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Effects of Endosulfan on Humoral and Cell-Mediated Immune Responses in Rats

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A great concern over genotoxic potential of environmental chemicals has been expressed in recent years. Endosulfan (6,7,8,9,10,10a-hexachloro-1,5,5a,6,9,9a-hexahydro, 6,9-methano-2,4,3-benzodioxathiepin-3-oxide), a polycyclic chlorinated hydrocarbon of cyclodien group, is a well known insecticide. It is widely used in agriculture and, in some countries, in public health. WHO (1984) classified endosulfan in the category of technical products that are moderately hazardous. Food is the main source of exposure of the general population to endosulfan (FAO/WHO 1975a). Several cases of suicidal and occupational poisoning have been reported (Demeter et al. 1977; Israeli et al. 1969; Tiberin et al. 1970; Kazen et al. 1974; FAO/WHO 1975b).

The physical, chemical as well as toxicological effects of endosulfan in experimental animals have been reported by various workers (WHO, 1984). However, the reports regarding the effect of endosulfan on immune system are not available. In view of its widespread use there is an urgent need to investigate the immunotoxicological effect of endosulfan in mammals for the safety evaluation of this insecticide (Vos 1977; Faith et al. 1980; WHO 1984). This has, therefore, prompted us to investigate the effect of endosulfan on immune system employing albino rats as the experimental animal. Included in this report are our preliminary findings on humoral and cell-mediated immune responses in rats exposed to sub-chronic doses of endosulfan.

MATERIALS AND METHODS

Technical grade endosulfan (purity 98%, consisting of alpha and beta isomers in the ratio of 70:30) was received through the courtesy of M/s. Hindustan Insecticide Ltd., India. Tetanus toxoid (Tetanus vaccine, Bio Vaccine Pvt. Ltd., Hyderabad), Freund's complete adjuvant Difco Laboratories, Detroit, MI) and tissue culture media RPMI-1640 (Centron Research Laboratories, Bombay) were used.

National Institute of Communicable Diseases colony bred Wistar male albino rats (Hissar strain) weighing 85-90 grams were housed four in a cage and individually labelled. The rats were randomly divided into four groups of 16 animals in each and fed a standard laboratory

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diet containing 0 (control), 10,30 or 50 ppm of endosulfan and water **ad libitum** for six weeks. To produce feeds containing 10, 30 or 50 ppm endosulfan, groundnut oil premixes were prepared by dissolving known concentration (10, 30 or 50 mg) of endosulfan in groundnut oil (30 ml). The premixes were incorporated into 1 kg. of diet and mixed manually for at least 30 minutes to ensure even distribution. The analysis of ten random samples of each diet batch for endosulfan residues by gas chromatography (Packard Gas Chromatograph equipped with electron capture detector) demonstrated an even distribution of endosulfan in various batches of test food, indicating uniform exposure of all animals to the toxicant. Endosulfan residues in diet varied by less than 10% from the expected levels. The control received an equal volume of vehicle in an identical manner. Food consumption, general condition and any other clinical symptoms were looked for daily. Body weights were recorded weekly.

Animals were immunized subcutaneously with tetanus toxoid (0.2 ml) mixed with equal volume of Freund's complete adjuvant after 25 days of pesticide exposure. Sterile liquid parafin (5 ml) was injected intraperitoneally in these immunized rats 48 hrs before terminating the exposure.

Blood samples were collected after six weeks of exposure from chloroform anesthetized rats by cardiac puncture without opening the abdomen. The serum was separated from the individual samples and kept at -20°C until analysed. Heparin was used in collecting whole blood for leucocyte migration inhibition (LMI) test. Peritoneal macrophages were collected by washing the peritoneal cavity with the media RPMI-1640 under aseptic condition for macrophage migration inhibition (MMI) test. The liver, spleen and thymus were removed immediately, blotted and weighed.

The serum antibody titer to tetanus toxoid was estimated by indirect haemagglutination technique according to Herbert (1979) using microtiter plates (Lexbro). A dilution of 1:3.5 of tetanus vaccine was used for the antigen coating. The antibody titers are expressed as $\log_2$ of the reciprocal of the first dilution where no visible agglutination was observed.

Zone electrophoresis of serum was carried out on an agarose (0.9% in Vernol buffer pH 8.6, 0.05 M) slide for 4-5 hrs in an anodic current (3 mA/slide) at 4°C. Slides were fixed in picric acid-glacial acetic acid reagent, washed, dried, stained with commasie brilliant blue for serum proteins and estimated by densitometer (Biochem Model M 77).

