Immunotoxicity of phosphamidon following subchronic exposure in albino rats

Sanvidhan G Suke, Rafat S Ahmed, A K Tripathi, Ayanabha Chakraborti & B D Banerjee*

Environmental Biochemistry Laboratory, Department of Biochemistry,
University College of Medical Sciences & G.T.B. Hospital (University of Delhi), Dilshad Garden, Delhi 110 095, India

Received 6 May 2005; revised 29 December 2005

Effect of subchronic doses of phosphamidon exposure on humoral and cell mediated immune (CMI) responses were studied in male albino rats using SRBC, ovalbumin and KLH as antigens. Humoral immune responses were assessed by estimating antibody titre against antigen and splenic plaque forming cells (PFC) assay. CMI responses were studied by using leucocyte migration inhibition (LMI), macrophage migration inhibition (MMI) and delayed type hypersensitivity (DTH) response. Results obtained in the present study revealed marked suppression of humoral and CMI responses in a dose dependent pattern. Hence, suppression of immune responses by phosphamidon even at subchronic doses is clearly an important aspect for its safety evaluation.

Keywords: Cell-mediated immunity, Humoral immunity, Organophosphate, Pesticide

Phosphamidon (2-chloro-2 diethyl carbamoyl-1 methylvinyl dimethyl phosphate) is a broad spectrum organophosphate insecticide widely used in agriculture. Like other organophosphates, the primary mechanism of action of phosphamidon is neurotoxic. They are designed to be effective inhibitors of acetylcholine esterase through interaction of nucleophilic active site serine of the enzyme to form a phosphorylated enzyme derivative. Acute intoxication leads to combination of muscarinic and nicotinic symptoms in different species, the severity of which varies depending on the dose, route and extent of exposure. Exposure of the general population to phosphamidon may occur through consumption of pesticide treated food, higher residues levels, by contact during applications, or by drift from aerial spraying. Several cases of suicidal or occupational poisoning have been reported. Hence phosphamidon 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 this pesticide. Despite this existing knowledge, it cannot be predicted with certainty how chronic exposure to phosphamidon affects human health. Possible effect of persistent exposure to phosphamidon on the functional integrity of immune system generated interest as an additional index to analyze long term health problems.

Although inadvertant alteration of immune responses by various organophosphate pesticides has been documented, at present little is known about the effect of phosphamidon on the immune system. Moreover, subchronic effect of phosphamidon on the immune system has not been clearly defined. Extensive and systematic studies on dose-time relationship in different experimental animals appear to be essential to establish the definitive immunomodulatory role of phosphamidon. Keeping this in view, the present study was designed to investigate the effect of subchronic doses of phosphamidon on humoral and cell mediated immune responses in albino rats.

Materials and Methods

Chemicals— Phosphamidon (technical grade, 94.1% purity) was obtained from Rallis, India. 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. Sheep red blood cells (SRBC) and guinea pig serum used as antigen and complement source respectively were
obtained in fresh from the central animal house of the institute. Keyhole limpet hemocyanin (KLH) was obtained from CAL Biochem; LaJolla, USA. All other reagents used were of analytical grade and obtained either from BDH or SISCO chemicals (Mumbai, India).

Animals and treatment— Wistar male albino rats (5 rats per cage) weighing 200-250 g were kept under standard laboratory conditions (12 hr light/dark cycle, 22° ± 2°C temperature and RH 70 ± 10%). Necessary approval was obtained from the institutional animal ethical committee for the study. Rats were randomly divided into four groups. Phosphamidon was dissolved in distilled water and animals were administered 0.174, 0.348, 0.696 and 1.74 mg/kg body weight phosphamidon/day for 28 days, using syringe and 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 was recorded weekly.

Immunization— Rats (10-12 animals/pesticide or antigen treatment group) were immunized with 2 x 10⁹ SRBC (ip, in 0.5 ml normal saline), or with ovalbumin (sc, 3 mg dissolved in 0.2 ml normal saline) and KLH (sc, 1 mg dissolved in PBS) mixed with equal volume of FCA. Sterile liquid paraffin (5ml) was injected (ip) in rats immunized with ovalbumin 48 hr before terminating exposure. 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), leucocyte 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).

