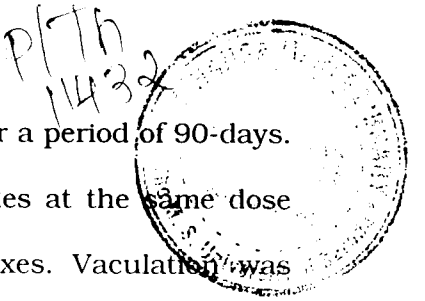
CHAPTER-6

EFFECT OF REPEATED SUBCHRONIC DIETARY EXPOSURE OF CHLORPYRIFOS AND LEAD COMBINATION AT CELLULAR LEVEL

Introduction

The evaluation of neurotoxicity of chemicals will be based primarily on behavioral and morphological observations and particularly on correlation between them. Many times correlation does not exist between behavioral findings and neuropathology. There are many factors that influence negative correlation between behavioral findings and neuropathology. Dose of the test substance, age of animals, species, duration of the test substance administration, timing of behavioral evaluations, sampling methods in pathology, reserve function capacity of nervous system and tolerance/or adaptation of the central nervous system are all probable factors that influence the negative correlation between behavioral findings and neuropathology (Broxup *et al.*, 1989).

Acute and subchronic or chronic studies of chlorpyrifos performed using rodent models by various laboratories reveal absence of marked histopathological changes in the nervous system (Wagner, 1999 and EPA 2000). There are reports about histopathological changes in adrenal gland (vaculation) after subchronic and chronic exposure and few reports about increased incidences of keratitis and hepatocellular fatty vaculation after chronic (2 years study) exposure (U.S. EPA, 2000). Yano *et al.* (2000) observed mild to moderate degree of vaculation of the parenchymal cells in the zona fasciculata of the adrenal cortex in male rats after exposure to



chlorpyrifos at dose levels of 5.0 and 15 mg/kg/day for a period of 90-days. However, similar changes were not observed in females at the same dose levels and at lower dose levels in either of the sexes. Vaculation was consisitent with accumulation of lipid. Other than in adrenal, they did not find any treatment related changes in other systemic organs (aorta, bone marrow, brain, caecum, cervix, coagulating glands, epididymides, esophagus, eyes, heart, kidneys, large intestine, larynx, liver, lungs, mammary gland, mediastinal tisssue, mesenteric lymphnode, mesenteric tissue, nasal tissues, ovaries, oviducts, pancreas, parathyroid glands, pituitary, gland, prostate, salivary glands, sciatic nerve, seminal vesicles, skeletal muscle, skin, small intestine, spinal cord, spleen, stomach, testes, thymus, thyroid gland, tibial nerve, tongue, trachea, urinary bladder, uterus, and vagina) after 13 weeks exposure to chlorpyrifos up to 15 mg/kg/day dose levels. Mattson *et al.* (1996) have reported that a adrenal cortical esterase, cholesterol esterase, is inhibited in vitro by micromolar concentrations of CPF-oxon. CPF and other OP were shown to inhibit in-vitro steriodogenesis stimulated by cyclic adenosine monophospahte or corticotrophin. Rosol *et al.* (2001) also reported impaired steriodogenesis in the adrenal cortex due to OP intoxication.

Nephropathy is a characteristic manifestation of lead toxicity. Two types of nephropathy, acute and chronic nephropathy, have been observed in humans. Acute nephropathy occurs during the early stages of excess exposure, especially in children. The characteristics of early or acute lead-induced nephropathy in humans include nuclear inclusion bodies, mitochondrial changes, and cytomegaly of the proximal tubular epithelial cells; dysfunction of the proximal tubules (Fanconi's syndrome) manifested as aminoaciduria, glucosuria, and phosphaturia with hypophosphatemia;

and increased sodium and decreased uric acid excretion. These effects appear to be reversible. Glomerular effects are either minimal or absent. The acute form is reported in lead-intoxicated children, whose primary exposure is via the oral route, and sometimes in lead workers (Davidson, 1996 and ASTDR, 1999).

