Bull. Environm. Contam. Toxicol. 27, 42-48 (1981)

Effects of Intraperitoneal Lead and Cadmium on the Humoral Immune Response of *Salmo trutta*

Julian G. O'Neill

Department of Life Sciences, Trent Polytechnic, Nottingham, England

Environmental stressors, especially pollutants, have been shown to be coincident with outbreaks of infectious bacterial diseases in fishes (SNIESZKO 1974). Although the bacterial species associated with these epidemics were found to be capable of producing disease symptoms, under laboratory conditions, it could be concluded that the bacteria were opportunists and invaded the fish at a late stage. A direct effect of a heavy metal pollutant on a latent infection of *Vibrio anguillarum*, harboured in the freshwater-adapted eel *Anguilla anguilla*, was shown by RØDSÆTHER et al. (1977). The introduction of a sub-lethal level of copper (30 to 60 μg dm^{-3}) into the water supply of these fish changed the commensal relationship between the bacterium and the host eel into one of disease and mortality.

Exposure to the heavy metals lead, cadmium and mercury has been found to affect adversely the immunological responses of mammals (KOLLER 1973). However, there have been few examinations of the effects of heavy metals or other pollutants on the immunological responses of fishes. GONCHAROV & MIKRYAKOV (1970) observed that the waterborne exposure of yearling *Cyprinus carpio* to non-toxic levels of phenol (12.5 mg dm^{-3}) reduced the agglutinin titres raised against *Aeromonas punctata*. Sub-lethal levels of methylmercury and copper (both 9 μg dm^{-3}) were also found by ROALES & PERLMUTTER (1977) to reduce the antibody responses raised against IPN virus and *Proteus vulgaris* in the blue gourami *Trichogaster trichopterus*. The latter authors cited the work of SAROT (1973) who had shown that the antibody production in the zebrafish, *Brachydanio rerio*, was reduced on exposure to zinc.

In the present study the effects of i.p. dosed lead (Pb) and cadium (Cd) on humoral antibody levels were examined in brown trout, *Salmo trutta*, immunised with *MS2* bacteriophage. Earlier work (O'NEILL 1979) had shown that the live *MS2* virus was a 'primary' immunogen and highly immunogenic, while non-pathogenic in fishes. Further, the antibody response could be quantified by a sensitive and reproducible technique. For the reasons outlined by OZOH (1979) the i.p. route of heavy metal challenge was utilised to remove the complications presented by the environmental modifications of the toxicity of these metals and their passage through the gills of the fish.

0007-4861/81/0027-0042 $01.40
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MATERIALS AND METHODS

Yearling *Salmo trutta*, 100 to 200g, obtained from the Severn-Trent Water Authority fish farm, Nottinghamshire, were held in 500 dm^3 polyethylene aquaria with a through-flow of well aerated and chlorine -free tap water, at a temperature of 15.5 ± 0.5°C. All the fish were marked with an opercular tag. A photoperiod of 12h light:12 dark was used and the fish were hand fed daily on Beta Trout Growers Diet 417, No. 5 (B.P. Nutrition). Each fish was i.p. inoculated with 10^9 plaque-forming units (PFU) of *MS2* bacteriophage in 0.1 cm^3 Freund's incomplete adjuvant water-in-oil emulsion. A second and third inoculum of 10^9 PFU *MS2* in 0.1 cm^3 teleost saline, pH 7.6, (O'NEILL 1979) was administered i.p. to each fish 7 and 15 weeks after the first. When handled the fish were gently netted and lightly anaesthe -tised in a 1g:200 dm^3 solution of tricaine methanesulphonate (Sandoz) made up in aquarium water. Weekly blood samples of not more than 0.1 cm^3 were taken by caudal venipuncture and were left to clot overnight at 1°C. The sera were decanted and assayed immediately in duplicate for *MS2* neutralisation antibody activity.

The bacteriophage *MS2* (picornovirus, group 1 RNA-phage) was grown using the Petri-plate and 'soft-agar overlay' method of EISENSTARK (1967), with *Escherichia coli* K12 as the host. The bacteriophage was isolated from the bacterium by ultrasonic disruption (20 kHz at 6nm) followed by 1 low-speed (20 min, 3000xg) and 2 high-speed (120 min, 40,000xg) centrifugations at 4°C. The bacteriophage pellets were deaggregated in teleost saline using ultrasonic disruption and stored at 1°C. A semi-micro viral plaque neutralisation assay procedure was modified from the Petri-plate methods of ADAMS (1959) and the neutralisation antibody titre of the sera was described as the reciprocal of the dose of serum required to produce 50% inactivation of the bacteriophage (SD_{50}). The full procedure has been described elsewhere (O'NEILL 1979).

