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Original article

An enzyme-linked immunosorbent assay for measuring anti-sheep red blood cells antibodies in lead-exposed toads

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Abstract

Introduction: Immune function assays to screen immunotoxic effects of xenobiotics has recently become of major interest. In the framework of our studies, we standardized methods to quantify the humoral response of an amphibian species (*Bufo arenarum*, Amphibia, Anura) exposed to sublethal lead (as acetate). **Methods:** The levels of agglutinins to heterologous red blood cells (RBC) were measured in serum from adult *B. arenarum*. Since agglutinin titers were very low, a noncompetitive enzyme-linked immunosorbent assay (ELISA) method was carried out. As toad serum showed marked nonspecific binding, we developed a new ELISA on microtiter plates for the quantitative determination of the heterophile antibodies. The method was based on that described by Hirvonen et al. [Vox Sang. 69 (1995) 341], employing sheep red blood cells (SRBC) sensitized with amphibian antibodies that were transferred to microplates; later the measurement of bound immunoglobulins was performed. Different variables such as the amount of antigen, blocking agents, and other experimental conditions (fixing solution and commercial plates) were studied. Toads ($n = 22$) received a weekly subcutaneous injection of 50 mg/kg lead (acetate) for 6 weeks, and the control ones ($n = 26$) were injected with Na acetate at the same time. **Results:** The anti-sheep RBC antibodies titers of adult toads were obtained with the improved ELISA method, being the absorbance range 0.12 to 1.58 AU (1/200 diluted serum). Titers from lead-exposed toads were also determined, being the final titers (expressed as $\bar{x} \pm$ S.E.M.) higher (0.79 ± 0.06 AU), than those of Day 0 (0.57 ± 0.06) ($P < .01$). **Discussion:** It was concluded that the ELISA technique we developed was useful for measuring the humoral immune response in this animal model and that in these preliminary studies, lead showed an immunostimulating action on the humoral immune system.
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Keywords: Amphibians; Antibodies; *Bufo arenarum*; ELISA; Hemagglutinins; Immunotoxicology; Lead (acetate); Methods

1. Introduction

Immunotoxicology has become of major interest in the last decade (Wester, Vethaak, & Muiswinkel, 1994). The development of a battery of immune function assays to screen potential immunotoxic compounds has been a major issue in the past years. Recently developed methods for toxicological assessment have implicated the immune system as a target after exposure to some xenobiotics and drugs. There are various immunological assays available for evaluating the effects of environmental stress (Anderson, 1990). The determination of circulating immunoglobulin levels in serum is a useful functional test of an immunological pathway in vivo. It can be measured in *naive* animals (total immunoglobulin, Ig) or after exposure to a specific antigen. The immune humoral response to red blood cells (RBC) can be monitored by agglutination tests (Wester et al., 1994). Nevertheless, enzyme-linked immunosorbent assays (ELISA), which can be considered sensitive and specific tests, were also applied for studying humoral immune response in different vertebrates (Arkoosh & Kaatari, 1990; Krzystyniak, Fournier, Trottier, Nadeau, & Chevalier, 1987). In order to be validated, these methods have to be standardized and the reference interval values for the species under study should be known.

The ectothermic vertebrates produce antibodies similar in structure and diversity to those of mammals (Schluter,

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Bernstein, & Marchalonis, 1997; Zapata, Varas, & Torroba, 1992). Amphibians are capable to form antibodies against several antigens, giving an anaphylactic response and rejecting grafts (Du Pasquier, 2001; Du Pasquier, Schwager, & Flajnik, 1989; Horton, 1994). In some earlier pioneer works on the detection of agglutinating or hemolytic activities, in the anurans *Rana esculenta* and *Calyptocephalus gayii*, an activity against several antigens (animal erythrocytes and bacteria) was found (Wollman, 1938). A natural heterohemoagglutinin was described in the serum of the toad *Bufo regularis*. This agglutinin for human erythrocytes appears to have anti (B + H) specificity (Balding & Gold, 1976). Jurd (1978) showed that adult *Xenopus* serum contain a natural factor capable of lysing and agglutinating RBC from many species. In addition, Fernández (1986) found mild and low levels of hemolytic and agglutinating activity against mouse RBC in serum of different species of native anurans of Argentina.

