P < 0.02$) reduced body weight (compared to the other groups) on day 15 of the study (Figure 2). Dexamethasone treatment also significantly reduced ($P < 0.01$) spleen:body weight and thymus:body weight ratios. No significant effects in these parameters were observed for any of the other treatment groups (Figure 3, Panels A and B). Some of the rats appeared listless with ruffled fur on the day after injection, but returned to normal appearance by two days after the injection regimens. At sacrifice, some animals showed mild gross lesions at the injection sites (skin ulcers), peritoneal adhesions, and mild peritonitis (probably from physical trauma due to the repeated injection regimens). However, there were no significant ($P > 0.05$) treatment related differences among groups for any of these findings. Alachlor treatment had no statistically significant effects on the DTH index (right -left foot weight). Dexamethasone treatment significantly reduced ($P < 0.01$) the DTH index, when compared to the other treatment and control groups (Figure 4).

The treatment regimens used in the current study had no statistically significant ($P > 0.05$) effects on the ability of the animals to raise antibodies in response to KLH immunization. Values ranged from about 80 to 120 percent of ELISA absorbencies found in standard positive sera (obtained from rats immunized previously with KLH in the absence of any other xenobiotic exposure, Figure 5).

NK cell cytotoxicity was also not significantly altered by the treatment and control regimens used in the present study. No statistically significant effects were observed between alachlor treated and either saline or propylene glycol control animal groups (Figure 6).

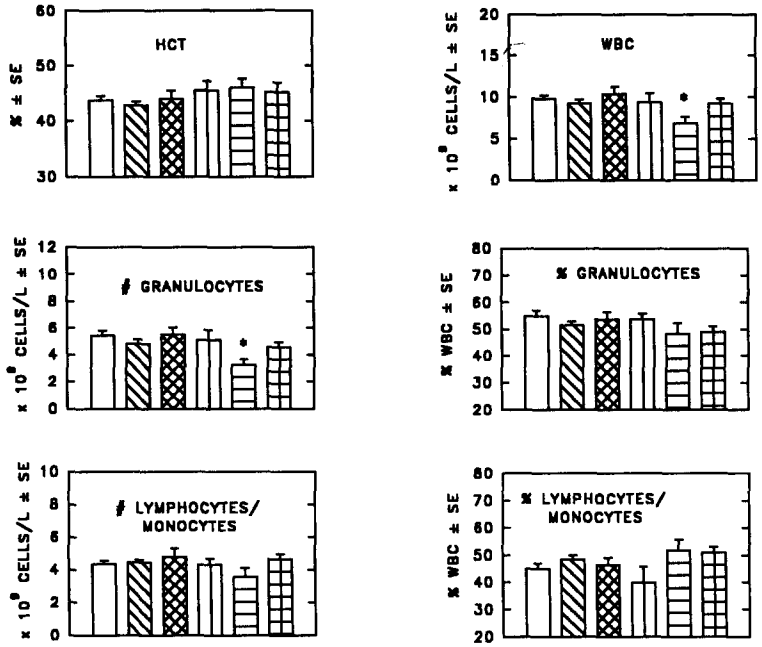

Figure 7. Effect of exposure to alachlor, dexamethasone and vehicles on blood cell counts ± SE. See bar fill legend on Figure 2.

Alachlor treatments had no statistically significant effects on hematocrit (HCT), total white blood cell counts (WBC), and the number and percentages of lymphocytes and granulocytes. In contrast to this, dexamethasone treatment yielded significantly reduced ($P < 0.05$) total white blood cell counts and numbers of granulocytes (Figure 7).

No significant differences ($P > 0.05$)in levels of alachlor-BSA-specific IgG antibodies were observed either between or within any of the groups reported in this study (not shown).

The dosages of alachlor used in the present study were derived from a study of the cytogenetic effects of alachlor in Wistar rats (Georgian et al. 1983). In that work, ip dosages of 5.0 mg/Kg proved to be fatal to all rats in 2-4 hrs. In the present study, dosages of up to 3.75 mg/Kg (ip) resulted in no fatalities nor weight loss at 14 days, however, some of the rats appeared listless with ruffled fur the day after injections, reverting to normal appearance one to two days after injection. However, statistical analysis indicated that this observation was not treatment related, and was considered to be due to the injection regimen. These findings suggest that the animals were being treated with dosages below the maximum these rats could tolerate for the present protocol, even though a highest dose of 75% of an acute LD_{100} dose was repeatedly administered.

Results of the present study indicate that alachlor (solubilized in propylene glycol), when given at 1.25, 2.50 and 3.75 mg/Kg to Fisher 344N rats on days -1, 6 and 13 of a 14-day study, has no statistically significant effects (compared to vehicle alone) on humoral

antibody to KLH, DTH, NK, alachlor ALA-BSA specific IgG antibody production, body weights, spleen:body weight ratio, thymus:body weight ratio and common hematologic parameters. The multiple immunoassay model was relatively comprehensive, and has been shown to be sensitive to xenobiotic effects on major types of immune responses, immunocyte populations and immunoregulatory pathways for detecting both immunosuppression and immunoenhancement (Exon et al. 1986). In addition, treatment with a moderate dose of dexamethasone (positive control) yielded statistically significant reductions on spleen:body weight ratios, thymus:body weight ratios, DTH, and some common hematologic parameters (compared to its PS vehicle control). The results for dexamethasone's effects on this multiple immunoassay model, in general, are consistent with those reported previously (Koller et al. 1983; Murray and Melanson 1987) and strongly suggest that all assays were functioning correctly.

