Mutagenesis vol.17 no.1 pp.55–61, 2002

Genotoxicity induced in CD-1 mice by inhaled lead: differential organ response

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Lead is perhaps the longest used and best recognized toxic environmental chemical and it is still being used recklessly. Lead (Pb) has been found to be capable of eliciting a positive response in an extraordinarily wide range of biological and biochemical tests; among them tests for enzyme inhibition, fidelity of DNA synthesis, mutation, chromosomal aberrations, cancer and birth defects. Since inhalation is one of the most important routes of environmental Pb exposure, in the present study a lead inhalation model in mice was implemented in order to detect the induction of genotoxic damage as single-strand breaks and alkali-labile sites in several mouse organs (nasal epithelial cells, lung, whole blood, liver, kidney, bone marrow, brain and testes), assessed by single cell gel electrophoresis (SCGE) or Comet assay. We found differences among the organs studied after a single and subsequent inhalations: in the organs analyzed we observed a positive induction of DNA damage after a single inhalation only in the liver and the lung. In subsequent inhalations the response was positive in all organs except the testicle, however, DNA damage induction over time was different for each organ. A correlation between length of exposure, DNA damage and metal tissue concentration was observed for lung, liver and kidney. Differences in DNA damage occurred in organs when lead acetate was administered acutely or sub-chronically. These results show that lead acetate inhalation induces systemic DNA damage but that some organs are special targets of this metal, such as lung and liver, depending in part on length of exposure, suggesting alternative organ processes to handle lead intoxication.

Introduction

Lead (Pb) is an abundant, ubiquitous, dangerous and important chemical (Mahaffey, 1990). It was used in ancient times and some of its toxic effects have been recognized for several centuries (Landrigan, 1990). Even though many of the toxic effects of lead were known at that time, lead was used as a gasoline additive in the 1920s (Rosner and Markowitz, 1985). Serious atmospheric lead pollution over the last 30 years was the result, and now damage has been identified even at very low lead exposure levels. Lead is among the top 10 US EPA priority pollutants (EPA, 1986a,b).

Lead (Pb) occurs in a variety of organic and inorganic compounds with a multitude of additional uses in plasters and ointments, the manufacture of colorless glass, protective paint for iron and steel, coloring rubber, matches, pigments in printing inks and paints, explosives, textile printing, process engraving, chemical reagents, as a mordant in dyeing, oxidizer, photographic sensitizer, rodenticide, for precious metals recovery from cyanide solutions and as a chemical intermediate in batteries (Wade *et al*., 1993). Specifically, lead acetate is used in dyeing and printing cotton, in varnishes, chrome pigments, the manufacture of pesticides, antifouling paints, analytical reagents, hair dyes and as an astringent and water repellent (Johonson, 1998).

Organic lead compounds include tetramethyl and tetraethyl lead, which were heavily used as a gasoline additive, but this use has been drastically reduced in the US beginning in the late 1970s (Wade *et al*., 1993). In Mexico tetraethyl and tetramethyl lead were added to gasoline until 5 years ago. Although population exposure to lead in this way has declined; chronic toxicity remains a major public health problem both in Mexico (Calderón-Salinas, 1996; Calderón-Salinas *et al*., 1996) and world wide, affecting millions of children and adults (Todd *et al*., 1996; Bogden *et al*., 1997).

There have been several articles concerning the genotoxic effects of this metal. Lead is considered a weak mutagen: some studies have shown that this compound is capable of inducing gene and chromosomal mutations, but not all studies have been positive (see revisions of Gebhart, 1984; Hansen and Stern, 1984; Kasprzak *et al.*, 1985; Johnson, 1998). A number of animal studies have demonstrated that exposure to lead salts is capable of inducing cancer (Azar *et al*., 1973; Kobayashi and Okamoto, 1974; Poirier *et al*., 1984; Koller *et al*., 1986; Mikalsen, 1990; Waalkes *et al*., 1995). Thus lead acetate and lead phosphate are listed as probable human carcinogens on the basis of rodent tests in the 7th annual report on carcinogens (IARC, 1987). However, it is clear that the carcinogenic potential of Pb compounds has never been studied by inhalation exposure. The inhalation route has been poorly evaluated, although for lead and for many other atmospheric pollutants inhalation is an important route because it is the main way of entry for several organisms.

Since inhalation is one of the main routes of environmental Pb exposure, the aim of this work was to evaluate possible genotoxic effects using the alkaline single cell gel electrophoresis (SCGE) assay in several CD-1 mouse structures (nasal epithelial cells, lung, whole blood, liver, kidney, bone marrow, brain and testis) after inhalation of 0.0068 μg/cc lead acetate. The SCGE assay is a sensitive procedure to quantify DNA damage [primarily single-strand breaks (SSB) and alkali-labile sites] in mammalian cells *in vitro* and *in vivo* (Singh *et al*., 1988; Sasaki *et al*., 1997). One of the advantages of the simultaneous assessment of DNA damage in many organs from the same animal is, as reported here, the comparison of their responses under identical conditions at the same time and in the same physiological state (Valverde *et al*., 2000).

