

HUMORAL IMMUNE ALTERATIONS CAUSED BY LEAD: STUDIES ON AN ADULT TOAD MODEL

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Abstract: HUMORAL IMMUNE ALTERATIONS CAUSED BY LEAD: STUDIES ON AN ADULT TOAD MODEL. Carolina E. Rosenberg; Nilda E. Fink; Alfredo Salibián. *Acta Toxicol. Argent. (2007) 15 (1): 16-23*. There is evidence that environmental metal levels affect the immune function. In the particular case of the impact of heavy metals, information available suggests that the immune system is a target for low-dose Pb exposure. Among vertebrates it was shown that amphibians are capable of forming antibodies against a variety of antigens, causing several responses such as anaphylactic response and rejecting grafts. In this study, the production of antibodies was assessed against sheep red blood cells (SRBC) in the anuran *Bufo arenarum* after six weekly injections of sublethal doses of lead (50 mg.kg⁻¹, as lead acetate). Natural antibodies (natural heteroagglutinins) were also quantified against SRBC. Both assessments were carried out employing an ELISA method developed to this end, measuring absorbance (A). For natural anti-SRBC antibodies in both control (C) and Pb treated (T) toads, there was a non significant tendency to increase the initial absorbances (C initial: 0.69±0.39 A; T initial: 0.54±0.30 A), relative to those registered at the end of the experiments (C final: 0.89±0.49 A; T final: 0.76±0.31A); the T/C ratios also did not show changes. The only significant difference was found between initial and final samples from lead-treated toads (p<0.014). The immune anti-SRBC antibody levels of toads immunized with SRBC showed a significant lower increase (p<0.05) in lead-treated animals (T final: 0.66±0.36 A), as compared to control toads (C final: 0.91±0.50 A) at the end of the experiment. It was thus concluded that the changes due to the assayed doses of Pb in the levels of antibodies cannot be explained on the basis of only one single action mechanism of the metal, but as the result of a conjunction of effects over different immunocompetent cell subpopulations. These different responses suggest that factors affecting animals exposed to a foreign stimulus are different from those influencing the response of wild animals.

Key words: Sheep red blood cells (SRBC) antibodies; Heavy metals immunotoxicology; Sublethal lead; *Bufo arenarum*.

Resumen: ALTERACIONES INMUNES HUMORALES CAUSADAS POR PLOMO: ESTUDIOS EN UN MODELO DE SAPO ADULTO. Carolina E. Rosenberg; Nilda E. Fink; Alfredo Salibián. *Acta Toxicol. Argent. (2007) 15 (1): 16-23*. Existe evidencia de que los niveles de metal ambientales afectan la función inmune. En el caso particular del impacto de metales pesados, la información disponible sugiere que el sistema inmune es un blanco para la exposición a bajas dosis de Pb. Entre los vertebrados, se ha mostrado que los anfibios son capaces de formar anticuerpos contra una variedad de antígenos, que causan diversas respuestas, tales como respuesta anafiláctica y rechazo de injertos. En este estudio, la producción de anticuerpos fue evaluada contra eritrocitos de oveja (EO) en el anuro *Bufo arenarum*, luego de seis inyecciones semanales de dosis subletales de plomo (50 mg.kg⁻¹, como acetato de Pb). Los anticuerpos naturales (heteroagglutininas naturales) fueron también cuantificados contra EO. Ambas evaluaciones fueron llevadas a cabo empleando un método de ELISA desarrollado a este fin, midiendo la absorbancia (A). Para los anticuerpos anti-EO naturales, tanto en sapos controles (C) como en sapos tratados con Pb (T), hubo una tendencia significativa a incrementar las absorbancias iniciales (C inicial: 0,69±0,39 A; T inicial: 0,54±0,30 A); la relación T/C tampoco mostró cambios. La única diferencia significativa se encontró entre las muestras inicial y final de los sapos tratados con plomo (p<0,014). Los niveles de anticuerpos anti-EO inmune de sapos inmunizados con EO mostraron un bajo incremento significativo (p<0,05) en los animales tratados con plomo (T final: 0,66±0,36 A), al compararse con sapos control (C final: 0,91±0,50 A) al final del experimento. De este modo, se concluye que los cambios debidos a las dosis analizadas de Pb en los niveles de anticuerpos no pueden explicarse sólo sobre la base de un único mecanismo de acción del metal, sino como resultado de una conjunción de efectos sobre diferentes subpoblaciones de células inmunocompetentes. Estas diferentes respuestas sugieren que los factores que afectan los animales expuestos a un estímulo externo son diferentes de aquellos que influyen la respuesta en los animales silvestres.