Chronic nephropathy occurs after exposure to high concentrations of lead. In occupational exposure studies, death due to kidney disease has occurred at blood levels of lead exceeding 62 µg/dL. Characteristics of chronic lead induced nephropathy include progressive interstitial fibrosis, dilation of tubules and atrophy or hyperplasia of the tubular epithelial cells, and few or no nuclear inclusion bodies, reduction in glomerular filtration rate, and azotemia. These effects are irreversible. The chronic form is reported mainly in lead workers, whose primary exposure is via inhalation (Davidson, 1996 and ASTDR, 1999). The protein or ligand bound lead which is filterable at the glomerulus is available for uptake into renal cells. Mitochondria are extremely sensitive to lead and, following chronic in-vivo administration, mitochondrial swelling, which is associated with decreased respiratory control, has been reported (Lock, 1995).

ATSDR (1999) reported that animal studies provide evidence of nephropathy similar to that which occurs in humans as when rats exposed to lead acetate in the drinking water through the dams during gestation and lactation and then until 9 months of age. Animals with higher blood lead concentration (26 µg/dL to 67 µg/dL) showed cytomegaly, intranuclear inclusion bodies, swollen mitochondria, and hemosiderin in the proximal tubule cells with no lesions in the glomeruli.

In another study, lead acetate was administered through drinking water for 2–3 months to evaluate renal function in male Wistar rats. Administration of approximately at 414 mg lead/kg/day (Blood lead, 105 μg/dL) did not cause any pathological changes. An increase in the urinary excretion of β2 micro-globulin was seen in animals that received 828 mg lead/kg/day (Blood lead, 196 μg/dL). Animals treated with 1,660 mg lead/kg/day (Blood lead, 320 μg/dL) exhibited increased urinary excretion of β2 micro-globulin, glucose, total proteins, lysozyme, and lactate dehydrogenase (LDH). Morphological changes observed primarily in the epithelial cells of the proximal tubules at 828 and 1,660 mg/kg/day groups were characterized by intranuclear inclusion bodies and enlarged nuclei. Hyperplasia and flattening of the proximal tubular epithelium were also observed. Similar findings were made in female rats as well (ATSDR, 1999).

Lead is a well-established immunotoxicant. Exposure of rats to lead *in utero* and continued for 7 weeks post-partum at low levels (25 and 50 ppm) produced altered humoral responses. The effect did not appear to result from altered B-lymphocyte function but rather from altered T-lymphocyte and/or macrophage activity. With respect to T-lymphocyte function, depression in T-lymphocyte mitogenic responses in similarly exposed young Sprague-Dawley strain rats was reported. Lead targets both T lymphocytes and macrophages particularly after early exposure. In a dose-response comparison among pregnant rats and rat pups exposed during gestation and assessed as young adults, developing fetuses were found to be susceptible to persistent immunotoxic effects of lead at doses that did not affect the pregnant dams. The nature of the immunotoxicity was similar to that reported for exposed adults; rats exposed *in utero* exhibited depressed Th1 function with a concomitant elevation in some Th2-dependent parameters. The primary

effect on T cells is a shift in the T helper (Th) cell functional balance, with Th1 function depressed and Th2 function elevated (Dietert *et al.*, 2001).

Prenatal and postnatal exposure of rats to 2.24 mg lead/kg/day as lead acetate in the drinking water (indirectly through the dams and then directly) until testing at 35–45 days of age, resulted in a mean blood lead level of 29.3 µg/dL and marked depression of antibody responses to sheep red blood cells, decreased serum IgG (but not IgA or IgM) levels, decreased lymphocyte responsiveness to mitogen stimulation, impaired delayed hypersensitivity reactions, and decreased thymus weights as compared to controls (ATSDR, 1999).

ATSDR (1999) has reported many studies on action of lead on nervous system. High levels of exposure to lead produce encephalopathy in several species, but blood lead data for this effect are generally not available. A number of histopathological studies of lead's effects on the nervous system of rats treated during early postnatal life with lead acetate or carbonate in the drinking water or diet through their dams or directly, for first 3 weeks of life resulted in variety of adverse effects at lead blood levels ranging from 258 to 400 µg/dL. These effects include reduction or delay in the development of the hippocampus or other hippocampal changes, reduction or delay in the development of the cerebral cortex, reductions in the number and size of axons in the optic nerve and demyelination of peripheral nerves. Cytoarchitectural changes have also been noted in limited studies of the eyes of monkeys chronically exposed to lead beginning at or shortly after birth (ATSDR, 1999).