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Materials and methods

Chemical reagents

Normal agarose, low melting point (LMP) agarose, ethidium bromide, Tris, Na_2EDTA, dimethyl sulfoxide (DMSO), Triton X-100 and RPMI-1640 medium were obtained from Sigma Chemical Co. (St Louis, MO). NaOH, lead acetate, absolute ethanol and NaCl were from Baker.

Animals

CD-1 male mice (from the Medical School Vivarium, UNAM, Mexico), 45 days old and weighing 30–35 g, were housed in hanging plastic cages under controlled lighting conditions (12 h light/12 h dark regime), relative humidity (50 ± 5%) and temperature (17 ± 2°C) and fed with Purina rat chow and water *ad libitum*.

Groups

Twenty-four males were allotted randomly to four experimental groups of six animals each (four exposed and two controls): group 1, single inhalation (acute treatment); group 2, 2 weeks of exposure, three inhalations; group 3, 3 weeks of exposure, five inhalations; group 4, 4 weeks of treatment, seven inhalations.

Exposures

Inhalations were performed in an acrylic box (50×30×20.98 cm) connected to an ultra-nebulizer (Ultra-Neb 99 DeVilbis) with a flux of 10 l/min, which produces particles of <1 µm. The exposure protocol was according to Fortoul *et al*. (1999). Briefly, 16 animals, four in each group, were placed in an acrylic box and lead acetate (0.0068 µg/cc) inhalations were performed for 60 min, twice a week (Monday and Wednesday). The control animals (eight) inhaled deionized water for the same period of time. After each inhalation the animals were returned to their cages.

Sampling

Groups of six animals (four exposed and two controls) were killed by cervical dislocation every week, 24 h after the last inhalation. Immediately, whole blood was obtained by intra-cardiac puncture with a preheparinized syringe and the brain, lung, nasal septum, liver, kidney, femur and testicles were removed. The organs were placed in cold saline solution (0.9% NaCl) until cell suspensions were prepared.

Cell suspensions

All organs, other than the nasal septum and femur, were washed twice with cold saline solution and placed in cold RPMI-1640 medium and minced with the help of cold scissors into 1 mm^3 pieces. Then the cell suspensions were kept at 4°C until a sediment was observed. The nasal septum was placed in 1 ml of cold saline solution, agitated in the solution with tweezers and then discarded. For bone marrow cells, 1 ml of cold saline solution was used to gently wash the femur medullar cavity to detach the cells and resuspend them. Viability of the cell suspension was determined using the trypan blue exclusion technique.

Single cell gel electrophoresis (SCGE) assay

An appropriate number of cells was obtained in 30 µl of cell suspension and mixed with 75 µl of 0.5% LMP agarose. The cells and LMP agarose at 0.36% final concentration were loaded onto microscope slides prelayered with 200 µl of 0.5% normal melting point agarose. The SCGE assay was performed as described by Tice *et al*. (1992). Briefly, after lysis at 4°C for at least 1 h [2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, supplemented with 10% DMSO and 1% Triton X-100), slides were placed in a horizontal electrophoresis chamber with running buffer solution (300 mM NaOH, 1 mM Na_2EDTA, pH >13). The slides remained in the electrophoresis buffer for 10 min to allow the DNA to unwind. Electrophoresis was performed for 10 min at 300 mA and 25 V, ~0.8 V/cm, and all technical steps were conducted using very dim indirect light. After electrophoresis, the slides were gently removed and rinsed with neutralization buffer (0.4 M Tris, pH 7.5) at room temperature for 15 min. The slides were dehydrated with absolute ethanol (15 min), after which they were air dried. Ethidium bromide (75 µl of a 20 µg/ml solution) was added to each slide and a coverslip was placed on the gel. Individual cells were visualized at 20× magnification under an Olympus BX-60 microscope with fluorescence attachments (515–560 nm excitation filter, 590 nm barrier filter) and the extent of migration (tail length value) was measured with a scaled eyepiece. To identify the tail the head of the comet was defined as the most brilliant circular region in the image. To evaluate DNA migration 100 cells/tissue/animal were scored for each condition.

Measurement of lead

As an exposure marker, lead concentration was measured in three different organs (lung, liver and kidney) after each treatment by atomic absorption spectrophotometry. Lead analyses were performed using a Perkin-Elmer 2380 atomic absorption spectrophotometer. Tissue samples of lung, kidney and liver were placed in acid-washed glass test tubes and solubilized with a mixture of nitric and perchloric acids for at least 5 h on a hot plate. Lead was quantified by the graphite furnace method. As an internal quality control, we analyzed blind random reference samples (SRM 1577b bovine liver) obtained from the National Institute of Standards and Technology. For lead recoveries were 92%.

