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Effect of Propoxur on Humoral and Cell-Mediated Immune Responses in Albino Rats

V. Seth,[1] B. D. Banerjee,[1] A. K. Chakraborty,[1] L. Institoris,[2] I. Desi[2]

[1] Environmental Biology Laboratory, Department of Biochemistry, University College of Medical Sciences and Guru Teg Bahadur Hospital, University of Delhi, Dilshad Garden, Shahdara, Delhi-110095, India
[2] Department of Public Health, University Medical School Szeged, H-6720, Szeged, Domter 10, Hungary

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Propoxur (2-Isopropoxy phenyl N-methyl carbamate) is a well known carbamate insecticide. It is widely used in agriculture and public health programs. Exposure of the general population to propoxur may occur through the consumption of food stuffs treated with this pesticide and/or harvested before residues have declined to acceptable levels, from domestic use or by drift from aerial spraying. Several cases of suicidal and occupational poisoning have been reported (Hayes 1991; Banerjee et al. 1998, 1999; Seth et al. 2001). Hence, propoxur has generated considerable concern regarding its subtle health effects.

Studies with laboratory animals, case reports, accidental or acute poisonings and epidemiological studies have provided significant information about the toxicological properties and pharmacodynamics of propoxur (WHO 1986, FAO and WHO 1989). Despite existing this knowledge, it can not be predicted with certainty how chronic exposure to propoxur affects human health. The possible effect of persistent exposure to propoxur on the functional integrity of the immune system has recently heightened interest as an additional index to analyse potential long term health problems (Banerjee et al 1996a; Banerjee 1999; Descotes 1998).

There are some limited and conflicting reports in the literature about inadvertent alteration of immune responses after acute or short-term exposure to carbamate pesticides such as carbaryl, carbfuron, aminocarb, aldicarb, methiocarb, prophom, zineb, and methyl-di-thio-carbamate (Repetto and Baliga 1996; Rodgers et al. 1992). In general, these assessments of pesticide-induced immunotoxicity were based upon extrapolation of multiple factors and/or variables including dose, duration of exposure, animal experiments and immunological parameters (Banerjee 1999; Banerjee et al. 2001).

At present, very little is known about the effect of propoxur on the immune system. Indeed, only a single report suggested both immunosuppression and immunostimulation by propoxur in mice (Gieldanowski et al. 1991). Such controversial results and scanty data need further systematic investigation to establish the definitive immunomodulatory role of propoxur. Moreover,

Correspondence to: B. D. Banerjee

extensive studies on dose-time relationship in different experimental animals are required in order to be able to extrapolate and predict the effect of this pesticide on human immune system. Keeping this in view, the present study was designed to investigate the effect of sub-chronic doses of propoxur on humoral and cell mediated immune responses in albino rats.

MATERIALS AND METHODS

Propoxur (99.4% purity) was obtained through the courtesy of M/S Bayer AG, Monheim, Germany. Ovalbumin, bovine serum albumin, o-phenylenediamine (OPD), tween-20 and goat anti-rat IgG conjugated with peroxidase were obtained from Sigma Chemical Co., St. Louis, USA. Freund's complete adjuvant (FCA), fetal calf serum (FCS), and tissue culture media RPMI-1640 were purchased from Difco Laboratories, Detroit, MI, USA. Sheep red blood cells (SRBC) and guinea pig serum used as antigen and complement source respectively, were obtained fresh from our central animal house. Keyhole lympet hemocyanin (KLH) was obtained from CAL Biochem; Lajolla, USA. Sources of other chemicals and materials presented in earlier papers (Banerjee and Hussain 1986; Banerjee et al. 1997).

Wistar male albino rats (4 rats per cage) weighing 200-250 gms were kept under a 12-12 hr light and dark cycle, 22° $\pm$ 2°C temperature and 70$\pm$10% humidity. Rats were randomly divided into four groups. Propoxur was dissolved in groundnut oil of pharmaceutical quality, and animals were administered 10, 30 and 90 mg/kg body weight propoxur/day for 30 days, using syringe and 20 gauge stomach tube. Each treatment group consisted of 10-12 animals. An equal number of animals in each group served as control and received vehicle treatments in an identical manner. Food consumption, general condition and any other clinical symptoms were observed daily and body weight were recorded weekly.

