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Immune Responses to *Aeromonas hydrophila* in Cat Fish (*Heteropneustis fossilis*) Exposed to Cadmium and Hexachlorocyclohexane

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Fishes are poikilothermic vertebrates hence all the body functions including immunological defence may be susceptible to adverse changes in the aquatic environment. Environmental pollutants, especially heavy metals and pesticides are known to contribute to outbreaks of infectious bacterial diseases in fishes (Sniesako, 1974). Pesticides enter the aquatic environment through intentional application, aerial drift or runoff from applications or accidental release and then become rapidly distributed. Major routes of pesticide movement into water are agricultural runoff from fields and grazing lands (Li, 1975). Several investigators have evaluated the toxicity of hexachlorocyclohexane (HCH) in fishes (Gopal et al., 1988; Watson et al., 1983), however, only sporadic observations on the humoral immune responses have been made. Cadmium has been demonstrated to affect the immune system of teleost (Viale and Calamari, 1984). The effects of sublethal concentrations of methylmercury and copper on the immune response of blue gourami (Trichogaster trichopterus have also been studied (Roales and Perlmuter, 1977). Attempts have been made to investigate the effect of the HCH and cadmium on the humoral immune responses to bacterial antigen using H.fossilis as test species. Attempt is also made to evaluate the vascular function at sublethal doses.

MATERIALS AND METHODS

H. fossilis obtained from natural resources were kept in glass Aquaria, and allowed to acclimate in chlorine free tap water at a temperature 20 ± 2°C for a period of two weeks, prior to heavy metal and pesticide exposure (Gopal and Misra, 1988). A photoperiod of 12 hr light; 12 hr dark was used and the fish were handfed on fresh goat liver. Healthy individuals of H.fossilis 20 ± 5 g wt were divided into four

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groups of 20 each. 1st group of fishes was exposed to sublethal concentrations of cadmium (0.2 mg/L) and group 2nd to HCH (1.3 mg/L). Group 3rd was exposed to both of the compounds in identical quantity. Animals of the 4th group served as control. All aquaria were aerated constantly and water was maintained for total hardness (206.9 mg/L), dissolved oxygen (8.2 mg/L), pH (7.2) and temperature 20°C. Tests in triplicate were carried out along with control.

A. hydrophila isolated from the maintained culture in our laboratory was grown in nutrient broth at 37°C for 48 hr and then heated at 60°C for 30 min. Subsequently the culture was centrifuged, pellet was washed with normal saline and reconstituted the same volume. An equal volume of Freund's Complete adjuvant was added. Each fish was inoculated intraperitoneally with 0.1 ml containing 10 cells in Freund's adjuvant (FCA) two wk after exposure to cadmium and HCH. Further, injections of the same cell concentrations were given after 7, 14, 21 and 28 d. Blood was collected by caudal vein puncture from 5 fish, left overnight at 1°C and the pooled sera was heat inactivated at 50°C for 30 min and kept at-20°C until required. O-antigen was extracted from the bacteria by heating at 60°C for 30 min. The treated bacteria were pelleted by centrifugation at 4000 x g for 2 hr and resuspended to give a concentration of 6% (w/v) in water. Aqueous solution of phenol (90% w/v) was added to this mixture at 65°C and stirred for 5 min. The preparation was centrifuged at 2000 x g for 1 hr at 0°C and the upper aqueous layer was dialysed for 48 hr against running tap water, then poured into 10 volumes of cold acetone at -20°C. The lipopolysaccharide precipitate was dissolved in water to give 3% (w/v) solution which was collected by centrifugation (100,000 x g) for 4 hr. The pellet was resuspended in half of the original volume of distilled water and then hydrolysed with 1% (v/v) acetic acid at 100°C. After 30 min centrifugation at 500 x g the supernate was dialysed at 4°C against distilled water for 48 hr (Talwar, 1983).