Recently, new information suggested that the immune system is a target for low doses of lead (McCabe, 1998), and there is evidence that environmental metal levels affect the immune function. Studies were carried out both in vivo and in vitro on rats, mice, rabbits, and fishes, as well as in human beings, allowing documentation of the effect of lead on humoral and cellular immunity (Queiroz et al., 1994). It has been postulated that lead toxicity may be due, at least in part, to an autoimmune response. These autoimmunity and hypersensitivity processes may be produced by an immune response deregulation.

In the present study, firstly, the antibody levels against RBC from different species in serum of *B. arenarum* were assessed, quantifying them through the study of their reactivity as natural heteroagglutinins. Secondly, due to the low titers obtained, a method of higher sensitivity and more appropriate for our aim had to be designed. With this aim, an ELISA method was studied, assessing different variables such as the amount of antigen (sheep red blood cells, SRBC), blocking agents, and other experimental conditions (fixing solution and different commercial plates). Finally, once the appropriate ELISA method was selected and standardized, serum antibody titers to SRBC in lead exposed *B. arenarum* individuals were obtained and were compared with those of the control toads.

2. Methods

2.1. Animals

Adult *B. arenarum* male specimens (average weight 120 g) were collected in the neighbourhood of La Plata, Argentina. Blood samples were obtained by heart puncture under general anesthesia achieved by immersion for 10–15 min in 1:1000 solutions of MS222 (ethyl *m*-aminobenzoate). Exuded serum was immediately centrifuged, aliquoted, and stored at −20 °C, until used.

2.2. Hemagglutinating assays

Hemagglutinating activity was determined against washed RBC from rat, sheep, chicken, and human A1, B, and O. Experiments were performed in U-microtiter plates (Nunc, Denmark) using 50 μl of serial 2-fold dilutions of serum, 50 μl of 0.6% NaCl solution, 50 μl of 1% bovine serum albumin (BSA), and 50 μl of 1% RBC suspensions in saline solution. Readings were taken by eye after 2 h incubation at room temperature and scores were assigned following those described by Dunsford and Bowley (1967).

2.3. Preparation of polyclonal antibodies to B. arenarum globulin

The preparation of globulin fraction was done according to Dunsford and Bowley (1967). Antibodies against globulin fraction were prepared in New Zealand white rabbits, after obtaining preimmune samples. Equal volumes of toad globulin fraction in complete Freund's adjuvant (Gibco Invitrogen, Carlsbad, CA, USA) were emulsified and injected subcutaneously. After 20 days, a second inoculation was done. Seven days later, a first bleed was obtained from the marginal ear vein. Twenty days later, an intramuscular inoculation was performed using an emulsion with Freund's incomplete adjuvant (Gibco Invitrogen). A total of 10 boosters were given at 20-day intervals, and bleeds were obtained to allow monitoring of antibody production. The antiserum titer was followed up by titration with immunodotting, according to Hawkes, Niday, and Gordon (1982), and ELISA was performed according to Elola and Fink (1996).

For characterization of rabbit antibodies to *B. arenarum* globulins, samples of normal toad serum were denatured by heating at 100 °C in SDS for 2 min. The samples (containing 30–40 μg protein) were run on 7.5% polyacrilamide–SDS gels. A normal human serum sample was run as a control. Ovoalbumin (44.23 kDa), BSA (71 kDa), phosphorylase B (105.7 kDa), and myosin (H-chain 196 kDa), were run as molecular weight standards (BRL, Gaithersburg, MD, USA). Some gels were stained with 0.125% Coomassie Brilliant Blue R-250. Others were 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 low-fat powdered skim milk 5% in LEADS–0.5% Tween 20, for 1 h at room temperature. They were incubated with the rabbit anti-*B. arenarum* globulin antiserum. Also, other transferred membranes were incubated with anti-*B. marinus* immunoglobulin (Whittington & Speare, 1996) as primary antibodies. The anti-*B. marinus* globulin was included in order to study the specificity of our antiserum by comparing it with other phylogenetically close species. As a methodological control, human serum anti-IgG against a homologous serum was employed. As secondary antibody, a goat anti-rabbit IgG antiserum conjugated with peroxidase was used. All sera were prepared in LEADS–Tween–low fat powdered