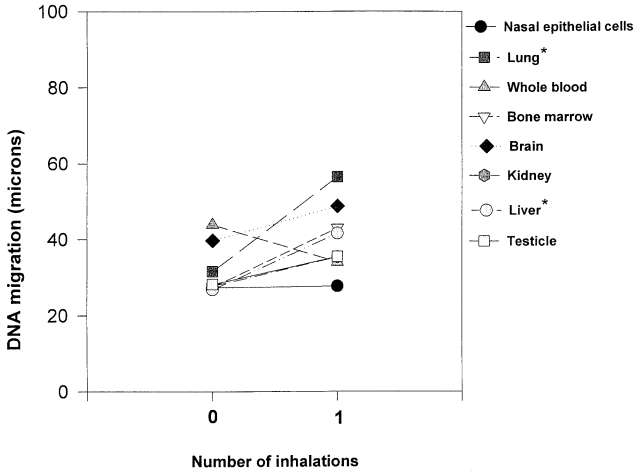

Fig. 1. Induction of DNA migration in several mouse organs after a single exposure. *Statistically significant as determined by the Mann–Whitney *U*-test ($P < 0.05$).

Statistical analysis

All the statistical analyses were performed with GraphPAD InStat software v.1.14. The Mann–Whitney *U*-test, which considers medians for analysis, was used to determine statistical differences between groups of animals for each organ. The unit of exposure and analysis used for statistical purposes was the animal, not the cells. Significance was considered at $P < 0.05$ (Kirk, 1999).

Results

Viability using classical trypan blue exclusion staining of the cell suspensions was >80% in all organs immediately after killing. DNA damage as SSB and alkali labile sites by lead acetate inhalation was analyzed in several mouse organs with respect to time of exposure.

Organs displayed different sensitivities after a single exposure. The distribution of damage from high to low susceptibility was: lung > bone marrow > liver >brain > kidney > testicle > nasal cells > leukocytes (Figure 1). However, only the lung and the liver had statistically significant differences with respect to their corresponding controls, probably due to the different cell populations within each organ.

An analysis of genotoxicity throughout the time of exposure showed a weak effect in the majority of the organs studied.

Testicle

This organ did not show statistically significant induction of damage throughout the time of exposure (Figure 2A).

Lung

This organ showed an increase in DNA migration after the first exposure and the damage remained of the same magnitude throughout the time of exposure. However, statistically significant differences were observed only at the first and third weeks of exposure, due to high variability during the second and fourth weeks of treatment (Figure 2B).

Kidney and liver

These organs showed a slight increase in DNA migration after the first exposure; in the kidney a statistically significant difference in DNA damage was observed at week two and remained at the same value until the end of the treatment,

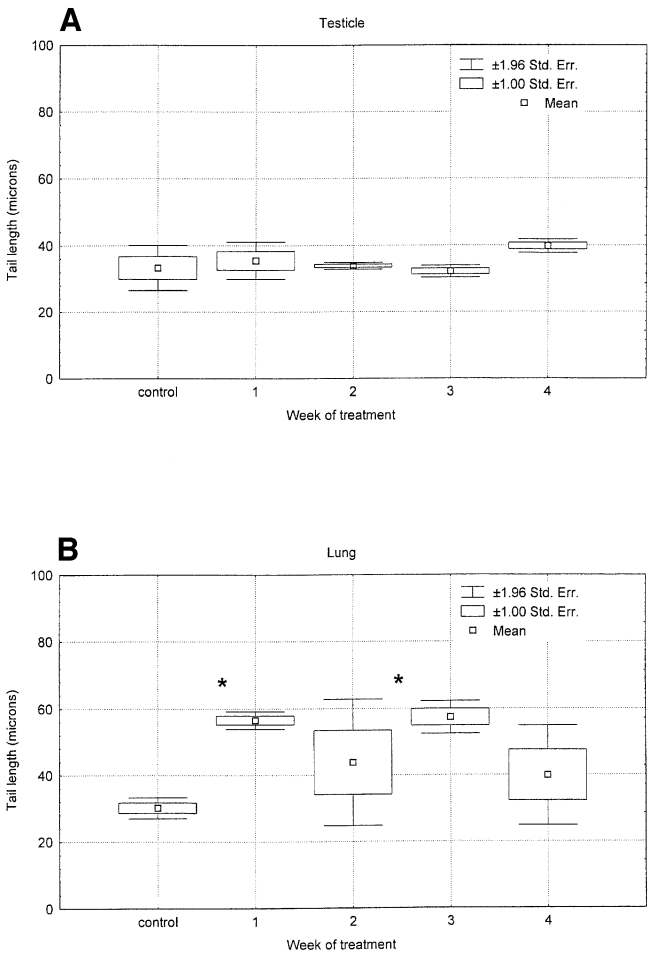

Fig. 2. Mean DNA migration (tail length) evaluated by SCGE assay in testicle (**A**) and lung (**B**) cells of four CD-1 male mice per week. *Statistically significant as determined by the Mann–Whitney *U*-test ($P < 0.05$).