SRBC collected in Alsevier's solution from the sheep kept in ITRC animal house facility, were washed three times in normal saline and resuspended to give a 2.5% suspension in phosphate buffer saline at pH 7.2. Equal volume of erythrocytes suspension and 1:20,000 tannic acid were mixed and incubated at 37°C for ten min. The cells were washed with PBS and resuspended to original concentration. Coating of the cells was carried out by mixing 1 ml of tanned cells (2.5%) to 4 ml of PBS at pH 6.4 and 1 ml protein solution in saline. After incubation for 10-15 min at room temperature the cells were centrifuged and washed with PBS containing 0.1% normal rabbit serum and were finally suspended in the same buffer to original volume. The test was performed

by adding 0.05 ml of diluted antiserum and the settling pattern was observed after 2 hr incubation. The antibody titers were reported as mean ± the standard error of log 2 agglutination titer. Results were evaluated statistically using Student's 't' test.

Blood was collected from twelve fish in each group from caudal vein. The erythrocyte count (per mm) was determined using a Neubauer double hemacytometer (Germany) and hemoglobin determined by Sahli's hematocytometer (Germany). The hematocrit was determined with microhematocrit pipette (U.S.A.). Brains, gills, liver, kidney, heart and spleen were removed, rinsed with physiological saline and immediately frozen for subsequent analysis. The tissues were homogenized in 2 vols of 25% methyl alcohol/water. HCH and its isomers were extracted successively with 10, 10, 5, 5 and 5 ml of hexane. The total hexane extract was cleaned up on a florisil column by using small volume of hexane to complete the transfer (Litterest and Miller, 1975).

Solvents were concentrated, dried with anhydrous Na_2SO_4 and transferred to 10 ml volumetric flask with hexane and made up to the volume. Analysis was carried out by using Varian Aerograph Series 2400 GC equipped with an electron capture detector. A glass column (6 x $\frac{1}{4}$ in) packed with 1.5% OV - 17 ± 1.95% QF1 on a 100-120 mesh chromosorb GH/P was used. The carrier gas was nitrogen at 60 ml/min. The injector, detector and column temperature were maintained at 210, 210 and 190°C respectively. The chart speed was 0.5 cm/min and the attenuation was fixed at 4×10^{-10}.

For the estimation of metals, wet acid digestion of the tissues was done and the concentration was determined in a Perkin Elmer Model 5000 Atomic Absorption Spectrophotometer. The instrument was set up for maximum sensitivity and a mixture of air and acetylene was used as a fuel with oxidising flame (Doyle and Pfander, 1975).

RESULTS AND DISCUSSION

The peak antibody titer observed in fish treated with Cd+ HCH were found significantly suppressed in comparison to the titers of fish exposed to Cd and HCH individually.

The immunosuppression effects of these chemicals even at low concentration, indicate potential vulnerability of fish from polluted habitat to diseases. Antibody responses among trout have also been established (Ingram and Alexnader, 1977, O'Neill, 1979).

Significant reduction of RBC in exposed animals and total count of leucocyte showed immunosuppressive responses in

Table 1. Effect of cadmium and lindane on the hematology of H.fossilis exposed to A.hydrophila

	Control fish		Cadmium treated		HCH treated fish		CD+HCH treated fish	
	N	X SD	N	X SD	N	X SD	N	X SD
Erythrocyte count (X 10 mm)	12	2.6±0.3	11	1.6±0.2	11	1.9±0.8	9	1.0±0.2
Leucocyte count (X 10 mm)	12	3.9±0.8	11	2.2±0.2	11	1.5±0.3	7	1.5±0.3
Hemoglobin count (g %)	12	12.8±0.7	11	11.5±0.5	9	9.8±1.8	7	10.6±1.4
Hematocrit (%) (PVC)	12	34.5±3.5	9	26.1±4.0	7	23.8±3.0	7	21.9±2.2
Plasma protein	12	8.3±0.4	9	6.0±0.6	7	6.9±1.6	7	5.9±0.6

Values are expressed as mean of six fish ± S.D.

fish treated with both compounds. Similarly, hemoglobin and hematocrit vales showed a significant reduction in the exposed fish. However, total plasma protein did not indicate any significant protein loss when treated individually while inhibition was recorded after synergistic exposure (Table 1). The general pattern of distribution shows that HCH concentration was higher in liver and kidney of exposed animals (Table 2,3). Accumulation of cadmium was higher in liver and gill of exposed fish (Table 4).

