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Morphological characterization of renal cell lines (BGM and VERO) exposed to low doses of lead nitrate

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Summary. The response to lead nitrate has been assessed in two cell lines of renal origin. The range of toxic concentrations was determined by Neutral Red assay after 24-h of exposure. Morphological changes in the Buffalo Green Monkey (BGM) and VERO cell lines after exposure to subcytotoxic doses (1.38 mM and 1.04 mM, respectively) equivalent to EC10 (effective concentrations 10%) of lead nitrate were evaluated at the ultrastructural level by transmission microscopy. The most notable finding in treated cells was the presence of inclusion bodies in the form of irregular granules of varying size in both cytoplasm and lysosomes. Cell membrane integrity was not affected. The number of phagolysosomes and myeline figures associated to the inclusion bodies was higher than in the control cultures. We conclude that the phagolysosomic mechanism fails to digest this metal ion and the BGM and VERO renal cell lines can be considered as useful tools for toxicological studies involving lead nitrate.

Key words: Vero, BGM, Lead, Cytotoxicity, Inclusion bodies

Introduction

Lead is the most ubiquitary heavy metal in the environment and all living beings are continuously exposed to it. In human, lead is considered the most abundant non-essential element (Goyer, 2001), and the cause of haematological, neurological and renal effects. In vivo and in vitro models have been widely used for lead kinetic studies after both acute and chronic exposures. Mitochondrial alterations in several types of cells have been described (Goyer, 1968; Goyer et al., 1968; Wielgus-Serafieska et al., 1980; Mahaffey et al., 1981; Legare et al., 1993; Cernochova and Kamarad, 1994). In addition, lysosomes have been described as lead deposit sites (Sternlieb and Goldfischer, 1976; Fowler, 1978; Nilsson, 1979; Prosi and Dallinger, 1988).

The morphological alterations observed in proximal tubular cells are responsible for lead nephrotoxicity (Klaassen, 2001), the presence of intranuclear inclusion bodies being the most frequent alteration described in both *in vivo* (Stiller and Friedrich, 1983; Boulahdour and Berry, 1996; Vicente-Ortega et al., 1996) and *in vitro* (Tang et al., 1996) models. However, it is still not clear whether the appearance of such inclusion bodies in the nucleus is associated with long or short exposure periods. Some authors have mentioned its presence in the nucleus after 24 hours' or less exposure (Choie and Richter, 1972), while others have not observed inclusion bodies until two months after exposure (Horn, 1970).

Renal cell cultures have been described as useful tools for studying heavy metal-induced cell damage and toxicity mechanisms (Cherian, 1985; Wilson, 1986), but few references to the BGM and VERO cell lines appear in the literature, although they have been successfully used in microbiological studies (Dahling and Wright, 1986; Romero et al., 2003).

In the present study, the morphological response of the BGM and VERO cell lines exposed to low doses of lead was analysed to assess the suitability of these lines for future toxicological research. The basal toxicity data were obtained using the neutral red (NR) cytotoxicity assay (Borenfreund and Puerner, 1984), and ultrastructural changes were evaluated after exposure to subcytotoxic doses equivalent to EC10 (1.38 for BGM and 1.04 mM for VERO), concentrations 1.37- and 1.66-fold lower than their corresponding EC50.

Materials and methods

Cell cultures

The BGM and VERO cell lines (renal cells from the African green monkey *Cercopitecus aethiop*s) were cultured in Eagles's minimum essential medium (MEM, Sigma) supplemented with 10% (v/v) foetal calf serum (FCS), 100 UI penicillin/ml and 100 mg

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streptomycin/ml. Cells were plated in 75 cm^2 bottles at a density of 1.5x 0^4 cells/cm^2 and incubated at 37 ºC in a CO_2 (5%) humidified incubator. The growth of this cell line followed three well-defined growth phases: latent, exponential and stable, allowing us to identify the optimal time for treatment (48 h after the beginning of subculture, just at the outset of the exponential growth phase).