Palabras claves: Anticuerpos de eritrocitos de oveja; Inmunotoxicología de metales pesados; Plomo subletal; *Bufo arenarum*.
Palavras chaves: Anticorpos de eritrócitos de carneiro; Inmunotoxicología de metais pesados; Chumbo subletal; *Bufo arenarum*.

INTRODUCTION

Immunotoxicology has become an issue of major interest over the last decade. Recently developed methods for toxicological assessment have involved the immune system as a target after exposure to some xenobiotics and drugs. The information on immunotoxicity studies in amphibian model is incomplete. There are various immunological assays available for evaluating the effects of environmental stressors on immunologi-

cal responses of amphibians (1,2). Among them, determining circulating immunoglobulin levels in serum is a useful endpoint in functional assays on the net result of a humoral immunological branch *in vivo*. This can be measured in wild animals (total immunoglobulins, Ig) or under experimental exposure to a specific antigen. Ectothermic vertebrates produce antibodies similar in structure and diversity to those of mammals (3,4). Amphibians are capable of forming antibod-

ies against a variety of antigens, causing an anaphylactic response and rejecting grafts (1,2,5-7). In earlier pioneer studies conducted on the anurans *Rana esculenta* and *Calyptocephalus gayii* on the detection of agglutinating or hemolytic activities against several antigens (animal erythrocytes and bacteria) were identified (8). A natural heterohe-moagglutinin was described in the serum of the *Bufo regularis* toad; this agglutinin for human erythrocytes appeared to have anti-(B+H) specificity (9). Jurd (10) showed that adult *Xenopus* serum contains a natural factor capable of lysing and agglutinating red blood cells (RBC) from many species. In addition, Fernández (11) found mild to low levels of hemolytic and agglutinating activity against mouse RBC in sera of different species of Argentine native anurans.

There is evidence that environmental metal levels affect the immune function. In the particular case of the impact of heavy metals on the immune system, the information available suggests that it is a target for low-dose Pb toxicity (12). Research including both in vivo and in vitro studies on animal models like rat, mouse, rabbit and fish, as well as humans, enabled documentation of the effect of Pb on humoral and cellular immunity (13-19). More recently, Chiesa et al. (20) have shown a significant increase in the heaviest fraction of serum globulins of *Bufo arenarum* injected with sublethal doses of lead, interpreting their finding as evidence of the compensatory immunostimulation effect of the metal.

Due to differences in the design of experimental protocols or tests used on different species, it is difficult to conduct an integrated analysis of the results obtained from studies of the effects of Pb on humoral immunity. For instance, the total level of antibodies in rat serum decreased after chronic exposure to the metal (21), but the same response did not occur when the assay was carried out on rabbits (22). Studies of the level of specific antibodies against an antigen in presence of Pb showed a reduction in serum titers in rats (23-26). In humans, special attention has been paid to occupational exposure, mainly in smelting industries, battery manufacturers, and mining activities. There is abundant information about the severe consequences of chronic exposure to Pb in people working in those industries. The relation between Pb concentration in blood (PbB) and some immunological parameters such as the levels of C3 and C4 components in the complement system, and IgG, IgA and IgM concentrations were studied in our country in adult human male exposed to the metal. Only C4 levels varied in line with PbB levels. In most cases, a negative Ig correlation was found, though this was positive in IgM, with very low PbB levels (27,28).

The levels of serum antibodies (IgG and IgM) in individuals exposed to Pb may decrease (29), or remain unchanged (17,30,31), although an increase in IgA in saliva was reported (30).