Two weeks after the third *MS2* immunisation dose groups of 5 trout were randomly selected and inoculated i.p. with 0.01, 0.1 or 0.3 mg Pb in 0.1 cm^3 saline per 100g body weight. A control group was inoculated with 0.1 cm^3 teleost saline adjusted to pH 3 with 4N HNO_3. The latter saline was used to prepare the Pb solutions from $Pb(NO_3)_2$. One week later groups of 5 trout were inoculated i.p. with 0.05, 0.1 or 0.2 mg Cd in 0.1 cm^3 saline per 100g body weight. The Cd solutions were prepared from $3CdSO_4.8H_2O$ dissolved in teleost saline pH 7. A control group was also inoculated with 0.1cm^3 of this saline. The Pb- and Cd-dosed fish were maintained in separate aquarium systems, though control and heavy metal treated fish were randomly mixed. The Cd-dosed trout were rechallenged 12 weeks later with 10^9 PFU *MS2* in 0.1cm^3 teleost saline. On termination of the experiments body weight change, haematocrit and serum protein concentration were determined.

RESULTS

Trout immunised with a tertiary dose of *MS2* bacteriophage were found to maintain a consistently high level of humoral antibody. The acid pH of the saline control inocula had no apparent effect on the titre. In week 10 of the experiment the control fish from the Cd -experiment were moved to a separate aquarium. This resulted in a small depression of antibody titre in these fish which then quickly returned to the original level. The i.p. inoculations of Pb (Fig. 1) and Cd (Fig. 2) were found to decrease the level of antibody in the immunised trout and the initial fall in titre was dose dependent for both metals. The highest concentrations, 0.3mg Pb and 0.2mg Cd, were found to depress antibody titre rapidly and a total mortality was recorded after 2 weeks for the Cd- and after 7 weeks for the Pb-dosed fish. Prior to the death of these fish a serum antibody titre was not detectable. This contributed to the rise in mean antibody titre which was observed in the surviving 0.3mg Pb-dosed fish from weeks 3 to 6. Although the lower concentrations of heavy metal reduced the level of antibody no mortality was recorded in these fish. Antibody titre was not totally depressed and a low-titre plateau was eventually reached. At this time short periods of increased antibody activity were observed, including a large but transient rise in the 0.1mg Pb group. The rechallenge of the surviving Cd-dosed trout with *MS2* was found, over a 4 week period, to produce a more than ten-fold increase in antibody titre in the 0.05mg Cd group. However, this elevated titre did not reach the level of the control fish and was not maintained. The group dosed with 0.1mg Cd demonstrated no significant increase in titre after *MS2* challenge.

The heavy metals were also found to affect parameters other than antibody titre (Fig. 3). A dose dependent reduction in body weight increase was observed in the Pb-dosed trout over the course of the experiment, with a decrease in weight of the 0.3mg Pb group. Haematocrits were reduced in both Pb- and Cd-dosed fish, again the Pb groups indicated a dose dependent response. Of the groups examined for serum protein only the 0.05 Cd group indicated a significant reduction in concentration.

DISCUSSION

It was apparent that single i.p. doses of Pb and Cd resulted in a substantial reduction of antibody titre in *MS2*-immunised trout and that within the time limits of the experiment there was no recovery. However, only in the case of the two lethal concentrations was antibody totally eliminated from the sera and death ensued.

The action of heavy metals on the immunological responses of fishes would be expected to be widespread. Pb and Cd ions will readily bind with and readjust the tertiary structure of a wide range of biologically active molecules (PHIPPS 1976). The Cd ion in particular has a high affinity for sulphydryl and hydroxyl groups. Such a direct action has been found to impair the activity of antibody