Culture conditions

To ascertain the best moment for adding the different concentrations of lead, cell growth and death were measured from the corresponding growth curve. Two cultures were shown with two different inocula (2500 and 5000 cells per well) suspended in 200 μl culture medium with calf foetal serum at 10%. Culture growth was analysed every 24 hours (Figs. 1, 2) using the Neutral Red technique (Borenfreund and Puerner, 1984). Neutral Red solution was added daily to each well and incubated at 37 ºC for three hours. The cells were fixed in 0.5% v/v formaldehyde in $CaCl_2$ and NR stain was extracted from the cells using 100 mL alcohol-acid. Absorbance was read with Multiskan MCC/340P plates at 560 nm with a 690 nm reference filter. The culture of 5000 cells per well was chosen because it gave a growth curve with a greater number of cells at each measurement time. The best moment for adding and withdrawing lead from the cultures was 48 and 72 hours after sowing, when the cultures were in full exponential growth and the cultures had not reached 100% confluence.

Test chemical

The lead nitrate was obtained from Sigma, and was prepared in purified and sterilised water (Milli-Q) at x 10 for each concentration to be evaluated. These solutions were added to MEM to obtain the final concentrations. The MEM used in the control cultures was prepared in the same form but by adding 10% purified water. Prior to carrying out the treatment, the osmolarity of these solutions (including the control) was measured so that any differences in cell viability after addition of the lead nitrate salt could be evaluated.

Neutral red cytotoxicity assay (NR)

The cells were sown in 96-well microtitre plates and the lead nitrate was added 48 h later. The initial concentration range assayed was between 0.5 and 3 mM, while controls consisted of medium with untreated cells and without cells. The cultures were tested using NR, which is selectively taken up by the lysosomes of living cells (Borenfreund and Puerner, 1984), thus pointing to the degree of viability. Twenty-four hours after the treatment, the NR solution was added to each well and incubated at 37 ºC for 3 h. Cells were fixed with 0.5% v/v formaldehyde in 1% $CaCl_2$ solution and NR dye was eluted from the living cells using 100 ml acid alcohol (1% v/v acetic acid in 50% ethanol) (Babich and Borenfreund, 1993). Absorbance was read on a

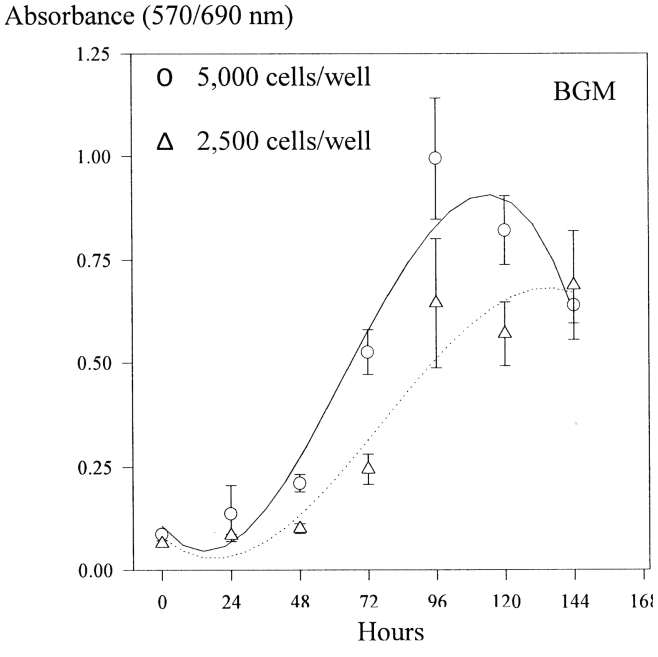

Fig. 1. Growth curve of BGM cell line. (triangle) Inocula of 2,500 cells per well. (circle) Inocula of 5,000 cells per well.