It has been suggested that Pb toxicity may be due, at least partially, to an autoimmune response, since the above mentioned type of disorders were observed in most of the affected target organs. Autoimmunity and hypersensitivity processes may be produced by a deregulation in the immune response. In both cases there is a change in the cellular T-helper 1 (Th1) and T-helper 2 (Th2) ratio that can be monitored, determining the pattern of cytokines produced by those two cellular types, i.e. interleukin 2, γ -interferon (Th1), and tumor necrosis factor or interleukins 4, 5 and 6 (Th2) (12). This study assessed the production of antibodies against sheep red blood cells (SRBC) in the anuran *Bufo arenarum* exposed to sublethal doses of lead (as acetate). Natural antibodies were also quantified against SRBC (natural heteroagglutinins).

MATERIALS AND METHODS

1 Animals

Eighty five adult *Bufo arenarum* male specimens (average weight 120 g) were collected in the neighborhood of La Plata, Argentina. Previous acclimatization was carried out keeping toads, individually, in plastic boxes containing tap-water, for a period of one week, at constant temperature ($20 \pm 2^\circ\text{C}$), and photoperiod (12D:12N). Blood samples were obtained by heart puncture under MS222 anesthesia, and received on heparin for lead measurement or without anticoagulant. Exuded sera were immediately centrifuged, aliquoted and stored at -20°C , until used within the following three months.

2 Preparation of polyclonal antibodies to *Bufo arenarum* globulin

Antibodies against globulin fraction, obtained by precipitation, were prepared in New Zealand white rabbits, after pre-immune serum sampling. An equal volume of toad globulin fraction was emulsified in complete Freund's adjuvant (Gibco Invitrogen Corp., Carlsbad CA, USA) and injected subcutaneously. After 20 days, a second inoculation was performed. One week later, a first exploratory bleed was performed to test antibody production. Later, an intramuscular inoculation was performed using incomplete Freund's adjuvant for the preparation of the emulsions. A total of 10 boosters were given every 20 days, while different bleeds from the marginal ear vein were carried out to monitor antibody production. The anti-serum obtained was titrated using immunodotting and ELISA (32,33).

For the characterization of rabbit antibodies to *Bufo arenarum* globulins by immunoblotting, samples of normal toads' sera were denatured by heating at 100°C in sodium dodecylsulphate (SDS) for 2 min. They were then run on 7.5% polyacrilamide-SDS gels. Gels were stained with 0.125% Coomassie Brilliant Blue R-250 or electroblotted onto nitrocellulose membranes at 0.4 A, for 1.5 h

in 25 mM Tris, 192 mM glycine, and 20% methanol, pH 8.3. Membranes were blocked with powdered 5% low fat skim milk in PBS-0.5% Tween 20, for 1 h at room temperature. An anti-*Bufo marinus* globulin antiserum was included in order to study the specificity of our antiserum by comparing it with another from other species of the same genus. These were therefore incubated with anti-*Bufo arenarum* globulin antiserum; others were incubated for comparison with anti-*Bufo marinus* immunoglobulin antiserum (34) as first antibodies. As a second antibody a anti-IgG rabbit serum, obtained from goat conjugate with horseradish peroxidase (HRP) (Sigma, Saint Louis MO, USA), was used. All sera were prepared in PBS-Tween-low fat powdered skim milk. The colour reaction was developed in presence of 4-Cl 1-naphthol dissolved in methanol with H₂O₂ in buffer Tris-NaCl. Membranes were washed with distilled water in order to stop the reaction and then dried.

3 Lead administration

Two Pb acetate and Na acetate solutions were prepared in distilled water. Experimental toads received a weekly injection of Pb acetate at a dose of 50 mg Pb . kg⁻¹, for six weeks, and control animals were injected at the same time with Na acetate. The injections were administrated in the dorsal lymph sac, at a rate of 0.6 ml . 100 g⁻¹ body weight. The dose of lead used was previously determined as sublethal at 20°C in our laboratory (35).