It was reported that prenatal exposure to lead, that continued for 20 weeks after birth resulted in altered normal developmental pattern of protein in

neurons from the central nervous system. The effects were not observed in rats exposed only as adults; however, brain lead levels were similar in the two groups. These results suggest that the rapidly developing rat brain may be more susceptible to the neurotoxic effects of lead than the brains of older rats (ATSDR, 1999).

The effect of lead on the distribution of calcium binding proteins in the hippocampus from monkeys was studied by a group of researchers. Monkeys were treated for 9 years (exposure beginning in utero) with lead in the diet at a concentration that provided approximately 4 or 7 mg lead/kg/day and resulted in lead blood levels of about 38 and 55 µg/dL, respectively, at 9 years of age. After a 32-month period of lead-free diet, PbB levels, while still higher than in unexposed monkeys, had decreased considerably. Immunohistochemical examination of the hippocampus revealed no significant differences in the pattern of distribution of three high-affinity calcium binding neuronal proteins, but there was a marked decrease in the immunoreactivity of the glial protein S100 in the high-dose group. The results were interpreted as confirming the hypothesis that glial cells are the main target of lead toxicity in the central nervous system (ATSDR, 1999).

MATERIALS AND METHODS

To study interaction between chlorpyrifos and lead at cellular level, at the end of treatment period, animals were sacrificed and the following organs were collected and preserved in 10% neutral formalin. Brain, pituitary, sciatic nerve, spinal cord (cervical, thoracic and lumbar), eye, muscle, liver, kidney, spleen, adrenal, thyroid and parathyroid and thymus. Histopathological examination was carried out for five males and five females each belonging control and, high dose groups of chlorpyrifos (group 5), lead (group 6) and chlorpyrifos plus lead (group 7). The dose levels, number of groups used, duration of treatment and experimental procedure are mentioned in the materials and methods of Chapter 5. Methodology used for tissue processing and slide preparation was as same as that described for histopathology of single dose study (Chapter 3).

RESULTS

The histopthological examination of organs such as brain, pituitary, sciatic nerve, spinal cord, (cervical, thoracic and lumbar), eye, muscle, liver, kidney, spleen, adrenal, thyroid, parathyroid and thymus were carried out assess the changes at cellular level and to decipher the correlation between neurobehavioral and biochemical findings with morphological changes at the cellular level.

The lesions observed in different organs of either sex of animals belonging to different groups were: liver (periportal MNC infiltration, hepatocellular vaculation/fatty change/altered cell focus, extramedullary hematopiosis, congestion and bile duct hyperplasia), kidney (cyst, calcification foci in renal pelvis, congestion, MNC infiltration/focal interstitial nephritis) spleen (lymphoid depletion/atrophy, extramedullary hematopiosis), brain (ependymal cell hyperplasia – focus), pituitary (Rache's cleft with secretion, cyst), adrenal (accessory cortical nodule/ accessory adrenal tissue, cortical tissue hypertrophy/ vaculation of cortex, congestion) thyroid (ultimobronchial cyst), thymus (cyst, starry sky appearance, lymphoid depletion/atrophy, epithelial cell hyperplasia) bone marrow (atrophy/hypocellularity).

The number of animals that exhibited particular lesions group wise is represented in the summary Table 1. Except in thymus, lesions observed in other organs are spontaneous/incidental findings and there was no specific pattern/correlation seen with regard to treatment.

Varying degree of starry sky appearance and lymphoid depletion were noticed more commonly in thymus of groups 6 and 7 animals. Two males (40%) of group 6 and three males (60%) belonging to group 7 exhibited lymphoid depletion/atrophy. In females, all five (100%) females belonging to group 6 showed lymphoid depletion/atrophy, and three (60%) animals showed starry sky appearance. In group 7, lymphoid depletion/atrophy were observed in four females (80%), starry sky appearance exhibited by two (40%) females (Table 1; Figure 1; Plates II and III). In some animals these degenerative changes were associated with epithelial hyperplasia (G6M - 20%, G7M - 20%, G6F - 60% and G7F - 40%).