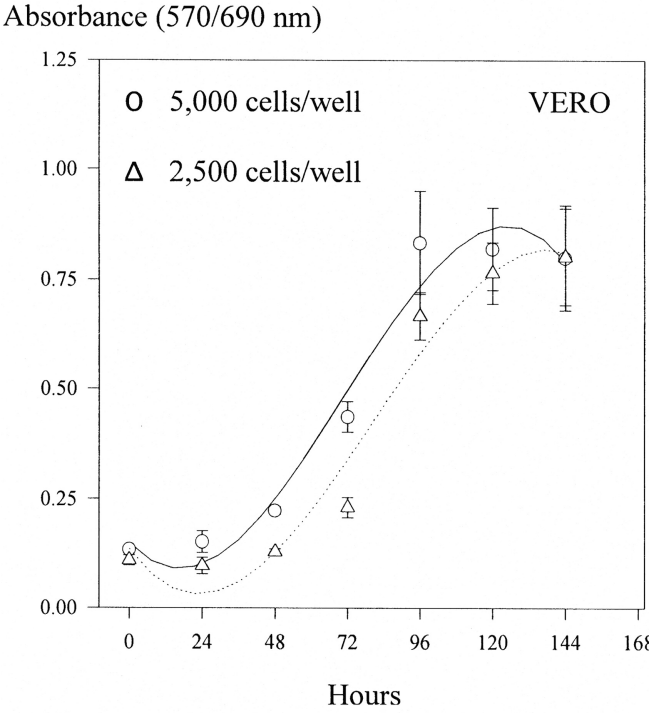

Fig. 2. Growth curve of VERO cell line. (triangle) Inocula of 2,500 cells per well. (circle). Inocula of 5,000 cells per well.

Multiskan MCC/340P plate reader at 560 nm with a 690 reference filter (Bayoumi et al., 1998). The mean absorbance for each concentration was expressed as a percentage of the value obtained for the blank wells and plotted against the lead nitrate concentration. The effective concentrations were determined from the dose-response curve by linear regression.

Transmission electron microscopy

The EC10 dose of lead nitrate (1.38 mM for BGM and 1.04 mM for VERO) was added 48 h after the cells were plated on 6 cm Petri dishes at a density of $1.5x10^4$ cells/cm^2. Cells were exposed to this toxic compound for 24 hr at 37 ºC. Control plates were prepared without lead nitrate. After exposure, the cells were trypsinized, collected and centrifuged (482 g, 10 min, +24 ºC). The pellets were immediately fixed in 3% (v/v) glutaraldehyde, postfixed in 1% (v/v) osmium tetroxide and stained with 4.8% uranyl acetate. After dehydration with a graded series of alcohol concentrations, the samples were rinsed in propylene oxide and impregnated with epoxy resins. The ultrathin sections were contrasted with uranyl acetate and lead citrate for the electron microscopy study. Electron micrographs were taken with a Zeiss EM 10C transmission electron microscope.

Results

Cytoxicity assays

The lead nitrate cytotoxic concentration ranges, as measured by the NR assay, were between 1.25 and 2.5 mM for the BGM cell line and 1 and 2.5 mM for the VERO cell line. The effective concentrations (Tables 1, 2) were obtained by linear regression from the dose-response curve. The small difference between the EC0 and EC100 demonstrated that the BGM and VERO cell lines were susceptible to the toxic activity of lead, VERO cells being slightly more susceptible than BGM cells.

Morphological findings

Untreated cells

Transmission electron microscopy showed that the BGM cells were rounded, with a well-defined outline and a variable number of microvilli. Spherical or oval mitochondria with well-defined transversal cristae, abundant smooth endoplasmic reticulum, few phagolysosomes and clearly recognisable vacuoles were characteristic of this cell line. Abundant chromatin, scarce peripheral heterochromatin and one to four nucleoli were observed in the spherical or oval nuclei, which were situated in a central position in the cell (Fig. 3).

The VERO cells presented variable morphology, a well-defined cell outline and microvilli in variable but normally scarce quantities. The cytoplasmic volume was large, showing well-recognisable organelles, but in smaller quantities that in the BGM cell line (Fig. 4). Spherical or long mitochondria with transversal cristae were frequently situated near the nucleus. A nucleolus was observed in the irregular and elongated nucleus. Heterochromatin formed clumps and great quantity of euchromatin could be seen. Free ribosomes were observed in the cytoplasm or associated to the endoplasmic reticulum. Autophagic inclusion bodies and vacuoles were also observed.

Treated cells

Following exposure to EC10 of lead nitrate, visual observation confirmed that the proportion of non-viable or altered cells was close to 10% in both cell lines. At

Table 1. Percentage of viable cells of the BGM cell line as a function of lead nitrate concentration with respect to the control.

CONCENTRATION (mM)	MEAN PERCENTAGE	STANDARD ERROR	STANDARD DEVIATION	MAXIMUM	MINIMUM
1.25	97.23	2.77	6.19	100.00	86.15
1.50	85.58	2.20	5.40	91.45	79.43
1.75	60.94	3.24	7.94	71.36	48.70
2.00	39.18	3.33	8.17	47.89	25.06
2.50	5.96	3.19	7.81	21.47	1.38

Table 2. Percentage of viable cells of the VERO cell line as a function of lead nitrate concentration with respect to the control.