Table 1
Agglutination titers of *B. arenarum* serum against RBC from different species[a]

Toad no.	RBC[b]					
	Human			Other species		
	A1	B	O	Sheep	Rat	Chicken
1	0	0	2	1	4	2
2	0	0	2	1	6	2
3	3	3	2	3	3	6
4	1	1	2	0	1	1
5	2	2	2	2	8	2
6	5	4	4	0	5	1
7	5	4	2	2	1	2
8	3	4	2	2	2	2
9	5	5	6	1	7	6
$\bar{x}\pm$S.D.	2.67± 2.06	2.56± 1.88	2.67± 1.41	1.33± 1.00	4.11± 2.57	2.67± 1.94

[a] Titer (T) was defined as the reverse of the last dilution in which agglutinating activity was observed and is expressed as Lg2 T. Data were obtained by duplicate.
[b] 1% RBC suspension in saline medium.

skim milk. The colour reaction was developed in the presence of 4-Cl-1-naphtol dissolved in methanol with H_2O_2 in Tris–saline buffer. Membranes were washed with distilled water in order to stop the reaction and were dried.

2.4. ELISA methods assayed for the study of B. arenarum humoral response

2.4.1. Method A

ELISA plates (Dynatech, Chantilly, USA) were sensitized with suspensions of $10-80 \times 10^6$ SRBC, in 100 μl of LEADS/well, incubating for 1 h at 4 °C. The supernatant was discarded and the bound antigen (SRBC) was fixed with 150 μl/well of 0.3% glutaraldehyde in LEADS for 30 min at 4 °C. Microplates were washed with LEADS and blocked under shaking with 200 μl/well of 2% BSA in LEADS (LEADS–BSA)–0.02% Na-azide, for 2 h at room temperature. The blocking solution was discarded and incubated with 100 μl/well of *B. arenarum* serum in dilutions of 1/100 up to 1/3200 in LEADS–BSA–0.02% azide, for 1 h at room temperature under mild shaking. After three washes with LEADS, 100 μl/well of rabbit anti-*Bufo* serum globulin diluted at 1/4000 in LEADS–BSA was incubated for 1 h at room temperature under shaking. After three washes with LEADS, 100 μl of goat anti-rabbit serum globulin conjugated with peroxidase and diluted 1/2000 in LEADS–BSA/well was added. It was incubated and shaken for 1 h at room temperature and washed five times with LEADS. 100 μl/well of 2 mg/ml *o*-phenylendiamine (in phosphate–citrate buffer with 30% H_2O_2, pH 5) was added. The reaction was stopped with H_2SO_4 4 N and the absorbance was read at 492 nm in an ELISA reader (Molecular Devices, Sunnyvale, CA, USA). Controls with rabbit preimmune serum were included in the analysis.

To optimise this protocol, the following changes were made for comparison: (a) fixing solution to 0.3% glutaraldehyde in LEADS–0.05% Tween 20 and 2% formaldehyde in LEADS–0.05% Tween 20; (b) blocking solution to 3% powdered skim milk, 1.5% gelatin, and 2% BSA in LEADS solutions; and (c) the commercial ELISA microplates to Kima (Piove di Sacco, Italy) and Dynatech (Chantilly, VA, USA) plates.

2.4.2. Method B

In order to minimize the nonspecific binding of *B. arenarum* serum observed with Method A, the technique of Hirvonen et al. (1995) was modified. SRBC were sensitized with toad serum in low ionic strength solution (LISS). The complex SRBC-toad serum was transferred to an ELISA plate, fixed with glutaraldehyde and then rabbit anti-*Bufo* serum globulin and goat anti-rabbit serum globulin (conjugated with HRP) were added. Finally, the absorbance was measured adding the substrate (*o*-phenylendiamine) and H_2O_2.

Some variations of the original method such as SRBC-serum incubation time and temperature were checked. In every plate run, the assay included titration of an anti-rabbit SRBC serum (positive control). Each sample was also tested without SRBC, anti-*B. arenarum* antiserum or conjugate-HRP and with preimmune serum.