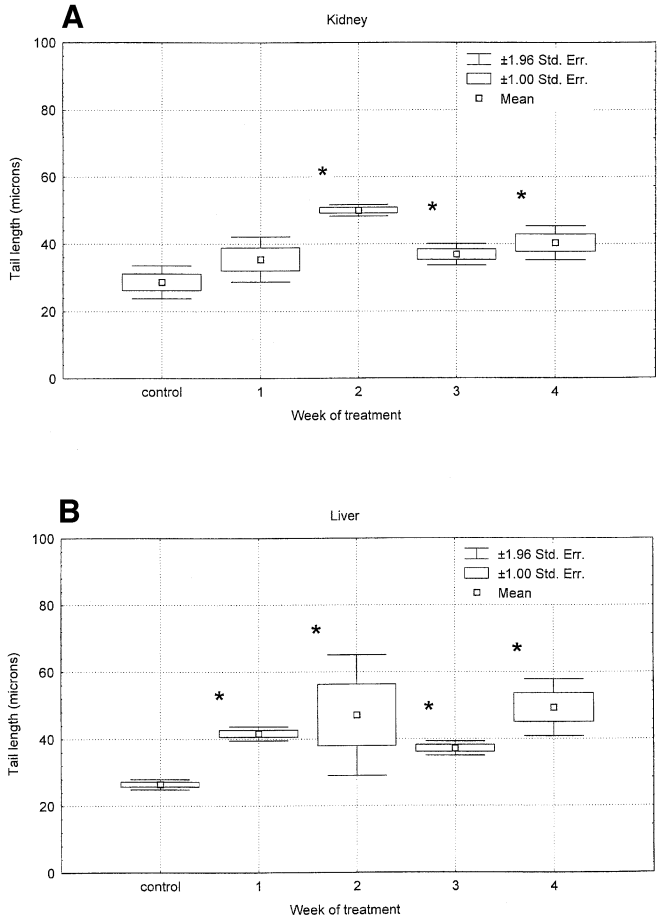

Fig. 3. Mean DNA migration (tail length) evaluated by SCGE assay in kidney (**A**) and liver (**B**) cells of four CD-1 male mice per week. *Statistically significant as determined by the Mann–Whitney *U*-test ($P < 0.05$).

while in the liver a difference in DNA damage was observed throughout the time of exposure (Figure 3A and B, respectively).

Brain and bone marrow

After the first 2 weeks of treatment these organs showed the highest damage induced, which decreased drastically at week three of treatment and returned again to the highest induction of DNA migration seen in the last week (Figure 4A and B). Both organs showed statistically significant differences in the second and fourth weeks of exposure.

Nasal epithelial cells and leukocytes

Although nasal epithelial cells are the first cells to come into contact with lead acetate, by inhalation and also via whole blood, including leukocytes, macrophages and plasma proteins, when the compound is distributed, nasal cells and leukocytes did not show any induction of damage after the first inhalation. The highest DNA migration values were observed during week two of treatment, but decreased slowly during the following weeks of exposure (Figure 5A and B).

Lead concentration in tissues

After each inhalation the metal concentration was determined in lung, liver and kidney throughout the exposure time, employing atomic absorption spectrophotometry. Figure 6 shows the correlation between lead concentration and the length of exposure in three different organs. In all organs a good correlation was found between time and metal concentration: for liver $r = 0.95$ and for kidney $r = 0.98$; in these cases we applied a linear correlation where the P value was < 0.05. In lung we observed a perfect correlation applying a polynomial fourth degree regression ($r = 1$); in this case the P value was also < 0.05.

The correlation between DNA damage, measured as tail length, induced by lead inhalation and accumulation of the metal in lung, liver and kidney is shown in Figure 7, when applying the equation $y = mx + b$, where m is the slope of the curve (biological effect), x is the lead concentration and b is the basal value of DNA migration (μm). It is possible to calculate that 1.0 μg of Pb induced an increase in DNA migration of 5.21 μm. The proposed formula for lung is $y = 5.21x + 32.99$. In the case of liver, 1.0 μg of Pb increased migration by 13.23 μm. The proposed formula is $y = 13.23x + 31.64$. For the kidney, 1.0 μg of Pb increased migration by 2.23 μm. The proposed formula is $y = 2.23x + 32.29$.

These data give the relationship between length of exposure and biological effect evaluated as SSB and alkali-labile sites under our experimental conditions.