Rats (10-12 animals/pesticide or antigen treatment group) were immunized with 2 x 10^9 SRBC (intraperitoneally, in 0.5 ml normal saline), or with ovalbumin (subcutaneously, 3 mg dissolved in 0.2 ml normal saline), and KLH (subcutaneously, 1 mg dissolved in PBS) mixed with equal volume of FCA. Sterile liquid paraffin (5 ml) was injected intraperitoneally in rats immunized with ovalbumin 48 hrs before terminating exposure (Banerjee and Hussain 1986).

Animals (10-12/group) were used for estimation of antibody titer (after 20 days of immunization with ovalbumin), splenic-antibody (IgM) forming cells (after 4 days SRBC immunization), leukocyte migration inhibition (LMI) and macrophage migration inhibition (MMI) tests (after 20 days of immunization with ovalbumin), and delayed type of hypersensitivity (DTH) reaction (after 14 days of immunization with KLH).

Blood samples were collected from chloroform anesthetized rats by cardiac puncture. Serum samples were kept separate at -20°C until analyzed.

Heparinized blood samples and peritoneal macrophages were collected for LMI and MMI tests respectively. The liver, spleen and thymus were removed immediately, blotted and weighed. Acetylcholine esterase (AChE) activity in RBC was assayed according to the method described by Ellman et al (1961). The rate of hydrolysis of acetylthiocholine by RBC hemolysate at pH 7.2 was measured as enzyme activity (KAU/l of packed cell volume) and results expressed as percentage of mean control values (Banerjee et al. 1999).

A Spleen cell suspension was prepared, from each rat and used for IgM plaque-forming cell (PFC) assay. In a polystyrene tube, the following sample preparation was made: 30 μl of splenocytes in HBSS, 70 μl of SRBC, and 500 μl of guinea pig complement. In a Cunningham chamber 200 μl of sample was pipeted. Duplicate were made; the chambers were sealed with liquid paraffin, and incubated for 60 min at 37°C. After incubation counts for PFC were made microscopically, and results were calculated as PFC/10^6 spleen cells (Institoris et al. 1995; Banerjee et al. 1997).

Serum antibody titer to ovalbumin was estimated by enzyme linked immunosorbant assay according to the procedure described by us earlier (Banerjee et al. 1996b). In brief, flat bottomed polysterene plates (Titertek) were coated with 12.5 μg of ovalbumin dissolved in 100 μl of 0.1 M sodium carbonate buffer (pH 9.6) at 4°C for 12 hr. Serial dilution of sera in PBS (0.15 M, pH 7.2, containing 0.05% tween-20, PBS-Tw) were prepared and 100 μl was incubated with coated wells for 1 hr at 37°C. After washings with PBS-Tw, the 100 μl of anti-rat IgG conjugated with peroxidase diluted in PBS-Tw (1:1000) was added and incubated at 37°C for 1 hr. The enzyme activity was determined at room temperature by addition of 100 μl of OPD (400 μg/ml) in sodium citrate buffer (0.1 M, pH 4.9 containing 1.5 μl of 30% H_2O_2/ml). The reaction was stopped with 8 N H_2SO_4 (50 μl) after 30 min and absorbance was measured at 490 nm. The antibody titer was expressed as $\log_2$ of the reciprocal of the highest serial two fold dilution of the serum showing three times the absorbance of normal serum.

LMI and MMI tests were assayed as described by us in detail earlier (Banerjee and Hussain 1986). Briefly, for LMI test blood (4.5 ml) was was added to 2 ml 3% dextran and leucocytes were separated. The leucocyte pellete was washed twice in RPMI 1640 media (pH 7.2), and a final suspension containing 15 x 10^6 leucocytes/ml was made. Similarly, for MMI test peritoneal exudate cell pellet was washed and a final suspension containing 15 x 10^6 cells/ml was prepared. The cells were pelletized in microcapillary tubes by centrifugation. The capillaries were cut at the pellet-medium interphase and 3-4 cell "explants" were mounted in perspex migration chamber with sterile silicon grease. The chambers were filled up with RPMI 1640 medium (containing 5% FCS) with or without ovalbumin (50 μg/ml for LMI or 125 μg/ml for MMI), closed with cover-slip and incubated at 37°C in humid atmosphere for 20 hrs. The area of migration of leucocytes or macrophages in control and test chambers was recorded with the aid of camera lucida and percentage migration inhibition was calculated.