The optimal conditions for this method were finally established as follows: in plates with U-bottom, 100 μl/well of an SRBC suspension at 20×10^6 in LISS (30 mM NaCl, 3 mM phosphate, 0.24 M glycine, 0.02% Na-azide, 1% BSA) were incubated, together with 100 μl of *Bufo* serum diluted 1/200 in LISS. After 30 min, the sensitized SRBC suspension was washed, then the RBC were resuspended in 250 μl of 0.2% BSA–saline and 100 μl was placed in each well in an ELISA plate, fixed with glutaraldehyde 0.3% and blocked with 2% BSA–saline. It was incubated for 1 h with serum anti-*Bufo* globulin in rabbit (1/4000, 100 μl/well). It was 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 the absorbance was read at 492 nm.

2.5. Lead administration

Two solutions, one containing lead acetate and the other containing sodium acetate, were prepared in distilled water.

Table 2
Immunodot and ELISA titers of the rabbit preimmune and anti-*B. arenarum* globulin antiserum

Methods	Preimmune serum		Immune serum	
	Dilution assay	Results	Dilution assay	Results
Immunodot (4)	1/20	negative	1/20 up to 1/4000	1/4000
ELISA (3)	1/20	negative	1/20 up to 1/16,000	1/4000

In brackets, number of experiments done with a pool serum from six toads. ELISA was carried out in duplicate.

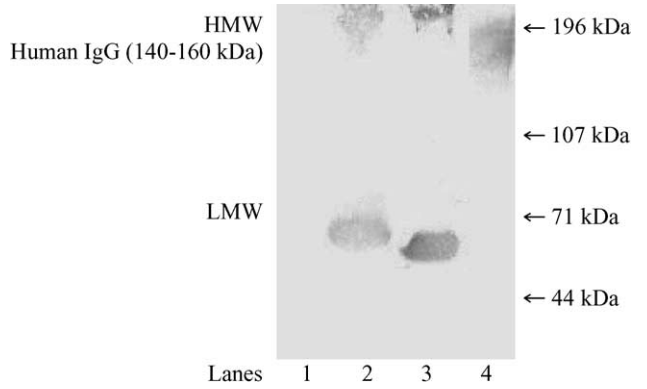

Fig. 1. Western blot of *B. arenarum* serum proteins. Lane 1: *B. arenarum* serum proteins against rabbit preimmune serum. Lane 2: *B. arenarum* serum proteins against rabbit anti-*B. arenarum* globulin serum. Lane 3: *B. arenarum* serum proteins against rabbit anti-*B. marinus* immunoglobulin serum. Lane 4: Human serum proteins against anti-human IgG serum, as control method. On the right side of the figure, molecular weights of standard proteins are shown. HMW and LMW: high and low molecular weight *B. arenarum* serum proteins, respectively.

Toads ($n=22$) received a weekly injection at a dose of 50 mg/kg lead for 6 weeks. Matched controls ($n=26$) received Na acetate. The injections were performed in the dorsal lymph sac, at a volume of 1 ml/160 g body weight. The dose of lead used has previously been determined to be sublethal at 20 °C in our laboratory (Arrieta, Rosenberg, Fink, & Salibián, 1999).

All reagents employed were analytical grade and were obtained from Sigma (Saint Louis, MO, USA), if otherwise not indicated.

2.6. Statistical analysis

Normality testing and parametric and nonparametric statistical tests were carried out using the StatgraphicsPlus statistical package (Manugistics, Rockville, MD, USA). H_0 was rejected when $P<.05$.

3. Results

3.1. Hemagglutinating assays

Agglutinating titers in toad serum against RBC from different species are shown in Table 1. In general, low titers were obtained against the RBC of all the species tested. The highest value was obtained against rat RBC. This low level of reactivity led us to standardize the more sensitive method (ELISA).

3.2. Characterization of rabbit antibodies to B. arenarum globulins

The immunodotting and ELISA data for rabbit anti-*Bufo* antiserum is shown in Table 2. The optimal dilution was 1/4000.