Quantitation of serum IgM and IgG was carried out by single radial immunodiffusion method (Mancini et al. 1965). Rat serum IgG and IgM were purified and antisera to these globulins were prepared according to Janah et al. (1970).

LMI and MMI were assayed as described by Bloom (1971). The final cell suspension was adjusted to contain 15 x 10^6 cells per ml. Cell viability (by trypen blue exclusion) was usually 99%. Concentrations of tetanus toxoid were adjusted to 25 µl/ml and 50 µl/ml for LMI

and MMI tests respectively. The area of migration was measured by traced camera lucida images on graph paper and the percentage migration inhibition was calculated according to the following formula:

$$\text{Percentage migration inhibition} = 100 - \frac{\text{area of migration in antigenic chamber}}{\text{area of migration in control chamber}} \times 100$$

The results are expressed as mean and their standard deviation (SD). Comparisons were made with control group using students t-test. A "p" value of 0.05 or less was considered to be significantly different from control.

RESULTS AND DISCUSSION

Effect of endosulfan on immune system has not been reported although their neurotoxic effects are well demonstrated in animals and humans (WHO 1984; Zaidi et al. 1985). Our interest in immunotoxic effect of endosulfan stemmed from neurotoxicity of this pesticide since a close dynamic relationship exists between nervous and immune system (Link 1979). Attempts were made to select exposure levels which did not produce overt toxicity. It was considered appropriate to incorporate endosulfan 10 to 50 ppm levels in the diet of experimental animals for the purpose of sub-toxic study (FAO/WHO 1968, 1983; Dikshith et al. 1984). Testing of sub-toxic effects upon immune responses is important in relation to human health aspects of pesticides particularly due to widespread use of endosulfan and its persistent in the environment (Street and Sharma 1975; Vos 1977; Faith et al. 1980; WHO 1984). Results of a preliminary study on immunotoxicological evaluation of endosulfan in rats are presented in this communication.

Rats exposed to endosulfan at the test dose levels for six weeks did not show any overt toxicity signs and symptoms. No significant differences were noted in body, spleen and thymus weights between control and treated rats (Table 1), suggesting that endosulfan at the test dose levels did not produce any stress responsible for the observed immunosuppressive effect in the present study. However, significant increase in liver weight was observed in rats exposed to 50 ppm endosulfan. Similar increase in liver weight in rats exposed to sub-chronic doses of endosulfan was observed by other workers (Gupta and Gupta 1977; Gupta and Chandra 1977; Dikshith et al. 1984). This increase in liver weight in endosulfan exposed rats was attributed to proliferation of smooth endoplasmic reticulum (Gupta and Chandra, 1977). Such alteration of the hepatic system may directly or indirectly influence the function of lymphatic system.

Serum antibody titer, immunoglobulin levels and globulin fractions were studied for the estimation of humoral immune responses. Rats exposed to endosulfan showed a significant decrease in serum antibody titer to tetanus toxoid in dose dependent pattern (Table 2). The serum immunoglobulin (IgG and IgM) concentrations and gamma-globulin level were significantly decreased in rats exposed to 50 ppm endosulfan (Table 2 and 3). Similar decrease in serum antibody titer, gamma-globulin fraction and immunoglobulin levels have been reported in

Table 1. Body and relative organ weights of tetanus toxoid stimulated rats exposed to different levels of endosulfan for six weeks*

Exposure level (ppm)	Body Wt (BW) (gram)	Liver Wt/BW ratio x 10^{-3}	Spleen Wt/BW ratio x 10^{-3}	Thymus Wt/BW ratio x 10^{-3}
0	130.00 ± 5.20	36.00 ± 3.00	3.52 ± 0.24	1.70 ± 0.31
10	134.00 ± 5.00	36.00 ± 4.00	3.33 ± 0.25	1.80 ± 0.40
30	132.00 ± 4.50	36.50 ± 3.50	3.55 ± 0.25	1.78 ± 0.43
50	126.60 ± 5.70	42.22 ± 4.49[a]	3.21 ± 0.43	1.66 ± 0.21

* The data presented as the mean ±SD of 10 to 14 rats in each group.
a Significantly different from control; $p < 0.05$

rats and rabbits exposed to organochlorine pesticides (Wassermann et al. 1969, 1972; Street and Sharma 1975; Vos 1977). These results indicate important changes in host immunity may occur after endosulfan exposure.