3.1 Quantitation of natural anti-SRBC antibodies

The optimal protocol established for the ELISA, modified after Hirvonen et al. (36) was as follows: in U-bottom plates, 100 µl/well of a SRBC suspension at 20x10⁶ in low ionic strength solution (LISS) (30 mM NaCl, 3 mM Na₂HPO₄, 0.24 M glycine, 0.02% azide, 1% BSA) was incubated, with 100 µl of *Bufo* serum successively diluted in LISS (dilution factor 2). After 30 minutes, the sensitized SRBC suspension was washed and then resuspended in 250 µl of 0.2% BSA-0.9% NaCl; 100 µl of the suspension were placed in each well in an ELISA plate, fixed with glutaraldehyde 0.3% and blocked with 2% BSA-0.9% NaCl. The plates were incubated during 1 h with anti-*Bufo arenarum* globulin serum in rabbit (1/4000, 100 µl/well), washed, and incubated for 1 h with the anti-rabbit globulin-HRP conjugate (1/2000, 100 µl/well). After washing, the substrate was added and read at 492 nm (32).

The assay included parallel titration of a commercial anti-SRBC rabbit serum as a positive control (Laboratorio Gutiérrez, Buenos Aires, Argentina). Each sample was also tested without SRBC, anti-*Bufo arenarum* antiserum and conjugate-HRP and with pre-immune serum.

3.2 Immunization and quantitation of anti-

SRBC antibodies in *Bufo arenarum*

For the immunization protocol, an exploratory experiment employing a small number of animals was performed, based on the following steps: a) pre-immune sera: blood obtained by cardiac puncture, and sera separated by centrifugation; b) immunization protocol: the toads received 7 subcutaneous weekly injections of a 30% SRBC suspension in 0.9% NaCl physiological solution. Immune sera were separated twice from blood obtained by cardiac puncture 7 days after the 3rd and 7th injection. The reactivity of antibodies against SRBC was measured with ELISA in successively diluted sera in order to determine the end point.

3.3 Pb concentrations in the samples analyzed

Serum samples were stored at -20°C up to the moment of processing. Whole blood aliquots were digested with HNO₃ in a water bath at 70°C, following standard methods. They were then filtered through nitrocellulose filters (MSI 0.45 µm pore), carrying a final volume of 10 ml with distilled deionized Nanopure MiliQ water (Pb content <0.006 mg/l).

Lead was determined by atomic absorption spectrophotometry (Varian Spectra AA 300) (Varian, Lexington MA, USA), by direct absorption in air-acetylene flame. The calibration curve was achieved by adding Pb nitrate solution to control samples with the same matrix as experimental samples, following the APHA-AWWA-WPCF specifications (37). The detection limit was 0.1 mg/dl. The lineal regression of the calibration curve was $y = -0.006 + 0.0575x$ ($r = 0.993$; $p = 0.007$). All the reagents used were of analytical grade. Results were expressed in mg/dl of blood.

4 Statistical analysis

Comparisons of antibody titers against SRBC of Pb-injected and control toads (ANOVA and *t* test), correlation test between blood Pb concentration and antibody titers, and normality tests were carried out using the StatgraphicsPlus statistical package (Manugistics Inc., Rockville MD, USA). H₀ was rejected when $p < 0.05$.

RESULTS

Data on immune response to Pb-injected animals are shown, and were obtained employing anti-*Bufo arenarum* Ig antiserum and developing an ELISA method to this end. Firstly, the response in lead-treated and control animals was followed up, applying an ELISA method on successive sera dilution; 1/200 dilution was then selected to perform statistical analysis. The efficiency of the immunization procedure and the specificity of the anti-Ig polyclonal antibodies were high (Fig. 1). Two bands were obtained, corresponding mainly to IgM, and light Ig antibodies. The titers obtained, followed up by immunodotting and ELISA, were 1/2000 and 1/4000, respectively.