Table 1

Dose: G1- 0; G5 (CPF) - 10; G6 (LA) - 500; G7 - (CPF+LA) - 10+500 ppm in diet

Organ/Lesion	Group 1		Group 5		Group 6		Group 7	
	M N=5	F N=5	M N=5	F N=5	M N=5	F N=5	M N=5	F N=5
Liver								
1) Periportal MNC infiltration	1	1	0	1	0	1	0	1
2) Hepatocellular vaculation /fatty change/altered cell focus	0	0	1	0	1	0	1	1
3) Extramedullary hematopiosis	1	0	0	0	0	0	0	0
4) Congestion	0	0	0	0	0	0	1	0
5) Bile duct hyperplasia	0	0	0	0	1	0	0	0
Kidney								
1) MNC infiltration/ focal interstitial nephritis	0	0	1	1	0	1	1	1
2) Calcification foci in renal pelvis	0	1	0	0	0	0	0	0
3) Cyst	0	0	1	0	0	0	0	0
4) Congestion	1	0	0	0	1	0	1	0
Spleen								
1) Lymphoid depletion/atrophy	0	0	1	1	1	1	0	1
2) Extramedullary hematopiosis	0	0	0	0	1	0	0	0
Brain								
1) Ependymal cell hyperplasia - focus	0	1	0	0	0	0	0	0
Pituitary								
1) Rache's cleft with secretion	1	1	1	1	0	1	0	1
2) Cyst	0	0	1	0	0	0	0	0
Adrenal								
1) Accessory cortical nodule/ accessory adrenal tissue	1	1	1	1	0	0	1	1
2) cortical tissue hypertrophy/ vaculation of cortex	0	1	0	0	0	0	0	0
3) Congestion	0	1	0	0	0	0	0	0

N = Number of animals examined histopathologically

Table 1 (continued)

Dose: G1- 0; G5 (CPF) - 10; G6 (LA) - 500; G7 - (CPF+LA) - 10+500 ppm in diet

Organ/Lesion	Group 1		Group 5		Group 6		Group 7	
	M N =5	F N = 5	M N = 5	F N = 5	M N = 5	F N = 5	M N = 5	F N = 5
Thyroid								
1) Ultimobronchial cyst	1	0	0	0	0	0	0	1
Bone marrow								
Atrophy/hypocellularity	0	0	1	0	0	0	0	0
Thymus								
1) Starry sky appearance	0	0	0	1	0	3	0	2
2) Lymphoid depletion/atrophy	0	0	0	0	2	5	3	4
3) Epithelial cell hyperplasia	0	0	0	0	1	3	1	2
4) Cyst	0	1	0	0	0	0	1	0