Discussion

The uptake and toxic effects in mammals of lead have been extensively investigated using chemical forms easily

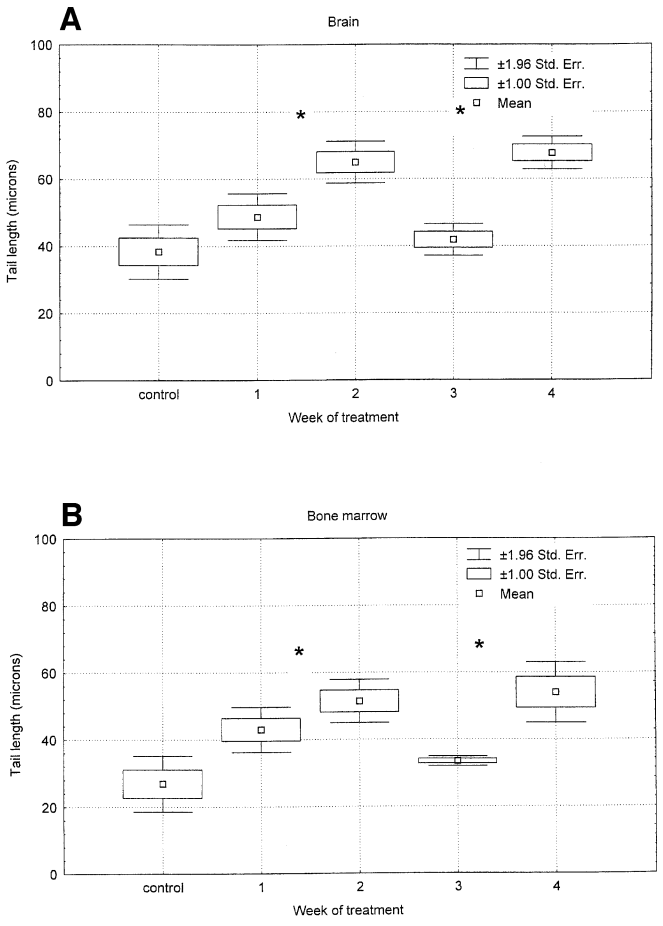

Fig. 4. Mean DNA migration (tail length) evaluated by SCGE assay in brain (**A**) and bone marrow (**B**) cells of four CD-1 male mice per week. *Statistically significant as determined by the Mann–Whitney *U*-test ($P < 0.05$).

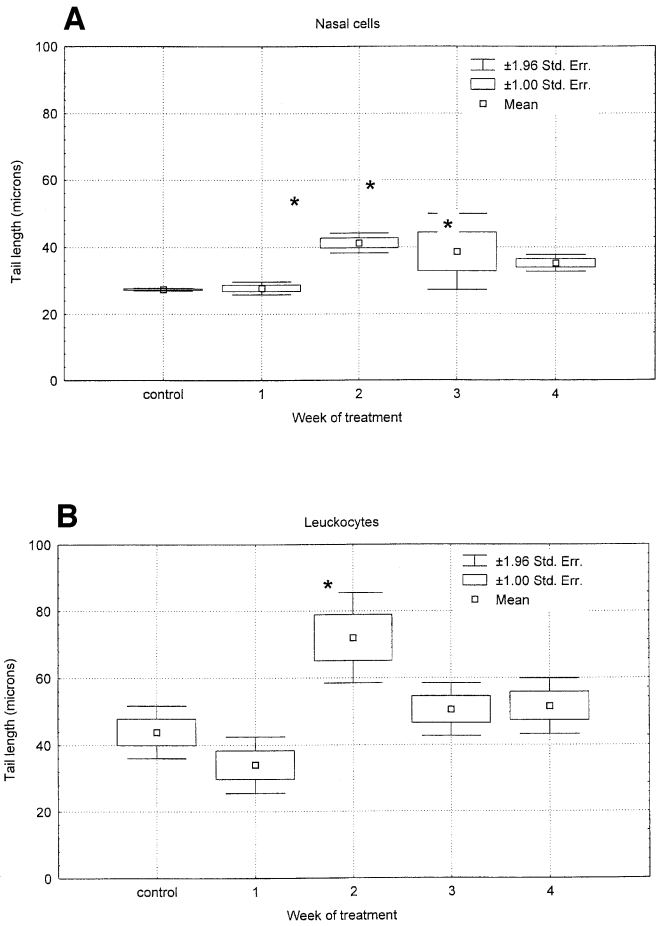

Fig. 5. Mean DNA migration (tail length) evaluated by SCGE assay in nasal cells (**A**) and leukocytes (**B**) of four CD-1 male mice per week. *Statistically significant as determined by the Mann–Whitney *U*-test ($P < 0.05$).

accumulated by the organisms (Regoli and Orlando, 1994). However, the bioavailability of lead due to environmental exposure depends on several factors. Factors influencing susceptibility may act at the site of exposure (usually by increasing or decreasing uptake), may effect the toxicodynamics of a metal (usually by complexing or covalent binding), may influence transport to a target organ or (theoretically at least) may influence some immunological, biochemical or cytological functional response at the target organ (Gochfeld, 1997). Susceptibility to any agent results from interactions between genetic enzyme polymorphisms and the environment; some toxicological study designs in animals can address susceptibility since in some degree different strains, species and genders can be incorporated into the bioassays. Information about inhalation exposure is poorly studied in this sense. For this reason we explored the possible role of lead acetate inhalation in the distribution and induction of DNA damage measured as SSBs, considering these factors and others such as the detoxification mechanisms.