CONCENTRATION (mM)	MEAN PERCENTAGE	STANDARD ERROR	STANDARD DEVIATION	MAXIMUM	MINIMUM
1.00	97.70	1.97	4.82	100.00	87.97
1.50	60.21	2.96	7.26	67.51	50.05
2.00	24.55	1.32	3.24	29.69	20.46
2.50	13.03	2.70	6.04	20.05	7.05

microscopic level the most relevant characteristic observed in both cell lines was the presence of electrodense clumps, often located close to the nucleus (Figs. 5, 6). In both cell lines, these inclusion bodies were observed as free granular deposits in the cytoplasmic matrix or sequestered into lysosomes. Inclusion bodies were also ring-shaped. In both lines, sporadic intranuclear inclusion bodies were also observed. Myeline figures were commonly seen close to these clumps, and were more evident in BGM cells (Fig. 5) than in VERO cells (Fig. 6). The rest of the organelles presented a very similar morphology to the control cells, except the mitochondria whose cristae were less well-defined than the control cells. A slight increase in the number of phagolysosomes was observed in both treated cell lines.

Discussion

The mean osmolarity of the medium used for both the treated and control cultures was close to that described as optimum for in vitro cell growth (270 mOsm) (Waymouth, 1970), strongly suggesting that both the basal cytotoxicity data and the morphological changes observed in the treated cells were solely due to the effect of the treatment.

The improved characterisation of established cell lines is important for physiological and toxicological research (Zimmerhackl et al., 1998). In this study, BGM and VERO cell lines were used in the evaluation of the effects of lead, showing a measureable response to the NR colorimetric assay. Moreover, their response to the toxic effects of lead could be observed at microscopic level. The usefulness of the BGM cell line for toxicological assays has recently been described by us (Romero et al., 2003).

The presence of intracytoplasmic inclusion bodies was the most characteristic feature of all the treated cultures. Migration of these inclusion bodies toward the nucleus has already been described by several authors (McLauglin et al., 1980; Kendall et al., 1981: Bonucci et al., 1983; Kendall et al., 1983; Vicente-Ortega et al., 1996). The inclusion bodies could occasionally be seen in the nuclei (Figs. 5,6) in the form of intensely electrodense spherical bodies, unlike those described by other authors (Goyer, 2001), who described bodies with a dense core and outer fibrillary region, although Vicente-Ortega et al. (1996) described them as being similar to those we observed (small irregular electrodense granules next to the perichromatic granules), next to other forms (dense bodies with a halo of fibrillae in the shape of a "radiant sun" or with a peripheral electronlucid halo, and irregular dense, randomly distributes bodies).

Controversy exists concerning the time lapse needed from lead exposure up to the moment that intranuclear inclusion bodies can be observed. Some authors defend the hypothesis that 24 hours of exposure is sufficient for these inclusion bodies to be seen in the nucleus (Choie and Richter, 1972). However other authors affirm that direct exposure to lead during two months is necessary to observe these inclusions (Horn, 1970). In our study, the lead was present in the culture medium in which the cells live, meaning that it is bioavailable in sufficient quantities to explain the presence of largish clumps of lead in the cytoplasm only 24 hours after exposure. It can be affirmed, then, that cultures of BGM and VERO cells are useful tools for studying the cellular kinetics of lead, as are cultures of neuronal and glial cells (Tiffany-Castiglioni, 1993).

The appearance of the above mentioned intracytoplasmic inclusion bodies contrasts with most