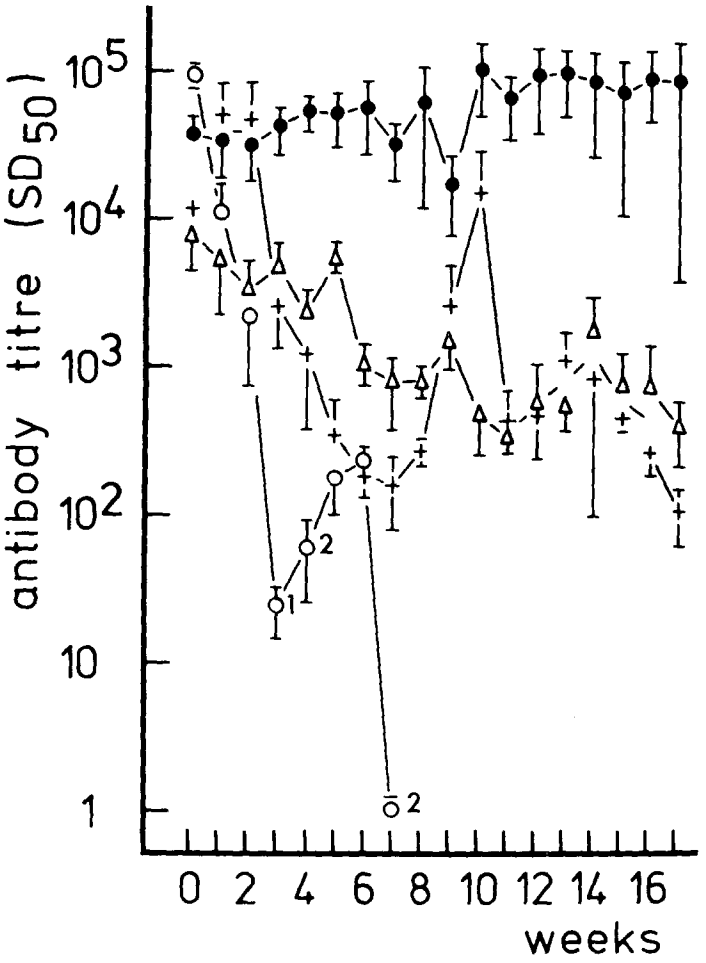

Fig. 1 Antibody titres produced by *MS2*-immunised *S. trutta* after i.p. inoculation at week 0 with 0.01 (△), 0.1 (+) and 0.3 (Ō) mg Pb, and in control fish (●). The SD_{50} values are means for 5 fish ± 2SE, except where inset numerals indicate the number of post-sample mortalities.

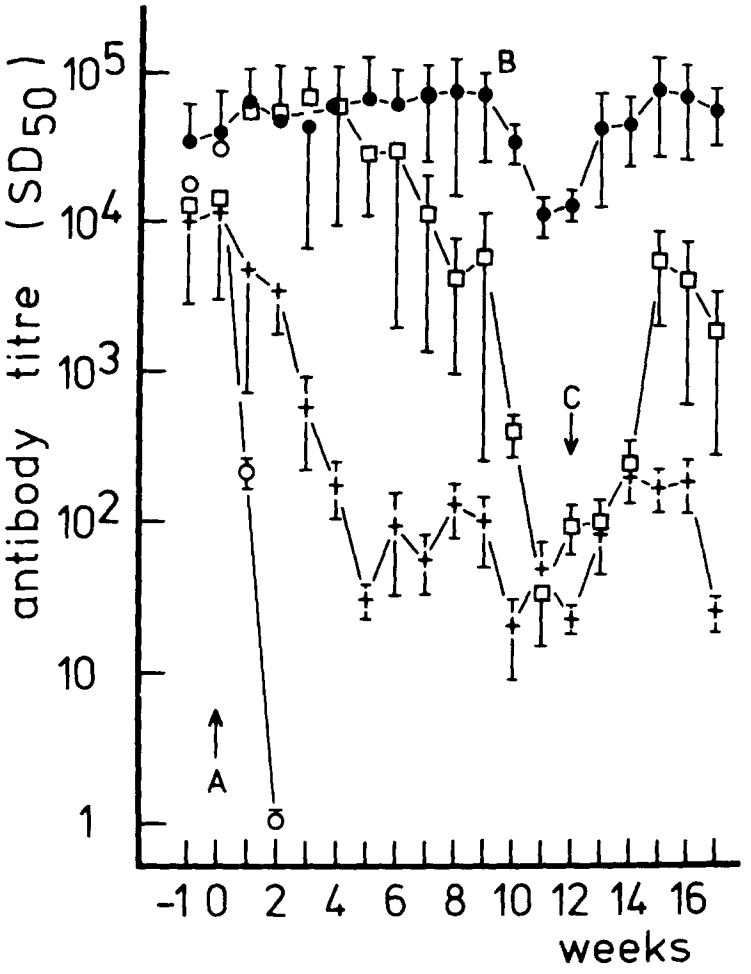

Fig. 2 Antibody titres produced by *MS2*-immunised *S. trutta* after i.p. inoculation at week 0 (A) with 0.05 (□), 0.1 (+) and 0.2 (○) mg Cd, and in control fish (●). The SD_{50} values are means for 5 fish ± 2SE. All 0.2mg Cd fish died after the +2 week sample.