Sampling— Blood samples were collected from chloroform anesthetized rats by cardiac puncture. Serum samples were kept separate at −20°C until analyzed. Heparin was used in collecting whole blood and leucocyte rich plasma was separated for (LMI) test as described earlier. Peritoneal macrophages were collected from rats and processed for the macrophage migration inhibition (MMI) test as described earlier. The liver, spleen and thymus were removed immediately, blotted and weighed.

Assay for acetylcholine esterase (AChE)— Acetylcholine esterase (AChE) activity in RBC was assayed according to the method described by Ellman et al. 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.

Humoral immune response

Humoral immune response was assayed by estimating the following parameters:

Antibody titre— Serum antibody titre to ovalbumin was estimated by enzyme linked immunosorbant assay (ELISA). In brief, flat bottomed polystyrene plates (Titertek) were coated with 12.5μg of ovalbumin dissolved in 100μl of 0.1M carbonate buffer (pH 9.6) at 4°C for 12 hr. Serial dilution of sera in PBS [0.15M, pH 7.2, containing 0.05% tween-20, (PBS-Tw)] were prepared and 100μl was incubated with coated wells for 1 hr at 37°C. After washing with PBS-Tw, 100μl 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 (400μg/ml) in sodium citrate buffer (0.1M, pH 4.9 containing 1.5µl of 30% H2O2/ml). The reaction was stopped with 8N H 2SO4 (50µl) after 30 min and absorbance was measured at 490 nm. The antibody titre was expressed as log2 of the reciprocal of the highest serial two fold dilution of the serum showing three times the absorbance of normal serum.

Splenic-plaque forming cells— 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—30μl of splenocytes in HBSS, 70μl of SRBC, and 500μl of guinea pig complement. In a Cunningham chamber, 200μl of sample was pipetted. The chambers were sealed with liquid paraffin, and incubated for 60min at 37°C. Then chambers containing samples were taken in duplicate. After incubation counts for PFC were made microscopically, and results were calculated as PFC/10⁶ spleen cells.

Cell mediated immune response

Leucocyte migration inhibition (LMI) test— For LMI test, the procedure followed was essentially similar to that described by us earlier. The final cell suspension was adjusted to contain 15 x 10⁶ cells/ml. Concentration of ovalbumin was adjusted to 50μg/ml for LMI test.
Macrophage migration inhibition (MMI) test—The peritoneal exudate cells from ovalbumin immunized rats were obtained and used in direct migration inhibition assay as described by us earlier\textsuperscript{12}. The concentration of ovalbumin in antigenic chamber was adjusted to 125 $\mu$g/ml.

Delayed type hypersensitivity—DTH reaction was challenged on 28\textsuperscript{th} day by injecting 17.5$\mu$g of KLH in 50$\mu$l PBS into the left (L) hind foot. Foot pad thickness was measured at 24 and 48 hr after challenge using a dial caliper (Mitutoyo, Japan). The right (R) hind foot pad was injected with 50$\mu$l of PBS that served as control\textsuperscript{16}.

Data analysis—Data was analyzed by ANOVA test using SPSS version 5 statistical program and effect of each treatment was obtained by using Tukey’s multiple comparison procedure at $P < 0.05$ \textsuperscript{17}.

Results
Exposure of rats to phosphamidon orally at the test dose level for four weeks produced no overt toxicity signs and symptoms. No significant difference was noted in mortality rate, body growth rate and food intake between control and treated rats (data not shown). However, activity of AChE in RBC was decreased in phosphamidon treated rats (90 ± 5\%, $P < 0.05$; 84.00 ± 8\%, $P < 0.05$ and 75.00 ± 10.00 \%, $P < 0.005$ for 0.348, 0.696 and 1.74 mg/kg body wt, respectively) as compared to control. No alteration in AChE activity (98.00 ± 5.0\%) was observed in rats exposed to (0.174mg/kg body wt phosphamidon).