Different extents of DNA migration, measured as tail length, were observed for different target organs; for a single inhalation lung was the most susceptible organ (Figure 1), while after 4 weeks of inhalation exposure the brain showed the highest level of DNA damage. Several modulating effects were present when genotoxicity induced by the treatment in each organ was analyzed and these modulators could be influencing the variability observed in each organ.

Nasal epithelial cells are the first epithelial tissue in contact with the metal, however, there was no statistically significant genotoxic effect until week three of treatment (Figure 5A). This could be a consequence of the pattern of deposition of inhaled lead in the respiratory tract, which is affected by particle size (in this study the particles generated were <1 μm), which modifies its deposition, which is ~20% (Chamberlain, 1985). The second organ in contact is the lung. The size of the metal particles we used were able to reach the alveoli (Skerfving, 1988; Wanner, 1993), however, induction of DNA damage in this organ showed a bimodal damage pattern (Figure 2B). This could be due to lead deposition in the alveoli, from whence the metal easily reaches the blood stream. Probably after induction of DNA damage (weeks one and three) the cells triggered a natural defense process, such as metallothionein synthesis induction (Klaassen and Liu, 1998), a mechanism by which cells and tissues are protected against further damage and by which restorative processes are initiated (Kushner, 1982). In addition, we did not observe extensive damage in the lung, probably because the rate of absorption of lead from particles deposited in the lung depends on the solubility of the chemical species of lead and our lead compound is highly soluble (Brune *et al.*, 1980).

After absorption via the pulmonary or gastrointestinal routes, lead is absorbed into the blood plasma and lymph. More slowly, but still within minutes, lead is transferred from the

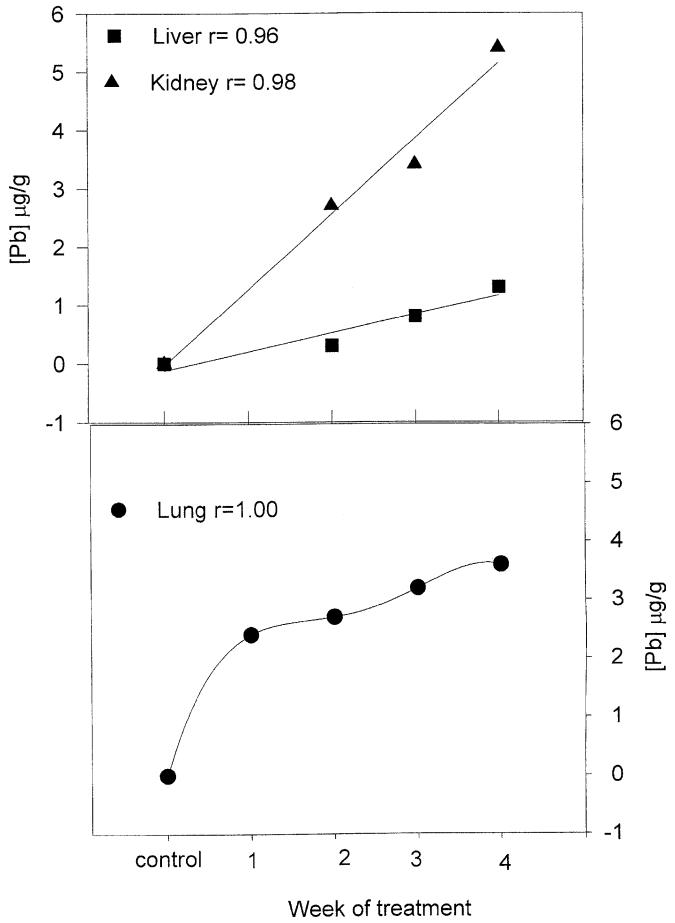

Fig. 6. Correlation between Pb concentration (µg/g) in several mouse organs and weeks of treatment. For liver and kidney the *r* value was determined by linear regression. A polynomial fourth degree regression was used for the lung.