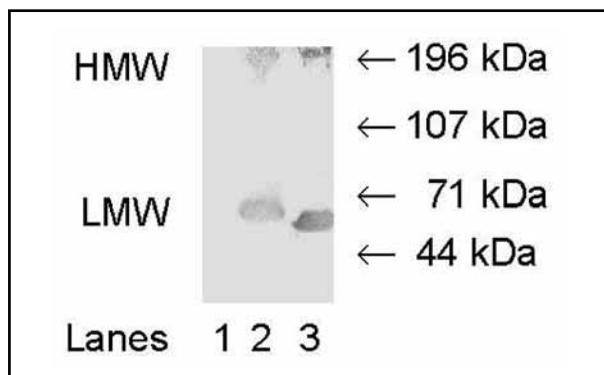


Figure 1. Specificity of antibodies obtained in rabbit to *Bufo arenarum* serum globulin fraction analyzed by immunoblotting. Lane 1: reactivity of preimmune rabbit sera (negative control). Lane 2: reactivity of *Bufo arenarum* antiglobulin antisera. Lane 3: reactivity of *Bufo marinus* antiglobulin antisera (positive control).

Table 1. Natural anti-SRBC antibody levels in adult male *Bufo arenarum*. ^a Initial time = day 1 of the experiment; final time = day 47 of the experiment.

Toads	Initial time ^a	Final time ^a
Control (C) (n=26)	0.69 ± 0.39	0.89 ± 0.49
Treated (T) (n=22)	0.54 ± 0.30 ^b	0.76 ± 0.31 ^c
T/C ratio	0.78	0.85

Data expressed as mean absorbances ± SD at 492 nm of sera diluted 1/200. Each pair of data (initial and final time) pertains to the same animal. Between brackets, number of toads. ANOVA $p < 0.03$; t test for mean comparison: b vs c: $p < 0.014$. Other comparisons were not significant.

Table 1 shows the average levels of natural anti-SRBC antibodies in *Bufo arenarum*, in both control and treated animals, at the beginning and end of the experimental period. When comparing initial and end absorbance changes, in samples of the same individual, the only significant difference was found in lead-treated toads, though significance was, in fact, only minimal. The treated/control ratios did not show changes. In comparing initial versus final absorbances for each group, a 29% increase was noted in controls and a 41% increase in Pb-injected toads.

The levels of anti-SRBC antibodies in 3 specimens of *Bufo arenarum* immunized with SRBC are shown in Table 2. In the three animals, titer increases between 30% and 90% were obtained. Table 3 shows the levels of immune anti-SRBC antibodies for *Bufo arenarum* immunized with SRBC, in both control and treated animals. Positive commercial control absorbance (anti-SRBC rabbit serum diluted 1/200) was 0.68 ± 0.23 (mean ± SD, n = 23). In this case also the treated/control ratios did not show significant changes. Table 4 shows the concentration of Pb in blood for *Bufo arenarum* after 6 weekly injections of 50 mg

Table 2. Results of an experimental approach to establish the protocol for *Bufo arenarum* immunization with SRBC. ^a Anti-SRBC antibody levels measured in duplicate in 3 adult male specimens of *Bufo arenarum*, immunized with weekly injections of 30% SRBC suspension.

Time	Toads		
	1	2	3
Initial (day 1, pre-immune control) (I)	0.96/ 0.94 ^a	0.58/ 0.65	0.79/ 0.78
Middle (7 days after the 3 rd injection)	1.18/ 1.17	0.81/ 0.84	1.05/ 1.10
Final (7 days after 7 th injection) (F)	1.26/ 1.23	1.16/ 1.17	1.25/ 1.28
F/I mean ratios	1.31	1.89	1.61

Data expressed as absorbances at 492 nm of sera diluted 1/200.

Table 3. Anti-SRBC antibody levels in adult male *Bufo arenarum* immunized with SRBC

Toads	Initial time*	Final time*
Control (C) (n=18)	0.52 ± 0.46 ^a	0.91 ± 0.50 ^b
Treated (T) (n=19)	0.42 ± 0.32 ^c	0.66 ± 0.36 ^d
T/C ratio	0.81	0.73

* Initial time = experimental day 1; final time = experimental day 47.

Data expressed as mean absorbances ± SD at 492 nm of sera diluted 1/200. Each pair of data (initial and final time) pertains to the same animal. In brackets, number of toads. ANOVA $p < 0.003$. t test for mean comparison: a vs b, b vs d, c vs d, $p < 0.05$. Other comparisons were not significant.