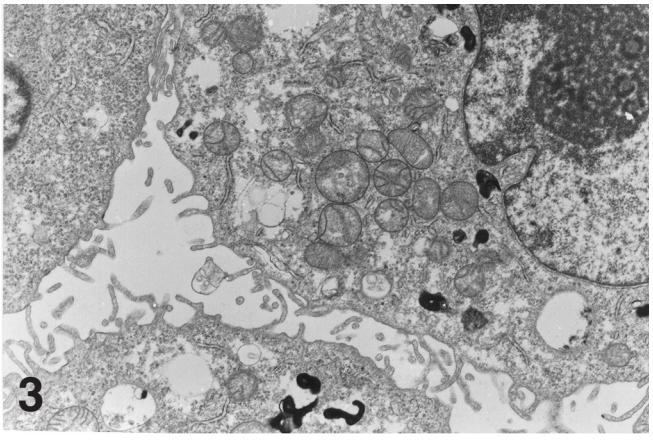

Fig. 3. Transmission electron micrograph of control (untreated) BGM cells. BGM cells have abundant cytoplasm, a well-defined cell outline and a variable number of microvilli. Spherical or oval mitochondria with well-defined vacuoles. Abundant chromatin in the nucleus. x 13,000

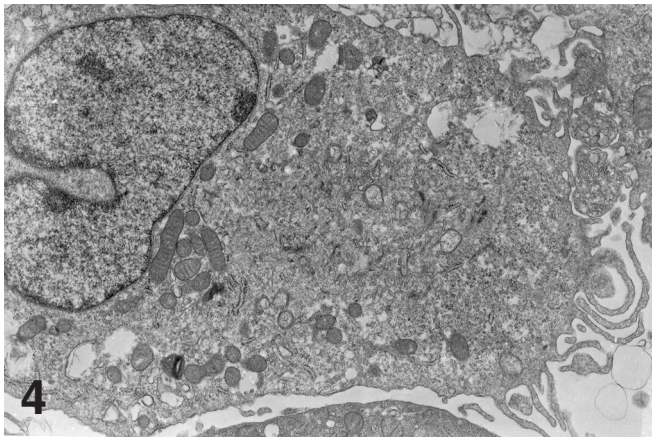

Fig. 4. Transmission electron micrograph of control (untreated) Vero cells. Vero cells present a variable morphology, a well-defined cell outline and microvilli in variable but normally scarce quantity. Abundant cytoplasmic volume with well-recognisable organelles. Spherical or long mitochondria are situated near the nucleus. Heterochromatin forms clumps and there is a great quantity of euchromatin. x 11,000

observations made in *in vivo* studies, which describe the constant presence of lead in intranuclear inclusion bodies (Stiller and Friedrich, 1983; Boulahdour and Berry, 1996), and only occasionally in the cytoplasm (Vicente-Ortega et al., 1996). The explanation for these differences from observations made *in vivo* may lie in any of the factors that depend on biological systems, such as lead transport, dispersion during transport, defence mechanisms, physiological barriers and excretion mechanisms, or in any factor related with the