Serum antibody titre to ovalbumin and IgM PFC response to SRBC were decreased significantly in the experimental animals exposed to 0.348, 0.696 and 1.74 mg/kg body wt of the pesticide. No significant effect on antibody titre or splenic PFC response was observed in rats exposed to 0.174mg/kg body wt phosphamidon (Table 1).

Table 2 summarizes the effect of phosphamidon on cell mediated immune response. Rats exposed to 0.348, 0.696 and 1.74 mg/kg body wt phosphamidon for 28 days showed significant decrease in LMI and MMI response in a dose-dependent manner. Similar dose response trend was also observed in case of DTH reaction. However, phosphamidon did not suppress cell mediated immune response at 0.174 mg/kg body wt of phosphamidon.

Discussion
Immunosuppression by pesticides and other chemicals of environmental importance is a concern in chronic toxicity assessment\textsuperscript{18}. There is paucity of information regarding dose dependent effect of phosphamidon on immune system. Epidemiological studies suggest that exposure to organophosphate pesticides can induce effects on central and peripheral nervous system, either after acute intoxication or as a result of low level long-term exposure\textsuperscript{10,19,20}. Central nervous system is an important regulator of immune function\textsuperscript{21} and hence it is important to study the immunomodulatory effect of this neurotoxic compound in experimental animals.

Attempts were made to select exposure levels, which did not produce overt toxicity or mortality. Selection of phosphamidon exposure levels in the present experiment was based on acute oral LD\textsubscript{50} of phosphamidon (17.4 mg/kg body wt) in rats and on general toxicity studies reported by different authors\textsuperscript{6}. Rats exposed to phosphamidon at test dose level for 28 days exhibited no symptoms of overt toxicity, delayed neurotoxicity or mortality. Phosphamidon at these doses is not reported to be neurotoxic in rats\textsuperscript{22}. No significant difference was noted in body wt, liver, spleen and thymus weight between control and treated rats (data not shown). These results suggested that

---

Table 1—Effect of phosphamidon on humoral immune response in albino rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Antibody titer $\ast$ (mg/kg body wt) (-log)</th>
<th>PFC/10$^6$ Spleen cells $\ast$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.20 ± 0.50</td>
<td>2500 ± 250</td>
</tr>
<tr>
<td>Phosphamidon (0.174)</td>
<td>10.50 ± 0.60</td>
<td>2600 ± 220</td>
</tr>
<tr>
<td>Phosphamidon (0.348)</td>
<td>8.50 ± 0.50$^a$</td>
<td>2020 ± 240$^a$</td>
</tr>
<tr>
<td>Phosphamidon (0.696)</td>
<td>7.50 ± 0.60$^a$</td>
<td>1750 ± 500$^a$</td>
</tr>
<tr>
<td>Phosphamidon (1.74)</td>
<td>5.60 ± 1.20$^a$</td>
<td>1100 ± 700$^a$</td>
</tr>
</tbody>
</table>

$\ast$Animals were immunized with ovalbumin; **Animals were immunized with SRBC; $^a$Significantly lower than control at $P<0.05$.

Table 2—Effect of phosphamidon on cell mediated immune response in albino rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LMI$^\ast$ (mg/kg body wt) (%)</th>
<th>MMI$^\ast$ (%)</th>
<th>Specific foot pad thickness $\ast$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>70.50 ± 5.00</td>
<td>65.00 ± 5.00</td>
<td>22.00 ± 1.5</td>
</tr>
<tr>
<td>Phosphamidon</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0.174)</td>
<td>70.50 ± 6.00</td>
<td>66.00 ± 5.50</td>
<td>21.50 ± 1.2</td>
</tr>
<tr>
<td>(0.348)</td>
<td>55.00 ± 8.50$^a$</td>
<td>50.00 ± 6.50a</td>
<td>18.00 ± 1.5$^a$</td>
</tr>
<tr>
<td>(0.69)</td>
<td>40.50 ± 10.6$^a$</td>
<td>40.00 ± 12.0$^a$</td>
<td>15.00 ± 2.5$^a$</td>
</tr>
<tr>
<td>(1.74)</td>
<td>25.00 ± 15.2$^a$</td>
<td>20.00 ± 10.0$^a$</td>
<td>10.00 ± 3.6$^a$</td>
</tr>
</tbody>
</table>

$^a$Animals were immunized with ovalbumin; $^a$Significantly lower than control at $P<0.05$. 