Table 4. Pb concentration in blood of adult male *Bufo arenarum* injected weekly with 50 mg Pb . kg⁻¹, for a period of 6 weeks.

Toads	Pb in blood (mg.dl ⁻¹)
Control (C) (n = 44)	2.2 ± 1.1
Treated (T) (n = 41)	8.2 ± 2.2
T/C ratio	3.73
p (C vs T) ^a	< 0.001

Data expressed as mean ± SD. In brackets, the number of toads. ^a t test.

Pb . kg⁻¹, compared with control toads that were given Na acetate.

The Pb concentration in blood of treated toads

increased almost four-fold. When comparing Pb level in immunized and non immunized animals, and anti-SRBC antibody titers (immune or natural) no significant correlations were found for each pair of data, in either the control or treated group ($p > 0.05$).

DISCUSSION

In amphibians, there are innate and adaptative immune responses that provide protection against foreign agents. The innate response includes antimicrobial peptides in the granular skin cells or in the gastrointestinal tract, phagocytic cells (macrophages and neutrophils), and complement system activated by both pathways. Also, they have NK cells that recognize and kill tumor cells or virus-infected cells. Valuable reviews of the amphibian immune system element have recently been published (1,2). The adaptive immune response in amphibians takes 7-14 days to develop after the detection of the antigens; it is slower and less specific than the response observed in mammals. The humoral-mediated immunity involves B-cells that in larvae and adults are differentiated mainly in spleen and secondarily in liver. There is likewise only weak recombination-activating gene (RAG) activity in bone marrow, indicating that B-cell differentiation has occurred. B-cells are not organized into distinct germinal centres, typical of ectothermic vertebrates, and may explain the lower diversity and binding affinity as compared to that found in mammals, though they do not have lymph node, but they have accumulations build up in adult gut and near the heart. These lymphoid areas in gut are B and plasma cells rich zones producing IgM, IgY, and IgX antibodies. In *Xenopus*, immunoglobulin genes organization and somatic diversification are similar to that found in mammals.

Information as to the effects of heavy metals on the immune system of amphibians is very limited (38-41), compared to the abundant amount of information regarding other species, mainly humans (19). In adult specimens of *Rana pipiens* and larvae of *Rana catesbeiana* exposed to sublethal doses of Cadmium, the immune response followed up by means of a hemoagglutinating assay showed an increase in the agglutination titers of SRBC (15,16). Pb may produce stimulation of certain immunological functions. It has been demonstrated that it can stimulate or inhibit several functions and structures of the immune system, depending on the dose and form of toxic administration.

It was observed that in *Bufo arenarum*, after weekly injections of 50 mg Pb . kg⁻¹, the production of natural antibodies increased significantly by 40%. In control toads of the experimental set, there were no significant differences between initial and final absorbances of sera during the experiment (Table 1). It is interesting to note that it has been proved that metal acts as a stimulating factor of B-lym-

phocytes in mice, producing an increase in the proliferative response of this lymphocytic subpopulation against mitogens (42,43) an increase in IgM production, and in the expression of class II histocompatibility molecules.

As mentioned previously, Pb has a stimulating effect on cellular lymphocytic populations, sometimes causing an increase in the production of antibodies. This effect, as well as other alterations produced by the metal, is highly dose-dependent. With low or mild concentrations it can be expected to stimulate the humoral immune response (44,45). With high doses instead, it can cause an inhibiting effect on the production of antibodies, possibly due to a direct toxicity of the metal on the B-lymphocytes (17).

There are several studies demonstrating an inhibition of the immune function caused by the metal. Müller et al. (46) showed, in mice chronically given Pb, that delayed hypersensitivity (DTH) against SRBC was suppressed, establishing a positive correlation between the inhibition to the primary and secondary responses to Pb concentration in blood. Waterborne Pb (acetate) produced in rats a decrease in the proliferative response to lymphocytes against mitogens, and in the DTH response (46,47). Luster et al. (21) reported that the response of antibodies against SRBC was depressed, while the proliferative capability of B-lymphocytes against LPS was not altered when rats were given different concentrations of Pb over a period of 7 weeks. In animals immunized with LPS, the IgM serum concentration increased, though not significantly. Williams et al. (48) showed that Pb bound to antibodies caused their inactivation in vitro.