In Fig. 1, the reactivity of antiserum against *B. arenarum* and human serum are shown. The reactivity of anti-*B. arenarum* antiserum was similar to that of an anti-*B. marinus* antiserum (Whittington & Speare, 1996), which reacted with two fractions, one of low and the other of high molecular weight. Whittington and Speare (1996) found similar results with their rabbit anti-*B. marinus* immunoglobulin serum. As they suggested, the two identified products could be a high molecular weight immunoglobulin and a low molecular weight one with similar specificity, or polymers and monomers of immunoglobulins.

3.3. ELISA methods for the study of B. arenarum humoral responses

3.3.1. Method A

We first established the optimal amount of SRBC per well. Fig. 2 shows the curves in relation to the SRBC amount. The higher values were obtained with 20×10^6 SRBC/well.

The results obtained for other methodological variables assayed are presented in Table 3. In the experiments where

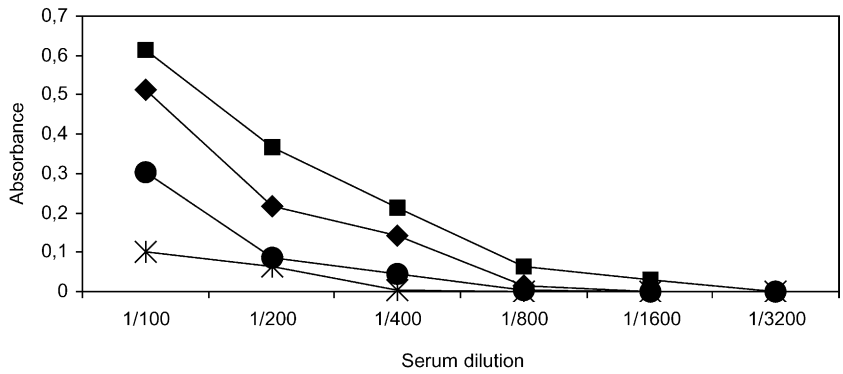

Fig. 2. Determination of the optimum concentration of SRBC antigen required for coating microtiter plates. Dilution of a pooled serum sample was used to sensitize SRBC (10×10^6 ◆–◆, 20×10^6 ■–■, 40×10^6 * –*, and 80×10^6 ●–●) and final absorbance at 492 nm was plotted. The final data were obtained from the differences between the sample Abs minus the blank Abs (corresponding to a nonantigen well).

Table 3
Comparison of the different variables in the ELISA standardization for anti-SRBC toad antibody titration (Method A)

Variable	Abs at 492 nm ($\bar{x}$ ± S.D.)	Statistical significance
Fixing solution (n = 4)		
2% Formaldehyde	0.69 ± 0.19	
0.3% Glutaraldehyde	0.78 ± 0.12	NS[a]
Blocking solution (n = 3)		
3% Powdered skim milk	0.37 ± 0.09	
1.5% Gelatin	0.35 ± 0.22	
2% BSA	0.81 ± 0.13	$P < .001$[b]
Commercial plates (n = 4)		
Kima	0.51 ± 0.03	
Dynatech	0.57 ± 0.09	NS[a]

In brackets, the number of serum used. Sera were diluted at 1/200 and assayed in duplicate.
[a] *t* test.
[b] ANOVA.

glutaraldehyde was used as fixing agent, the values were higher than those obtained with formaldehyde. Although nonstatistically different ($P > .05$), we chose glutaraldehyde because it was recommended by Johnstone and Thorpe (1982). From the tabulated data, it can be deduced that the best blocking agent was 2% BSA. There was no difference between the two commercial plates tested, so they were used indiscriminately.

3.3.2. Method B

Nonspecific binding and other control data run in the experiments are described in Table 4. The background (well diluent alone) was 0.05 so we considered 0.075 (1.5 times the background value) as the lower limit of the assay. The need to transfer the sensitized SRBC into a new microtiter plate was noted; this transfer step reduced the nonspecific binding of proteins to plastic. The decrease in the nonspecific binding was marked for Method B, being more than 50% ($P < .05$). Also, a small but significant difference ($P < .01$) was obtained between Methods B and A when no toad serum was added (Table 4).