---
phosphamidon at these levels did not produce any physical stress responsible for observed immunosuppressive effect in the present study. However, activity of ACHE decreased in phosphamidon treated rats in a dose dependent manner as compared to control, except in rats exposed to 0.174 mg/kg body wt phosphamidon.

IgM PFC response to SRBC decreased significantly and found consistent with reducing antibody levels to ovalbumin, indicating the same threshold for suppressing immune response to both antigens in rats. Animals exposed to phosphamidon and subsequently immunized with ovalbumin showed marked decrease in the ability of antigen sensitized cells to release migration inhibition factors which prevent leucocyte and macrophage inhibition in vitro in a dose dependent pattern, suggesting impairment of effector mechanisms during immune response. In the present study, increase in footpad thickness in response to antigenic challenge in immunized rats decreased after phosphamidon exposure. Although it appears that the depression of cell mediated immunity extends to the primary humoral response, more light may be thrown in this direction by studying the response to thymus independent antigen. Taken together, the present study revealed suppression of humoral and cell mediated immune responses in the experimental animals exposed to subchonic doses of phosphamidon. Adverse effects on immune function could place the host in a more vulnerable position towards various pathogens. Immunotoxicity of phosphamidon was observed in this study at dose levels which have been reported earlier not to cause any other toxicological effects. It is apparent that complete understanding of toxicity of phosphamidon is necessary to study human health hazards and to establish guidelines for setting no observable adverse effect levels (NOAEL). It is emphasized that threshold level of the chemical below which no effect would be seen depends on the method of testing for immune responses, animal species, endocrine and nutritional status of the host and type of antigen against which the responses are studied.

It is clear from the present study that the immune system may be a sensitive target for phosphamidon. It is important to keep in mind that there are few compounds for which the exact molecular target or mechanism of immunotoxicity are known and indeed for many compounds there are probably multiple targets and mechanisms of action. The explanation for immunosuppressive effect of phosphamidon, therefore, may be at many levels. Our interest in immunotoxic effect of phosphamidon stemmed from neurotoxicity and oxidative stress induced by this pesticide, since a dose dynamic relationship exists between nervous system, oxidative stress and immune response. Furthermore, ACHE activity in RBC was significantly inhibited in rats exposed to 0.348 to 1.74 mg/kg body wt phosphamidon indicating a possible role of ACHE, gamma glutamyl transpeptidase and glutathione in producing immunosuppression following organophosphate exposure. Hence, immunosuppression by phosphamidon may be a consequence of toxic chemical stress induced cholinergic stimulation and its effects on immune cells (lymphocytes) function. Phosphamidon may also influence physiological and pathological conditions, hormonal functions, nutritional/oxidative stress and hepatic metabolism of other endogenous and immunoregulating substances. It may also act directly or indirectly on lymphoid cells, immunoglobulin metabolism T/B cell-macrophage cooperation and macromolecular biosynthesis.

In conclusion, our studies showed for the first time a direct evidence of immunosuppression by subchronic doses of phosphamidon and this suppression was found to increase in a dose dependent pattern. The exact mechanism by which phosphamidon causes immunosuppression is not yet clear, but close interaction between neurological and immunological function provides many potential targets for toxicity. A better understanding of these interactions and more clearly defined endpoints in different species remains a priority for future.

Acknowledgement
One of the authors (SGS) is grateful to CSIR, New Delhi for financial support.

References
5. Tarbah F A, Kardel B, Pzers S, Temme O & Daldrup T, Acute poisoning with phosphamidon: determination of