In rats, a single dose of 1 mg Pb/100 g body weight increases the susceptibility to endotoxins of *Escherichia coli* (49) by a factor of 100,000. It has been demonstrated that lower Pb concentrations have a mitogenic effect on lymphocytes, stimulating their proliferation. However, at concentrations higher than 200 mg.l⁻¹ in drinking water, the metal has an immunosuppressive activity. The ATP and IP3 levels were increased in the lymphocytes from rats exposed in vitro to 50 mg.l⁻¹ Pb (acetate). This increase was independent of antigen receptor activation (50).

The number of antibody-producer spleen cells (mainly IgG) against SRBC diminished in mice exposed to different Pb (acetate) concentration in drinking water, with this response being interpreted as commitment of memory B-cells (51). In further studies, the same authors (45) showed that when the metal is administered in a single intranasal or intraperitoneal dose produced an increase in the primary immune response, and a decrease in the secondary response to the same antigen.

These facts may help to interpret the results reached on the amphibian model. Our results may contribute to a better understanding of the effects

of sublethal Pb on the immune system of amphibians. The natural antibodies are mainly IgM and can be considered as a primary response. Immune antibodies instead were quantified after a 6 weeks immunization protocol with the antigen; at the end of this period, all the toads displayed a positive response. Anti-SRBC immune antibody titers increased over time. In both groups, control (C) or treated (T) toads, the increase in antibody titers at the end of the experimental period was statistically significant, but the final titers were higher in C than in T (Table 3). In this case, the metal would have produced a lower increase in Ig production, comparable to the IgG in mammals, may be as a consequence of a failure to produce a response.

An alteration in the regulation of the secondary response to B-cells would explain the inhibition in the production of immune antibodies against SRBC, perhaps due to a deleterious action on the Th lymphocytes. Likewise, it has been suggested that Pb would alter the relation between the Th cell subpopulation Th1 and Th2, increasing the production of the cytokine Th2 pattern and diminishing the Th1 cell functions (20).

Thus it is concluded that the increase due to the assayed doses of Pb in the levels of natural antibodies cannot be explained on the basis of only one single action mechanism of the metal, but as the result of a conjunction of effects over different immunocompetent cell subpopulations. In contrast, the immune antibody levels in treated toads (Table 3), at the end of the experiments, showed a non-significant tendency, despite a significant lower increase as compared to controls. These different responses suggest that factors affecting animals exposed to a foreign stimulus are different from those influencing the response of wild animals. Finally it is worth mentioning that disturbances in amphibian populations caused by prolonged exposure to heavy metals, even at sublethal concentrations, can lead to important ecological consequences (52). In addition, such consequences can trigger a cascade of adverse secondary effects, affecting species at other trophic levels. Thus numerical decrease in amphibian populations, either larvae or adults, could mean substantial changes in other populations trophically related to them. A growing number of authors have been reporting evidences showing that numerous amphibian populations are declining worldwide (53), as well as in Latin America (54,55). Both natural and human-associated causes have been suggested to explain such declines. Obviously, no single cause can be unequivocally identified as the cause of the phenomenon, and the synergic effects of several environmental pollutants cannot be disregarded. Among those causes, the anthropogenic augmentation of heavy metals in the ecosystem compartments was clearly identified.

The adverse biological impacts of Pb are the consequence of its intrinsic toxicity as well as its long

environmental and biological half-life. It is found extensively dispersed in practically all parts of the ecosystems. Since toads have a biphasic developmental cycle, first in water and later on land, they are species particularly at risk of exposure to Pb.

In general, studies devoted to the effects of Pb on non-mammalian vertebrates are scarce (41). The scarcity of information as compared to other groups would seem to be more pronounced when referring to the impact of the metal on the amphibian immune system.

In this context, prolonged exposures to Pb may cause alterations to the immune system which, in turn, may lead to an increase in the susceptibility of animals to infections and infective factors.

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