Table 2. Effect of endosulfan on serum antibody titer and immunoglobulin concentrations in rats immunized with tetanus toxoid*

Exposure level (ppm)	Haemagglutination $-\log_2$ titer	IgM (mg/ml)	IgG (mg/ml)
0	14.40 ± 1.12	0.70 ± 0.12	15.50 ± 2.00
10	14.52 ± 1.20	0.65 ± 0.17	15.80 ± 1.08
30	12.68 ± 1.20[a]	0.68 ± 0.10	14.38 ± 1.80
50	10.10 ± 1.75[b]	0.56 ± 0.15[a]	12.15 ± 2.00[b]

* Values are the mean ±SD of 10 to 12 rats per group.
[a] Significantly lower than control, $p < 0.05$; [b] $p < 0.01$

Table 3. Effect of endosulfan on serum protein levels of tetanus toxoid stimulated rats*

Exposure level (ppm)	Albumin	α-globulin	β-globulin	γ-globulin
0	44.45 ± 5.50	26.20 ± 5.00	13.00 ± 2.70	16.24 ± 2.50
10	47.50 ± 4.20	24.10 ± 2.10	13.30 ± 2.20	15.20 ± 2.10
30	47.50 ± 3.80	25.30 ± 1.80	12.10 ± 2.50	15.20 ± 2.45
50	50.70 ± 5.00	25.00 ± 1.50	12.14 ± 2.00	12.20 ± 2.20[a]

* Values represent the mean percentage of total protein content ±SD of 10-12 rats per group.
[a] Significantly different from the respective control, $p < 0.002$.

The effect of endosulfan on cell-mediated immune response was evaluated with the help of macrophage migration inhibition (MMI) and leucocyte migration inhibition (LMI) test. Rats exposed to endosulfan and subsequently immunized with tetanus toxoid showed marked decrease in LMI and MMI responses in dose related pattern (Table 4). Although it appears that the depression of cell-mediated immunity extends to the primary humoral response, however, more light can be thrown in this direction by studying the response to a thymus independent

antigen.

The results of the present study reveal a suppression of humoral and cell-mediated immune responses in rats exposed to sub-toxic doses of endosulfan. This suppression was found to increase in a dose dependant pattern. Suppression of humoral and cell-mediated immune responses by organochlorine pesticides has been reported by various workers (Street and Sharma 1975; Vos 1977; Faith et al. 1980). Adverse effect of endosulfan on immune function could place the host more vulnerable position against various pathogens.

Table 4. Effect of endosulfan on cell-mediated immune response in rats immunized with tetanus toxoid*

Exposure level (ppm)	LMI Mean ± SD (%)	MMI Mean ± SD (%)
0	36.25 ± 6.25	43.80 ± 5.40
10	35.48 ± 5.25	46.28 ± 7.14[a]
30	29.18 ± 7.05[a]	36.50 ± 6.87[b]
50	25.90 ± 5.66[b]	30.37 ± 7.10

* Ten to twelve rats were used in each group.
a Significantly lower than control, $p < 0.05$; b_p < 0.01.

Immunotoxicity of endosulfan was observed at dose level (30 ppm) which has been reported not to cause any toxicological effects (FAO/WHO 1983). It is apparent that a more complete understanding of the toxicity of endosulfan is necessary to study human health hazards and establish guidelines for acceptable residues in the environment. It is emphasized that the 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 (Street and Sharma 1975; Vos 1977). More extensive and systematic studies on dose-time relationship in different experimental animals appear to be essential in order to evaluate the effect of endosulfan on immune system of mammalian host. Since there are numerous functions associated with immune system it is necessary to study multiple parameters to properly evaluate the immune function.

It is clear from our preliminary study that the immune system may be a sensitive target for endosulfan. The explanation for immunosuppressive effect of endosulfan may lie at many levels. Like all other organochlorine pesticides, endosulfan may influence physiological and pathological condition, hormonal function, nutritional status, hepatic metabolism of other endogenous and immunoregulatory substances (Vos 1977; Faith et al. 1980). It may also act directly or indirectly on lymphoid cells (Street and Sharma 1975), lymphoid cell distribution, immunoglobulin metabolism, T-cell/B-cell-macrophage co-operation and macromolecular biosynthesis (Vos 1977). It is now important to elucidate the phenomenon in order to understand its mechanism of immunosuppression and the possible health hazards due to continued use of endosulfan. Further investigations are in progress to study the nature of toxic effects of endosulfan on primary and secondary immune cytokinetics, lymphocyte-mediated cytotoxicity,

lymphoid cell distribution and reticuloendothelial system. These studies would contribute to the understanding of the mechanism of action of endosulfan at the cellular level and could be utilized for a meaningful extrapolation of poisoning in humans.

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