Table 2. Distribution of HCH and its isomers (ppm) in vital tissues of H.fossilis exposed to HCH and cadmium for 30 d

Name of organ	Alpha HCH	Beta HCH	Gamma HCH	Total HCH (ppm)
Brain	0.26±0.02	0.40±0.06	0.38±0.01	1.04±0.09
Gill	0.70±0.09	0.34±0.06	0.25±0.02	2.29±0.17
Liver	6.53±1.02	4.54±0.89	2.19±0.88	13.26±2.79
Kidney	3.37±0.46	0.72±0.08	3.29±0.28	7.36±0.82
Heart	1.98±0.34	2.91±0.26	2.02±0.32	6.91±0.92
Spleen	2.00±0.24	3.33±0.82	1.68±0.23	7.01±1.29

Values are expressed as mean of six fish ± S.D.

Cadmium has been found to be responsible in reducing antibodies which play a significant role in the outbreaks of bacterial diseases (Jones et al., 1971). Pb and Cd ions have high affinity for sulphydryl and hydroxyl groups. This direct action impairs the activity of antibody complement (Hemphill et al., 1971) and interferon (Gainer, 1974). Cadmium is responsible in reduction of the B-like cells, as well as the loss of helper and memory cell activity (Phipps, 1976). An initial finding of neutralisation of serum antibody have been shown in Cd treated fish. Our findings showed significant reduction of antibody titers in animals exposed to hexachlorocyclohexane. Instead of their immunosuppressive responses they are also able to damage erythropoietic tissues in spleen and kidney (Gardner and Yevich, 1970). Haemopoietic system of fish have been found to be a target for other heavy metals (Chipmen et al., 1958; Braker, 1969; Rucker and Amend, 1969). Our observations indicate that immunocompetent cells and erythropoietic cells have been affected due to reduced level of erythrocytes leucocyte, hemoglobin, haematocrit and plasma

Table 3. Distribution of HCH and its isomers (ppm) in vital tissues of H.fossilis exposed to HCH for 30 d

Name of organ	Alpha HCH	Beta HCH	Gamma HCH	Total HCH (ppm)
Brain	0.85±0.12	0.70±0.11	0.40±0.08	1.95±0.31
Gill	0.49±0.06	0.28±0.08	0.33±0.06	1.11±0.20
Liver	4.24±0.88	4.08±0.64	2.39±0.62	10.71±2.14
Kidney	5.08±0.66	0.32±0.04	0.90±0.10	6.30±0.80
Heart	1.67±0.48	0.96±0.82	0.84±0.12	3.46±1.42
Spleen	0.38±0.06	0.11±0.02	0.68±0.08	1.17±0.16

Values are expressed as mean of six fish ± S.D.

Table 4. Distribution of cadmium in vital tissues of H.fossilis exposed cadmium and lindane for 30 d

Name of organ	Control	Cd (ug/gm)	Cd+HCH (ug/Cd/gm)	Lindane
Brain	0.81±0.06	2.08±0.22	1.03±0.26	0.29±0.06
Gill	0.53±0.08	52.14±11.2	11.73±3.20	0.42±0.09
Liver	2.77±0.06	49.63±8.80	27.90±3.80	0.17±0.06
Kidney	1.07±0.12	0.59±0.08	7.56±1.82	0.39±0.06
Heart	0.14±0.02	0.03±0.00	0.04±0.01	0.14±0.02
Spleen	0.05±0.01	0.05±0.01	0.93±0.23	0.03±0.00

Values are expressed as mean ± S.D.

protein concentration. A variability of hematological anomalies have been described in fresh water fish following exposure to copper (Christensen et al., 1972). The continuous exposure of cadmium along with HCH resulted in the accumulation of hepatic cadmium and HCH due to low potentiation of various hepatic metabolizing enzymes in fresh water

teleost. It is suggested that immune responses of fish may serve as a sensitive indicator for chemical pollution of aquatic habitat. Also, the immunotoxicity leading to decreased resistance to diseases by low levels of pollutants, should be taken into account while developing regulatory standards.

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