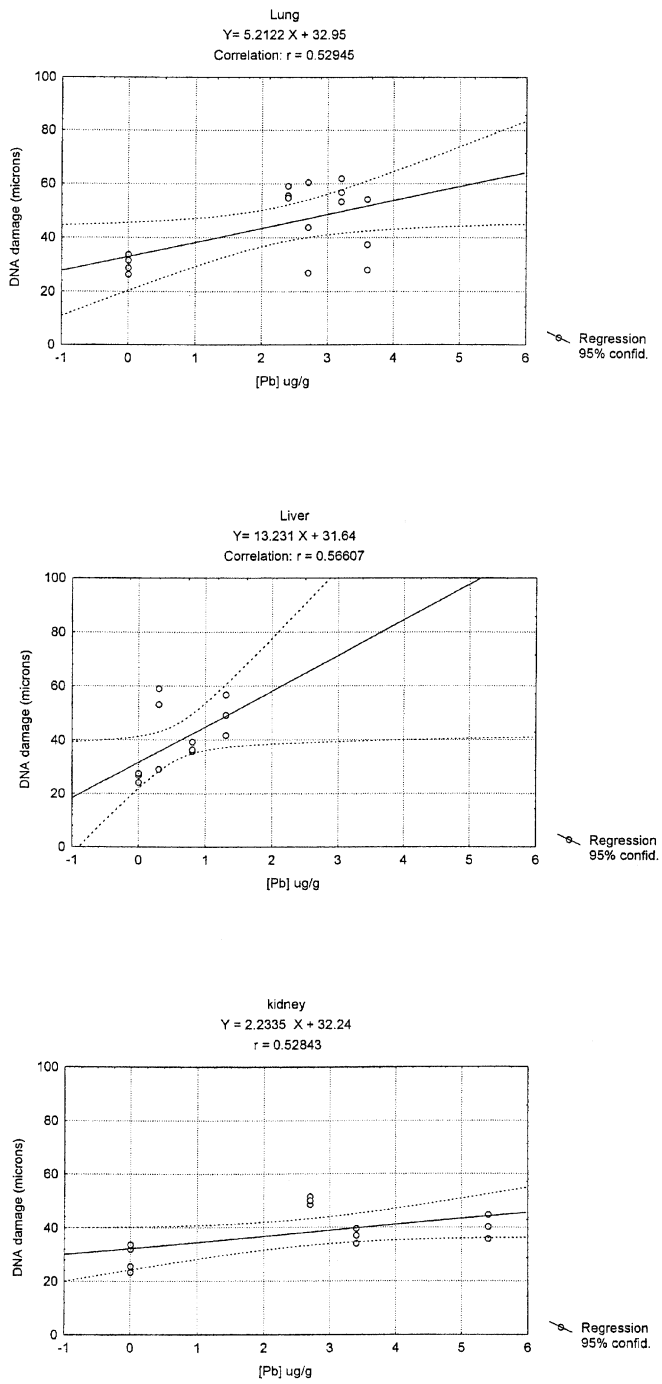

Fig. 7. Correlation between DNA migration and Pb concentration (µg/g) in several mouse organs. The *r* value was determined by linear regression.

plasma into blood cells. Once in the blood, 99% of the lead content is contained within red cells and only ~1% is associated with plasma proteins (Skerfving, 1988). This is the principal reason why lead acetate inhalation could not be seen to induce significant DNA damage when evaluated by the Comet assay, because this technique only detects damage in nucleated cells (leukocytes). In red blood cells the lead is bound to hemoglobin and other components such as δ-aminolevulinate dehydratase (ALAD), the second enzyme in the heme group biosynthetic pathway, which is inhibited as a result of lead replacing zinc (Johonson, 1998). When lead exerts this effect it probably generates oxidative stress and induces DNA damage by an indirect pathway, a process which could explain the behavior observed in week two of exposure (Figure 5B). The absorbed lead is distributed to different organs in the plasma. The liver and the kidney attain the highest concentrations (Skerfving *et al*., 1988). Nevertheless, it is important to note that, unlike Onalaja and Claudio (2000), who employed an animal model of variants of the ALAD gene and determined that ALAD plays an important role in bioaccumulation of lead in various tissues (blood, liver, kidney and brain) and therefore may play a pivotal role in the toxicokinetics of lead, our study was in a strain of mice which did not have this feature, which is the probable reason why we did not observe high levels of lead accumulation and DNA damage. The liver was the organ in which we found statistical differences from weeks one to four of treatment, maybe as a consequence of its function and blood supply (Figure 3B). However, throughout the exposure time the magnitude of DNA damage was not the highest, probably as a consequence of lead-induced metallothionein gene expression in the liver (Waalkes and Klaassen, 1985; Maitani *et al*., 1986; Yiangou *et al*., 1991; Johonson, 1998). When the synthesis of this group of acid serum proteins, which may belong to the acute phase family (Yiangou *et al*., 1991), increases these factors could modulate lead genotoxicity.

The kidney is a target organ for lead exposure (Pereza *et al.*, 1998). However, unlike the studies of Pereza and collaborators, in our work, employing inhalation exposure, we observed a

high level of lead accumulation throughout the exposure time but did not find extensive induction of DNA damage. In this respect, in accordance with Gochfeld (1997), we suggest that endogenous antioxidant levels may influence susceptibility to some of the toxic effects of lead and therefore we did not detect significant induction of DNA damage. In addition, there are indications that metallothioneins and heat shock proteins are stimulated by lead (Yiangou *et al*., 1991). These proteins have been considered as antioxidants and are probably involved in detoxification or modulating damage processes.