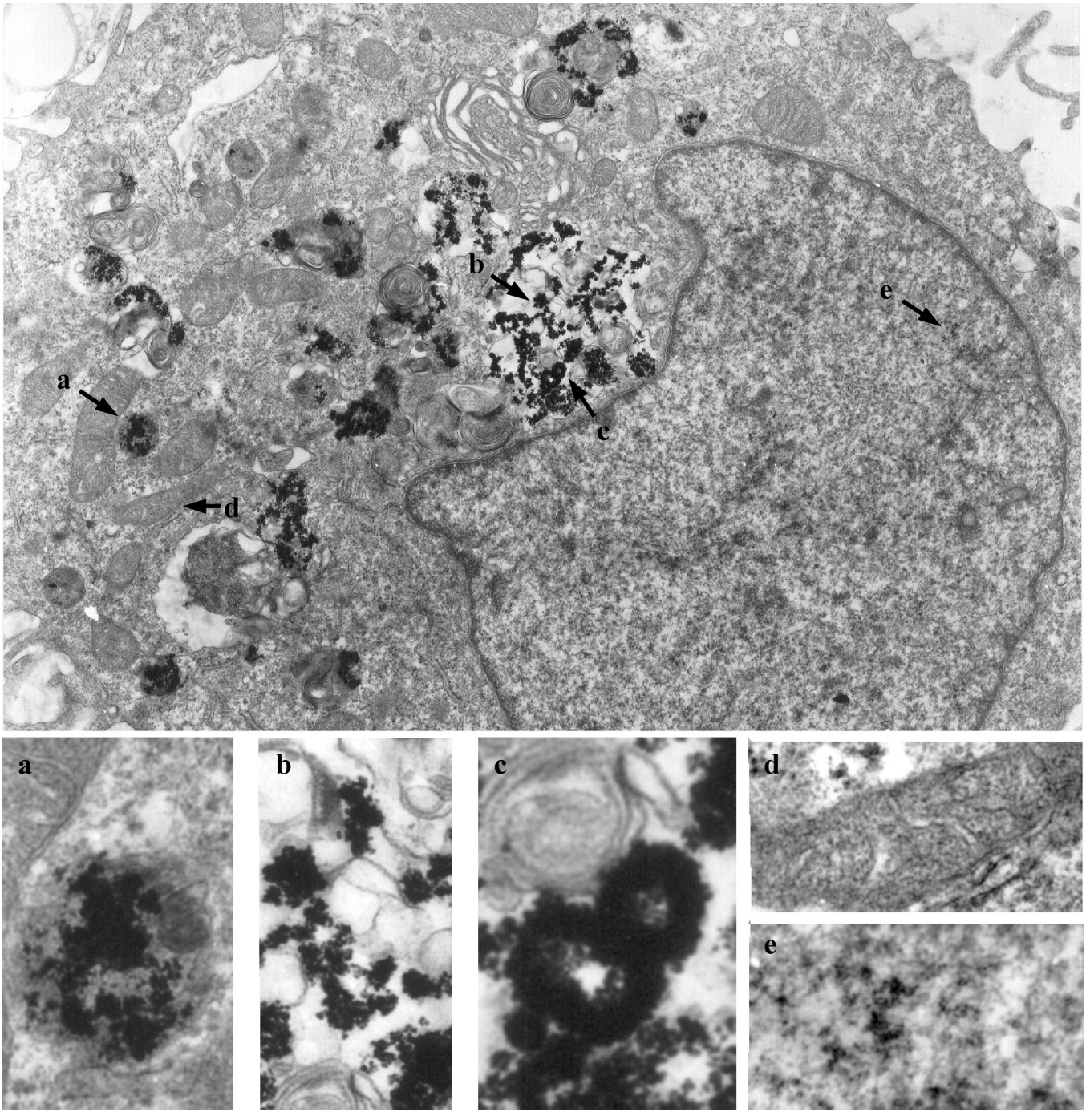

Fig. 5. Transmission electron micrographs of BGM cells exposed to 1.38 mM (EC10) lead nitrate for 24 h. Intracytoplasmic inclusion bodies are in lysosomes (**a**, x 125,000) or free in the cytoplasmic matrix (**b**, x62,500). Ring-shaped intracytoplasmic inclusion bodies are free in cytoplasmic matrix (**c**, x 93,750) and myelin figures are associated to inclusion bodies. Loss of mitochondrial cristae (**d**, x50,000) and intranuclear inclusion (**e**, x 62,500).