Table 4
Nonspecific binding and other controls for anti-SRBC toad antibody titration by two ELISA methods

Variable of control	Abs at 492 nm ($\bar{x}$ ± S.D.)		Statistical significance[a]
	Method A[b]	Method B[b]	
No SRBC (11)	0.53 ± 0.12	0.25 ± 0.12	$P < .05$
No *Bufo* serum (11)	0.12 ± 0.01	0.17 ± 0.02	$P < .001$
No conjugate-HRP (3)	0.05 ± 0.01	0.05 ± 0.01	NS
With preimmune serum (11)	0.06 ± 0.01	0.05 ± 0.01	NS

In brackets, the number of experiments. Sera were diluted at 1/200 and duplicate assayed.
[a] *t* test.
[b] For description, see Methods section.

Table 5
Antibody titers against SRBC and blood lead levels in control and lead-exposed toads serum

	Lead concentration (mg/dl)	Antibody titer * (absorbance at 492 nm)
Control toads (n = 26)		
Day 0	3.33 ± 0.08	0.70 ± 0.08[a]
After 6 weeks	1.68 ± 0.11	0.89 ± 0.10[b]
Lead-exposed toads (n = 22)		
Day 0	2.96 ± 0.06	0.57 ± 0.06[c]
After 6 weeks	7.18 ± 0.30	0.79 ± 0.06[d]

Data expressed as $\bar{x}$ ± S.E.M.; a versus b, a versus c, and b versus d, nonsignificant; c versus d, $P < .05$ (Mann–Whitney test).
* Serum diluted 1/200 and measured at the beginning and at the end of the experimental time.

The screening dilution of serum was selected after the evaluation of several curves corresponding to single individuals. A 1/200 dilution was chosen as appropriate for the screening considering it would give readings at approximately the middle of the absorbance scale. Under this condition, the absorbance range was between 0.12 and 1.58 AU. The standard deviation of the differences between replicates mean for each sample was 0.0015 ($CV_{diff\ mean} = 3\%$). The adult reference value at a 1/200 serum dilution expressed as $\bar{x}$ ± S.E.M. ($n = 23$) was 0.78 ± 0.08 AU. The mean levels of natural anti-SRBC were low but the range was large.

In Table 5, antibody titers and blood levels in control and lead-exposed toads are shown. The production of natural antibodies increased significantly by 39%. This significant increase was observed when serum from toads exposed to lead at the beginning and at the end of the experiment was compared; it is interesting that, at the same time, the blood lead concentration results increased by 142%. In nonexposed control animals, there was a nonsignificant lower titer increase (29%), and the blood lead concentration decreased by 49%. As animals were sampled from the field, the blood levels of noninjected control toads may be attributed to a complex process of environmental pollution. Although there is a decrease after keeping them for 2 weeks in metal-free medium, the clearance of the metal is not completed.

4. Discussion

With the objective to extend our previous immunotoxicological studies in an animal model (Rosenberg, Arrieta, Fink, & Salibián, 2000), several studies were conducted for monitoring humoral responses in *B. arenarum*.

Firstly, we have detected natural antibodies as hemoagglutinins to RBC of foreign species in normal adult amphi-

bian serum. For the toads under study, natural agglutinins to human and some animal RBC at low or negative titers have been detected previously but with fewer positive samples than found in the present study (Caferra, Micucci, & Palatnik, 1975; Palatnik & Caferra, 1971). In agreement with Fernández (1986), we obtained a positive reactivity with rat RBC.

Secondly, SRBC were selected as the antigen for the ELISA standardization in order to develop a more sensitive assay, taking into consideration that SRBC are extensively used as the standard antigen to monitor the humoral immune response measured by agglutination tests (Wester et al., 1994).