N = Number of animals examined histopathologically

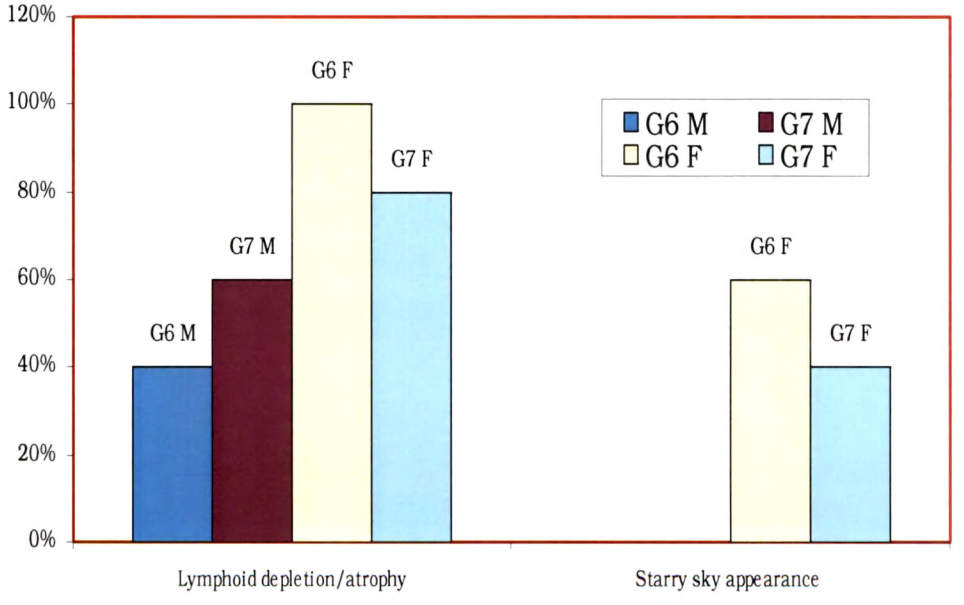


Figure 1. Degenerative Changes of Thymus

Plate II

Figure A: Thymus from animal belonging to control group showing normal structure of cortex and medulla with normal population of cells **(H & E 10X)**.

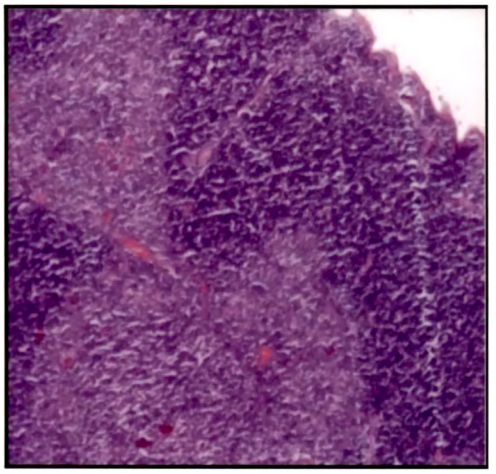
Figure B: High magnification of thymus from animal belonging to control group showing normal lymphoid population in cortex **(H & E 40X)**.

Figure C: Thymus from animal belonging to lead treated group (500 ppm) showing lymphoid depletion **(H & E 10X)**.

Figure D: Thymus from animal belonging to lead treated group (500 ppm) showing lymphoid depletion with epitheloid cell infiltration (arrow) **(H & E 40X)**.

PLATE II

A



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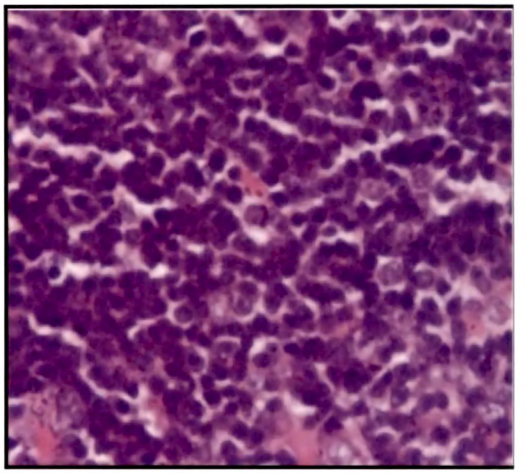

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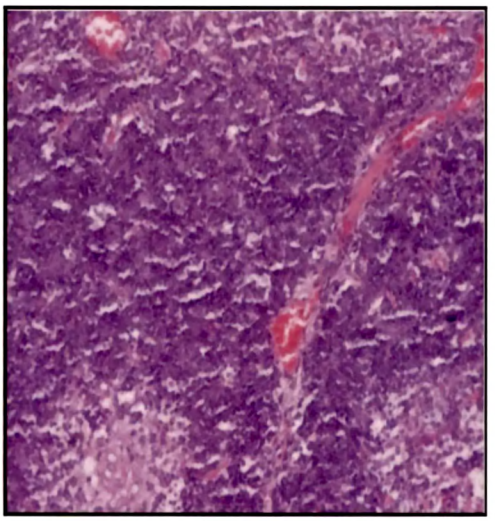


D

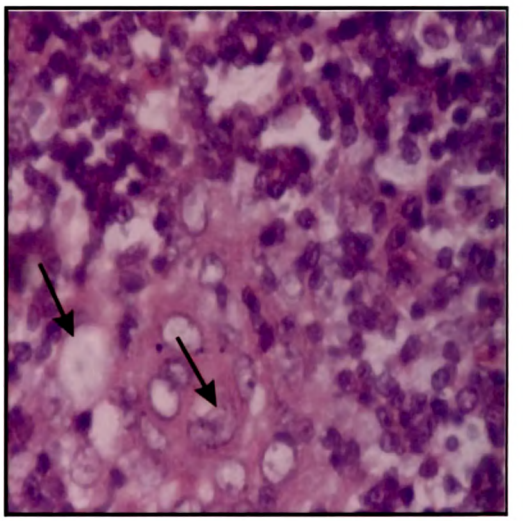


Plate III

Figure A.