DTH reaction was challenged on 28th day by injecting 17.5 μg KLH in 50 μl PBS into the left (L) hind foot. Foot pad thickness was measured at 24 hrs and 48 hrs after challenge using a dial caliper (Mitutoyo, Japan). The right (R) hind foot pad was injected with 50 μl PBS and this served as the control. The results of DTH reaction were expressed as the percentage increase in foot pad thickness (L-R) over the vehicle treated control values (Institoris et al. 1995).

Data were analyzed by ANOVA test using SPSS version 5 statistical program and the individual effects of treatment were obtained by using Tukey's multiple comparison procedure at $p < 0.05$ (Wayne 1987).

RESULTS AND DISCUSSION

There is a paucity of information in the literature regarding the dose-dependent effect of propoxur on immune system. Attempts were made to select exposure level, which did not produce overt toxicity or mortality. It was considered appropriate to administered propoxur 10, 30 and 90 mg/kg bw orally to rats for the purpose of subchronic/chronic toxicity studies. Selection of propoxur exposure levels in the present experiment was based on our earlier determination of acute oral LD_{50} of propoxur (191 mg/kg bw) on six weeks male Wistar albino rats, and, on the results of neurotoxicity, behavioral toxicity and oxidative stress studies reported by different authors (WHO 1986; FAO and WHO 1989; Banerjee et al.1998, 1999; Seth et al. 2001; Banerjee et al. 2001). Determination of subchronic/chronic effects of propoxur on immune response is important to understand risk to humans due to wide spread use of this pesticide, its persistence in the environment and several instances of human poisoning (FAO and WHO 1989; Banerjee et al. 1998, 1999; Seth et al. 2001).

Rats exposed to propoxur at the test dose levels for 4 weeks exhibited no symptoms of overt toxicity, delayed neurotoxicity, or morality. Consumption of food and water was the same for treated and control rats. No significant difference was noted in body, liver, spleen and thymus weights between control and treated rats (data not shown). These results suggest that propoxur at these levels did not produce any physical stress responsible for the observed immunosuppressive effect in the present study. However, activity of AChE in RBC was decreased in propoxur treated rats (85.00 $\pm$ 8.00%, $p<0.05$ and 77.00 $\pm$ 10.00%, $p<0.05$ for 30 and 90 mg/kg bw respectively) as compared to control. No alteration in AChE activity (98.00 $\pm$ 5.00%) was observed in rats exposed to 10 mg/kg bw propoxur.

Serum antibody titer and splenic-PFC were studied for assessment of humoral immune response. Rats exposed to propoxur showed a significant decrease in antibody titer to ovalbumin in a dose dependent pattern (Table 1). IgM-PFC response to SRBC was also significantly decreased and found to be consistent with reducing antibody levels to ovalbumin, indicating the same threshold for suppressing immune response to both antigens in rats.

Table 1. Effect of propoxur on humoral immune response in albino rats.

Treatment (mg/kg bw)	Antibody titer[ab] ($-\log_2$)	PFC/10^6 spleen cells[ac]
Control	10.1 ± 0.5	2400 ± 200
Propoxur (10)	8.6 ± 0.5[d]	2160 ± 205[d]
Propoxur (30)	7.0 ± 0.5[d]	1800 ± 250[d]
Propoxur (90)	5.5 ± 1.0[d]	1255 ± 700[d]

a. Values are the mean ± SD of 10 rats per group; b. animals were immunized with ovalbumin; c. animals were immunized with SRBC; d. significantly lower than control; $p < 0.05$.

These results indicated important changes in humoral immunity may occur after propoxur exposure.

Effect of propoxur on cell mediated immunity was evaluated with the help of LMI and MMI tests and DTH reaction. Rats exposed to propoxur and subsequently immunized with ovalbumin showed a marked decrease in LMI and MMI responses and DTH reaction in a dose dependent pattern (Table 2). Our study demonstrates suppression of cell mediated immunity, this is itself may be responsible for the suppressed humoral response (mentioned above). However, further studies are required to assess changes in humoral response to thymus independent antigens (Banerjee 1987, 1999; Banerjee et al. 1996a, 1997).

Table 2. Effect of propoxur on cell mediated immune response in rats.