To our knowledge, this is the first ELISA method standardized to quantify the antibodies to xenogenic RBC in an anuran species. Studies previously made in *Xenopus* employed agglutinations assays (Jurd, 1978). Also, in studies on adult *R. pipiens* and larval *R. catesbeiana* intoxicated by prolonged low-level cadmium exposure, the response of the tadpole immune system was followed up by means of a hemagglutination assay (Zettergren et al., 1991). In other lower vertebrates such as fish, an ELISA method has been described for the titration of serum antibody to a specific antigen such as trinitrophenyl (Arkoosch & Kaattari, 1990); this technique was described as an extremely sensitive tool for the assessment of the immunocompetence of small animals where the volume of serum from each individual may be very small. In another fish, the Atlantic salmon *Salmo salar*, the humoral immune response was monitored using an ELISA against a soluble artificial antigen, a vitamin C substitute (Sandnes, Hansen, Killie, & Waagbo, 1990). In amphibia, Whittington and Speare (1996) described a sensitive ELISA for the detection of antibodies in the toad *B. marinus*, using antiserum against amphibian immunoglobulin, which allowed the study of the humoral response. Later an adaptation of that method was done for the detection of antibodies against iridoviruses in serum of the same toad (Whittington, Kearns, & Speare, 1997). Hirvonen et al. (1995) developed a method for the quantitative determination of human anti-D in a preparation of immunoglobulins and plasma donor samples or for diagnostic purposes in maternal immunization during pregnancy. It was based in previous work where an enzyme linked antiglobulin test in microplates (Leikola & Perkins, 1980) or in tubes (Riley, Ness, Taddie, Barrasso, & Baldwin, 1982) was standardized. Using the ELISA proposed by Hirvonen et al., and after some modification of the original method, we were able to diminish the nonspecific binding showed by *Bufo* serum and to determine sensitively the levels of a heterophile antibody as anti-SRBC in a normal adult population.

It is worth mentioning that it has been demonstrated that lead acts as a stimulating factor for B-lymphocyte in mice, producing an increase in the proliferative response to mitogens (Shenker, Matarazzo, Hirsch, & Gray, 1977), an increase in the production of IgM, and in the expression of class II histocompatibility molecules. The density increase of these molecules on the B-lymphocyte surface was the same of that obtained with other common activators of this lymphocyte subset. An increase in the expression of these molecules facilitates the interaction between B-cells and T-helpers, implying that this stimulating activity of lead will produce a better B−T interaction, and hence, an increased differentiation of B-cells (McCabe & Lawrence, 1990). This may also help interpretation of the possible mechanisms involved in the effect of lead on the humoral response in toads. This interpretation is difficult, since there is not much information related to the effects of heavy metals on the amphibian immune system (Devillers & Exbrayat, 1992; Schuytema & Nebeker, 1966; Zelikoff & Thomas, 1998; Sparling et al., 2000).

It is well known that agents increasing intracellular concentration of calcium or activating protein kinase C, such as calcium ionophores or phorbol esters, respectively, also produce an increase in the expression of surface class-II molecules. Coincidently, it has been demonstrated that lead modulates intracellular calcium concentrations and calcium-mediated processes in several cellular types, and that it also binds to and activates protein kinase C more efficiently than calcium (Belloni-Olivi, Annadata, Goldstein, & Bressler, 1996; Markovac & Goldstein, 1988; McCabe & Lawrence 1990; Simons, 1986). These actions may explain lead's ability to stimulate certain cell types such as lymphocytes.

Several related studies have been carried out in vitro. Lawrence (1980) found that lead not only produced an increase in human B-lymphocyte proliferation, but also that it could exceed the effects of immune suppression caused by cyclic AMP. Since blastogenic changes depend upon extracellular concentrations of several ions (among them calcium), it can be anticipated that divalent lead, which can function in a similar way to calcium (or substitute for it) may have a stimulating effect on B-lymphocytes (Bendich, Belisle, & Starusser, 1981). In mouse splenic cell cultures, differentiation of B-cells and production of IgM induced by LPS increased after the addition of lead solutions to the cultures. It was suggested that this stimulation was originated, at least in part, by a direct action of lead on B-cells (Lawrence, 1981a, 1981b, 1981c). In human lymphocytes exposed to lead, an increase in the production of immunoglobulin was observed (Borella & Giardino, 1991), and the B-lymphocyte set was stimulated, resulting in an increment of the proliferation index against LPS. T-lymphocytes and their function were also affected by lead. Bendich et al. (1981) showed in rats that the intake of lead acetate mixed with food produced an increase in the response of T-lymphocytes to concanavalin-A. Based on the above, it can be said that lead has several and varied immunostimulating actions, either directly on B-lymphocytes or indirectly through cytokine release from other stimulated cells.

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