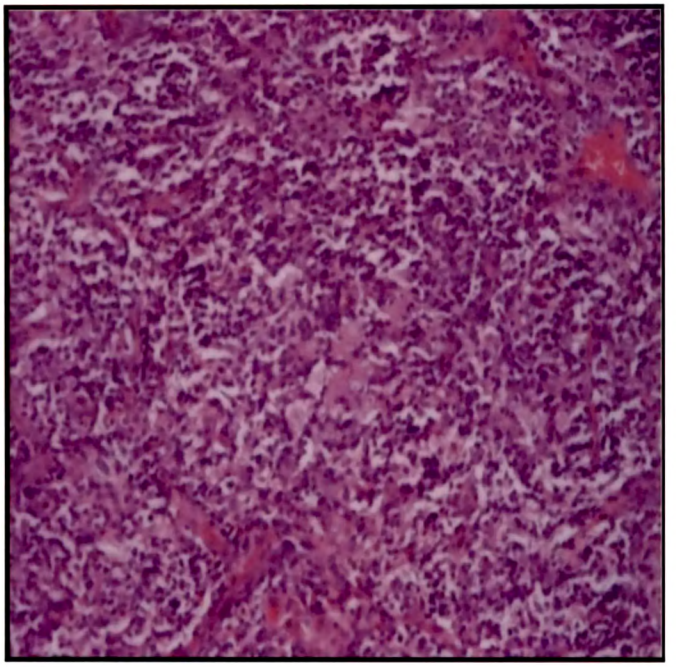
Atrophied thymus of lead and chlorpyrifos treated animal (Chlorpyrifos – 10 ppm + Lead - 500 ppm) showing severe lymphoid depletion leading to loss of demarcation between cortex and medulla **(H & E 10X)**.

Figure B.

High magnification of thymus of lead and chlorpyrifos treated animal (Chlorpyrifos – 10 ppm + Lead - 500 ppm) showing lymphoid depletion in medulla with epitheloid cell infiltration (arrow) **(H & E 40X)**.

PLATE III

A



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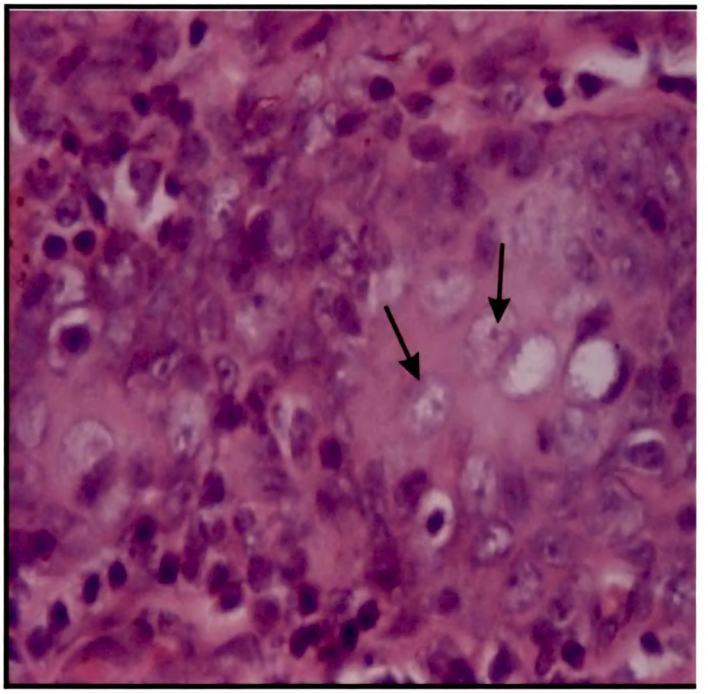

DISCUSSION

To establish relationship between neurobehavioral and biochemical findings with histopathological findings, the microscopic examination of neuronal organs [brain, pituitary, sciatic nerve, spinal cord, (cervical, thoracic and lumbar), eye] and systemic organs (liver, kidney, spleen, adrenal, thyroid and parathyroid thymus and muscle) was carried out after sub-chronic dietary exposure to chlorpyrifos and lead acetate for a period of 90 days in both sexes of Wistar rats (Table 1). The spontaneous and/or incidental lesions observed in the listed organs were seen only in a few animals and did not reveal any specific pattern related to treatment.