(B) Control fish moved to a separate aquarium.

(C) Rechallenge of Cd fish with 10^9 PFU *MS2*.

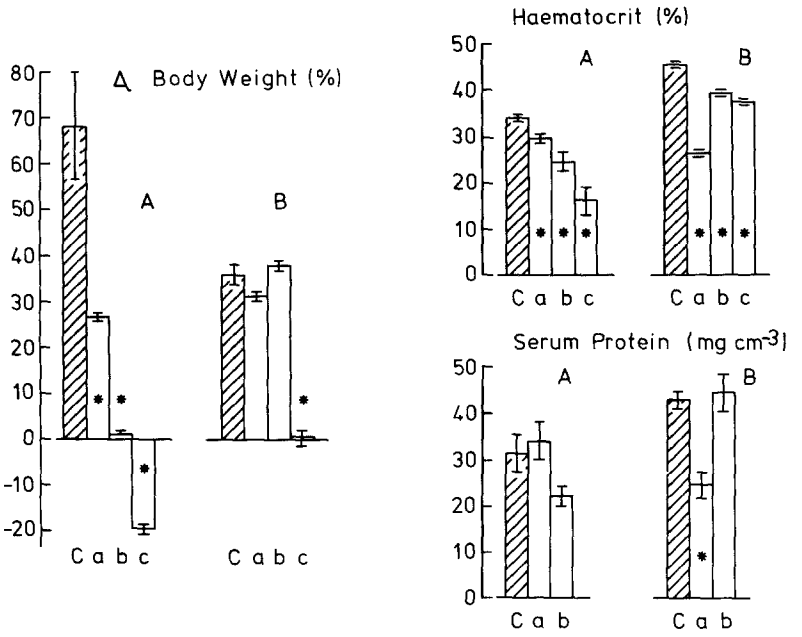

Fig. 3 Body weight change, haematocrit and serum protein concentration in *S. trutta* i.p. inoculated with:
(A) (a) 0.01, (b) 0.1 and (c) 0.3 mg Pb.
(B) (a) 0.05, (b) 0.1 and (c) 0.2 mg Cd.
(Hatched bars-C) Control values. Mean values are for 5 fish ± SE.
(*) Significant difference from control values, P=<0.001.

(JONES et al. 1971), complement (HEMPHILL et al. 1971), and interferon (GAINER 1974) molecules after Pb- or Cd-exposure. An initial neutralisation of serum antibody activity may have occurred in the Pb- and Cd-dosed trout, though this alone would not explain the long-term suppression of antibody titre. The general metabolic and enzymic processes of fish are also affected by heavy metal exposure (CHRISTENSEN 1975) and would be expected to impair growth. Indeed, GONCHAROV & MIKRYAKOV (1970) found that metabolic depletion, produced by starvation, could reduce antibody titre in *C. carpio*.

Pathological changes have been observed in the erythropoietic tissues of the spleen and kidney of fishes exposed to heavy metals (GARDNER & YEVICH 1970). In the present study the observed anaemia indicated such an action in the Pb- and Cd-dosed trout, and a direct action on the immunocompetent cells in the spleen and kidney cannot be ruled out. KOLLER & KOVACIC (1974) concluded that the reduced number of antibody producing cells observed in Pb-exposed mice were caused by a suppression of the clonal expansion of B-lymphocytes, and MÜLLER et al. (1979) found that Cd-exposure suppressed those

responses mediated by T-lymphocytes and macrophages. A reduction in the number of B-like cells, as well as the loss of helper and memory cell activity, could have been responsible for reducing antibody titre in Pb- and Cd-dosed trout. A reduction in the number and activity of immune effector cells would also account for the Cd-dose dependent suppression of the antibody response after a rechallenge with *MS2*.

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Accepted April 29, 1981