One of the organs in which we found greater DNA damage was bone marrow, however, statistically significant differences were only observed at week two of exposure (Figure 4B). Some authors have reported that lead causes many adverse health effects, including toxicity in the hematopoietic system (Moore and Goldberg, 1985; Pereza *et al*., 1998). It has also been reported that this metal interferes with mitochondrial energy metabolism, which is necessary to reduce ferric to ferrous iron before insertion of this metal into the porphyrin ring; when iron deficiency is present, ferrochelatase is more sensitive to these effects of lead, which results in depression of hematopoiesis. Therefore, iron supplementation may prevent this toxic effect of lead on hematopoiesis. With respect to these findings, our results do not represent the expected effect, probably because the distribution of lead by the inhalation exposure route differs from that by ingestion.

The brain is the organ most studied in lead toxicity (Johonson, 1998; Fortoul *et al*., 1999) because lead can, to some extent, pass the blood–brain barrier (Skerfving *et al*., 1988). Judging from animal experiments, the degree of passage of lead into the nervous system is higher in young organisms than in adults. In our study it was the organ with the most DNA damage after four weeks of exposure (Figure 4A). This result was expected due to the neurotoxicity of lead. Lead is similar enough to calcium to mimic it in some circumstances. *In vitro* studies have revealed the capacity of lead to compete with calcium for entry into synaptosomes, and picomolar concentrations of lead can activate protein kinase C (Cooper *et al*., 1984; Markovac and Goldstein, 1988; Jadhav *et al*., 2000). Lead also has inhibitory and enhancing effects on various receptor-activated and voltage-activated ion channels in the cell. These properties could be related to the neurological and behavioral effects reported (Johonson, 1998; Jadhav *et al*., 2000).

Unlike the previously described organs, in testis we did not observe induction of DNA damage throughout the treatment (Figure 1A). Lead has been implicated as a reprotoxic substance (decreased fertility and increased perinatal death) in experimental oral exposure of animals to high levels of lead (WHO, 1980). Sperm abnormalities have been noted in lead workers (Lancranjan *et al*., 1975). The changes observed by the Comet assay are not related to the alterations mentioned above, since the Comet assay only evaluates the induction of DNA SSBs and not the function of the organ.

A good correlation between length of exposure and metal concentration was found in the organs where lead was measured, except for the lung, in which accumulation fitted a polynomial non-linear regression curve. In this organ we observed a high accumulation of Pb after the first inhalation, which remained elevated after four weeks. This could be due to the induction of a detoxification response (Klaassen and Liu, 1998). For liver the r value was 0.95 and for kidney r was 0.98, with a linear response; in all cases the correlation was significant ($P < 0.05$) (Figure 6). The results observed in liver and kidney suggest that the length of exposure is an important factor in the bioaccumulation of lead, as reported by others. Each organ displayed different lead bioaccumulation and the distribution from high to low was kidney > lung > liver. These results are in agreement with Onalaja and Claudio (2000) and despite their employment of strains of mice differing in expression of the ALAD gene, the relationship between kidney and liver was the same. On the other hand, the lead levels observed in these organs were lower in our study than in that of Onalaja and Claudio, probably due to the ALAD genotype.

Compared with our previous cadmium study (Valverde *et al*., 2000) we found a weak correlation between DNA damage, measured as tail length, induced by lead acetate inhalation (0.0068 μg/cc) and accumulation of metal in lung, liver and kidney (Figure 7). The r value for lung was 0.49, for liver $r = 0.48$, while kidney had $r = 0.22$. This suggests that induction of DNA damage is independent of bioaccumulation of lead and is probably a reflection of the relationship between lead and additional factors, such as natural defense processes; mainly induction of acute phase proteins, metallothioneins, heat-shock proteins, calcium-dependent signaling and levels of antioxidants, such as glutathione (Yiangou *et al*., 1991; Jadhav *et al*., 2000).

As a whole, DNA damage in several CD-1 mouse organs was observed, however, the magnitude of damage was smaller than that observed after cadmium inhalation, supporting the claim of those who classify lead as a weak genotoxic agent. Nevertheless, the concentrations used in this work were similar to those mentioned in the air quality standard for this metal (WHO, 1980) and were lower than those employed by other authors (Gebhart and Rossman, 1991; Johonson, 1998), indicating that even at low levels of exposure by inhalation this metal could induce DNA damage and should be considered as a risk for living organisms. It is important to determine whether the inhibition of DNA repair processes described for this metal (Hartwig, 1994) could play a role in the accumulation and stability of DNA damage, resulting in initiation of the carcinogenic process associated with this metal.

Acknowledgements

We gratefully acknowledge M.C.López, I. Sánchez and I.López for their technical support. This study was partially supported by CONACyT project 3180P-M. M.V. was the recipient of a fellowship from DGEP and CONACyT.

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Received on December 11, 2000; revised on June 28, 2001; accepted on August 28, 2001