The degenerative lesions (starry sky appearance and lymphoid depletion/atrophy - Table 1; Figure 1; Plates II and III) observed in thymus of group 6 and group 7 animals are similar to the findings with single dose study (Chapter 3). The severity and incidences of lesions were higher in females of groups 6 and 7 as compared to their respective male counterparts. In group 6, all five (100%) females examined showed lymphoid depletion/atrophy and in group 7, 80% of the examined females showed same lesion (Table 1). No interactive synergism or potentiation effects were noticed. In some animals, lymphoid depletion in medulla/cortex was associated with varying degree of epithelial hyperplasia. Atrophy/lymphoid depletion associated with epithelial hyperplasia might be characteristic of atrophic thymus.

The effects of lead exposure on local and systemic immune functions were demonstrated after subacute (14 or 28 days) exposure to lead nitrate through inhalation route in mice. Local and systemic immune function

parameters were studied in the immunized, lead-exposed mice. Significantly higher concentration of lead in the liver, spleen, thymus, lung, and kidney were observed in the lead exposed animals as compared to the control group in both the 14-day and 28-day exposure groups. Absolute weight of spleen and thymus was significantly decreased in lead-exposed animals as compared to the controls. Different cells of the immune system such as leukocytes, circulating antibodies, and antibody forming cells were decreased in different lead exposed groups, thus suggesting immunosuppressive role of lead. With respect to systemic immunosuppression, only the thoracic lymph node response was suppressed after intravenous immunization, suggesting lack of systemic immunosuppression after exposure through inhalation. The immunosuppressive effects were more pronounced in mice exposed for 28 days as compared to those exposed for a 14 days due to higher concentration of lead in different tissues, and thus emphasizing a fundamental aspect of toxicology – significance of dose (ATSDR, 1999).

Dietert *et al.* (2001) reported that immunosuppression results from altered T-lymphocyte activity rather than altered B-lymphocyte function. Rats exposed to lead during pregnancy and postpartum at low levels (25 and 50 ppm, for 7 weeks) showed altered humoral responses; however, they suggested that the effect did not seem to result from altered B-lymphocyte function but rather from altered T-lymphocyte and/or macrophage activity. Lead targets both T lymphocytes and macrophages after early exposure. The primary effect on T cells is a shift in the T helper (Th) cell functional balance, with Th1 function depressed and Th2 function elevated. The nature of the immunotoxicity was similar to that reported for exposed adults; rats exposed *in utero* exhibited depressed Th1 function with a concomitant elevation in

some Th2-dependent parameters. The degenerative changes observed in the thymus of lead treated animals (group 6 and group 7) in the present study could also be related with the purported changes in T-lymphocytes as there were no changes observed in total and differential leucocyte counts.

SUMMARY

Chlorpyrifos, a well known organophosphorus insecticide elicits toxic action mainly by inhibiting cholinesterase enzymes. Heavy metal, Pb a well known neurotoxicant, and its systemic toxicity are also well established. Groups of Wistar rats were exposed to chlorpyrifos, lead and chlorpyrifos plus lead at two different lower dose levels via diet for 90 consecutive days. At the end of 90 days of exposure period, animals were sacrificed and organs of nervous system (brain, pituitary, spinal cord and nerve) and some systemic organs (liver, kidney, eyes, adrenals, thyroid and parathyroid, thymus, spleen, muscle, bone marrow, femur and sternum were) processed for microscopic examination. Except for thymus, no other organs showed treatment related changes. Animals treated with lead acetate alone at 500 ppm or a combination of lead acetate (500 ppm) and chlorpyrifos (10 ppm) showed degenerative changes (lymphoid depletion/atrophy/starry sky appearance) related to treatment. No interactive effects due to chlorpyrifos and lead acetate were evident.