It is not the intent of this report to indicate that alachlor has no immune modulating potential in humans. Orally administered alachlor is extensively and exhaustively metabolized in rats to at least 23 metabolites such as mercapturates, methylsulfoxides, sulfones, mono- and di-hydroxlated compounds, glcuronides and a phenol sulfate (Sharp 1988). Rats eliminate alachlor metabolites about equally (1:1 ratio) in urine and feces (Sharp 1988). In contrast to this, monkeys excrete alachlor in 10:1 ratio (urine:feces) to five major metabolites (two mercapturates, a cysteinyl conjugate, a glucuronide and a thioacetic acid conjugate) (Sharp 1988). These five monkey alachlor metabolites have also been identified in rat excretion studies (Sharp 1988), but as noted above, quantitatively in different amounts and by different routes.. This information, in addition to results of excretion studies in humans (Dubelman and Cowell 1989), suggest that monkey and human profiles are essentially equal, while rodent species appear to metabolize and excrete alachlor quite differently than primates. Therefore, the potential exists for primate specific metabolite(s) of alachlor to be immunotoxic or have other immune mediated effects which would not be identified in a rodent immunoassay.

In conclusion, results of the present study of the immunotoxic potential of alachlor in a rodent model suggests that immunologic effects of alachlor are minimal, in comparison to immune modulation caused by a therapeutic level of dexamethasone, and are likely not related to alachlor's carcinogenic effects in rodents.

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REFERENCES

Biagini, RE, Klincewicz, SL, Henningsen, GM, MacKenzie B, Gallagher, JS, Bernstein, IL and Bernstein, IL (1990) Antibodies to morphine in workers occupationally exposed to opiates at a narcotics manufacturing facility and evidence for similar antibodies in heroin abusers. Life Sciences 47:897-908

Daly IW, McCandless JB, Jonassen H (1981) A chronic feeding study of alachlor in rats, (unpublished) submitted to EPA by Monsanto Co., Project No. 77-2065, EPA Accession No.91050

Dean, JH, Luster, MI, Boorman, GA, Lauer, LD, Luebke, RW (1980) The effect of adult exposure to diethylstilbestrol in the mouse: Alterations in tumor susceptibility and

host resistance parameters. J Reliculoendolhel Soc 28:571-583
Dean, JH, Luster, MI, Booorman, GA (1982). Immunotoxicology. In: Sirois P, and M. Rola-Pleszczynski (eds) Research Monographs in Immunology: Immunopharmacology 4:349-398.
Dubelman S and Cowell JE (1989) Biological monitoring technology for measurement of applicator exposures. In: Wang, RGM, et al. (ed) Biological Monitoring for Pesticiide Exposure, ACS Symposium Series 382, American Chemical Society, Washington, DC, p 240.
Exon, JH, Patton, NM, Koller, LD (1975) Hexamitiasis in cadmium-exposed mice. Arch Environ Health 30:463-464.
Exon, JH, Koller, LD, Kervliet, NI (1979) Lead-cadmium interaction: Effecls on viral-induced mortality and tissue residues in mice. Arch Environ Health 34:469-475
Exon JH, Koller LD, Talcott PA, 0'Reilly CA, Henningsen GM (1986) Immunotoxicity Testing: An Economical Multiple Assay Approach. Fund Appl Toxicol 7:387-397
Federal Register, Alachlor; Notice of Intent to Cancel Registrations; Conclusion of Special Review, Vol. 52. No. 251, Thursday, December 31, 1987, p. 49480
Georgian, L, Moraru, I, Draghicesu, I, Ghizellea, G (1983) Cytogenetic effects of alachlor and mancozeb. Mutat Res 116:341-348
Henningsen GM, Exon JH and Koller LD (1984) A Sensitive Delayed-Type Hypersensitivity Model in the Rat. J Immunol Meth 70:153-165
Koller, LD (1979). Effects of environmental chemicals on the immune system. Adv Vet Sci Comp Med 23:267-295.
Koller, LD, Exon, JH, Henningsen, GM, Osborne, CA (1983) Multiple immunoassays in a one rat system. Toxicologist 3:56
Murray, MJ, Melanson, PA (1987) Species comparison of immune function assays commonly included in immunotoxicity assessment. The Toxicologist 7:225
Sharp, DB (1988) Alachlor. In: Kearny PC and Kaufman DD (ed) Herbicides: Chemistry, Degradation and Mode of Action, vol 3. Marcel Dekker, New York, pp301-333.
Vos, JG (1977) Immune suppression as related to toxicology. CRC Crit Rev Toxicol 5:67-101.
Ward, EC, Murray, MJ, Lauer, LD, House, RV, Irons, R, Dean, JH (1984) Immuno-suppression following 7,12-dimethylbenzalanthracene exposure in B6C3FI mine. Effects on humoral immunity and host resistance. Toxicol Appl Pharmacol 75:299-308
Weed Science Society of America, Herbicide Handbook, 4th ed. (1979) Champaign, Ill, p. 9.

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