Treatment (mg/kg bw)	LMI[ab] (%)	MMI[ab] (%)	Specific foot pad thickness[ac] (%)
Control	69.6 ± 4.6	66.0 ± 4.0	21.2 ± 1.3
Propoxur (10)	50.5 ± 5.1[d]	45.5 ± 5.5[d]	18.0 ± 1.5[d]
Propoxur (30)	35.3 ± 5.8[d]	35.0 ± 2.0[d]	15.2 ± 1.5[d]
Propoxur (90)	22.0 ± 3.0[d]	21.0 ± 2.5[d]	11.5 ± 3.5[d]

a. values are the mean ± SD of 10 rats per group; b. animals were immunized with ovalbumin; c. animals were immunized with KLH; d. significantly lower than control; $p < 0.05$.

The results of the present study revealed a suppression of humoral and cell mediated immune responses in rats exposed to subchronic doses of propoxur. This suppression was found to increase in a dose dependent pattern. Suppression of humoral and cell mediated immune responses by carbamate pesticides other than propoxur has been reported by various workers (WHO 1986; Repetto and Baliga 1996). Adverse effect of propoxur on immune function could render exposed individuals more vulnerable to various pathogens.

Effect of propoxur on the immune system of rats has not been reported although their neurotoxic effects are well documented (FAO and WHO 1989; Lima et al. 1995; Lifshitz et al. 1997). Our interest in immunotoxic effect of propoxur stemmed from neurotoxicity and oxidative stress induced by this

pesticide, since a close dynamic relationship exist between nervous system, oxidative status and immune responses (Koner et al. 1998; Banerjee 1999; Felten and Felten 1994). Recently we have also observed altered activities of antioxidant enzymes, AChE and gamma glutamyl transpeptidase (GGT) and glutathione (GSH) level in lymphocytes or blood of human poisoning cases with propoxur (Banerjee et al. 1999; Seth et al. 2001). Furthermore, in the present study AChE activity in RBC was significantly inhibited in rats exposed to 30 or 90 mg/kg bw propoxur. A possible role of AChE, GGT and GSH in producing immunosuppression following carbamate or organophosphate exposure has been suggested elsewhere earlier (Banerjee et al. 1999; Koner et al. 1997; Rodgers et al. 1992). Hence, immunosuppression by propoxur may be a consequence of toxic chemical stress-induced cholinergic stimulation, and its effect on immune cells (lymphocytes) function.

Immunotoxicity of propoxur was even observed at a dose level 10 mg/kg bw, which has been earlier reported not to cause any toxicological effects (WHO 1986; FAO and WHO 1989). It is apparent that a more complete understanding of toxicity induced by repeated low doses of propoxur is required to establish guidelines for acceptable residues in the environment and "no observed adverse effect level". It is emphasized that the threshold level of the chemical below which no effect would be seen depends upon the method of testing for immune responses, animal species and endocrine and nutritional status of the host, age, stress and type of antigen against which the responses are studied (Banerjee et al. 1996; Banerjee 1999; Descotes 1998). Since there are numerous functions associated with immune system, it is necessary to study multiple parameters in order to comprehensively evaluate the effect of propoxur on immune response and in particular its role in modifying immunocompetence.

It is clear from this preliminary study that immune system may be a sensitive target for propoxur. The explanation for immunosuppressive effect of propoxur may lie at many levels. Like all other carbamate pesticides, propoxur may influence physiological and pathological conditions, hormonal functions, nutritional/oxidative status and hepatic metabolism of other endogenous and immunoregulating substances (Banerjee et al. 1997; Banerjee 1999; Descotes 1998). It may also act directly or indirectly on lymphoid cells, immunoglobulin metabolism, T/B cell-macrophage cooperation and macromolecular biosynthesis (Rodgers et al. 1992; Banerjee 1999).

It is now important to elucidate the phenomenon in order to understand the mechanism of immunosuppression and the possible health hazards due to continuing use of propoxur. Further investigation are in progress on primary and secondary immune cytokinetics, lymphocytes-mediated cytotoxicity, lymphoid cells distribution and relationship between anticholine esterase toxicity and immune responses. Studies concerning the effect of propoxur exposure on immunological memory in terms of secondary immune responses might be of particular importance. These studies may contribute

towards understanding of the mechanism of action of propoxur at the cellular level and could be utilized for a meaningful extrapolation in human poisoning(s).

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