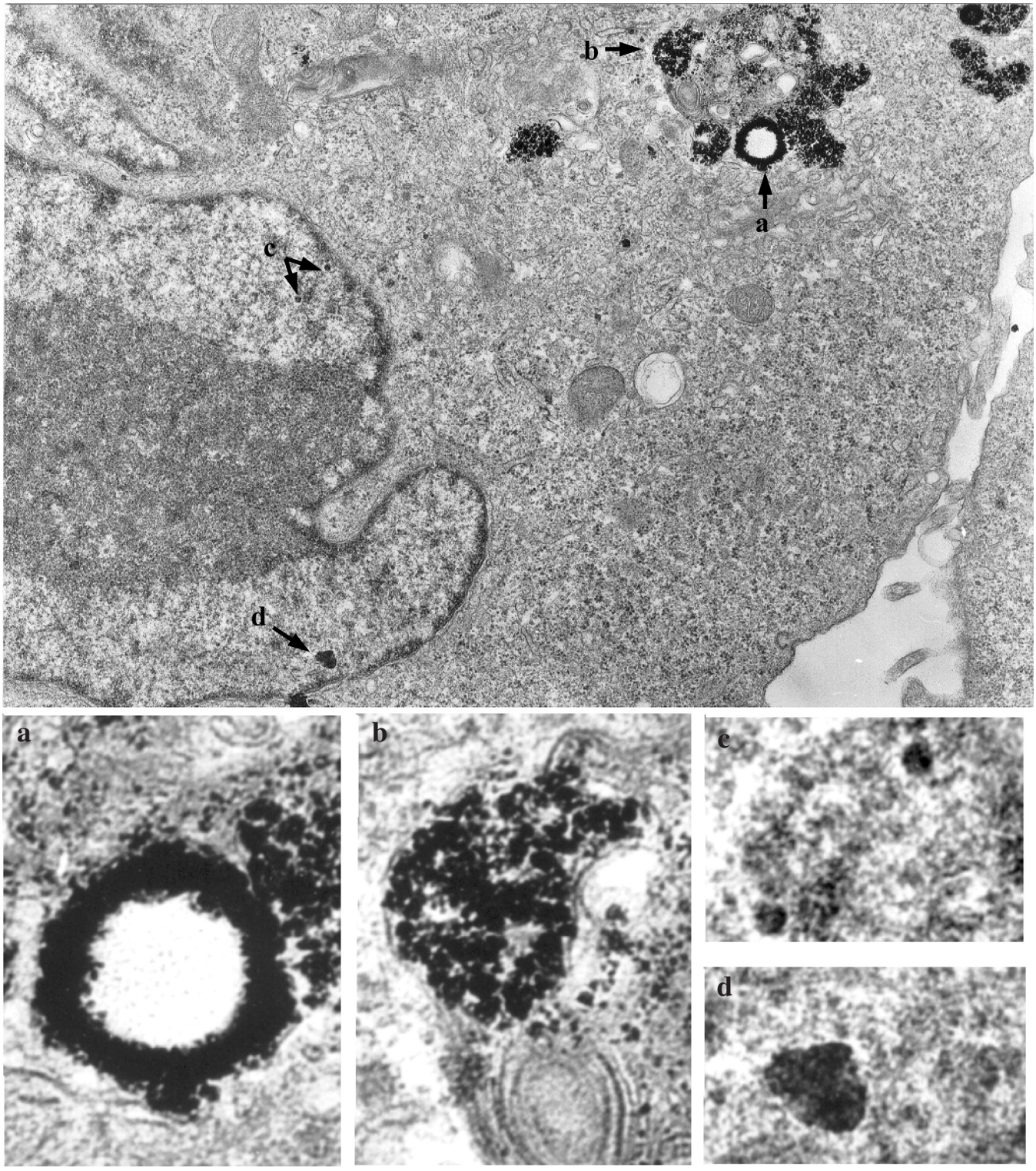

Fig. 6. Transmission electron micrographs of Vero cells exposed to 1.04 mM (EC10) lead nitrate for 24 h. Ring-shaped intracytoplasmic inclusion bodies (**a**, x 102,500) and free clumps (**b**, x102,500) are common in the cytoplasmic matrix and myelin figures are associated to some inclusion bodies. Occasional intranuclear inclusion (**c** and **d**, x102,500)

experimental study, such as exposure time, doses and the cell line used. Some authors have suggested that the scant presence of lead in the cytoplasm is due to the increased activity of the phagolysosome apparatus (Stiller and Friedrich, 1983). If this is so, the quantity of lead entering the cell would determine the greater or lesser activity of the phagolysosomic apparatus. Our observations lead us to think that it is precisely the quantity of bioavailable lead that determines its final destination and, consequently, its effect. The deposits of lead that appear free in the cytoplasm tend to come together, forming large clumps and seem to be in some way associated with membranous structures. This association with membranes coincides with the observations made by Vicente-Ortega et al. (1996) who describe the lead as being associated with polysomes and the rough endoplasmic reticulum. As has been described in other studies (Sternlieb and Goldfischer, 1976; Fowler, 1978; Nilsson, 1979; Goyer, 1983; Prosi and Dallinger, 1988), it is possible that the lead inside the cells may be sequestered by the lysosomes. This would result in the lead being difficult to digest, leading to the formation of myelin figures (Figs. 5, 6). We may therefore speak of the cells' failure to digest this metal ion.

Other morphological alterations were not seen in the rest of the organelles, except the loss of clearness in the outline of the mitochondrial crests in the BGM line. Numerous studies, both *in viv*o and *in vitro*, show lead to be an element capable of producing morphological and biochemical alterations at mitochondrial level, both in renal and other cell types (Wielgus-Serafieska et al., 1980; Mahaffey et al., 1981; Laszczyca, 1989; Goyer, 2001). It is possible that such morphological alterations may appear late after a longer exposure time to lead, or that they may be produced slowly and not be immediately visible.

In conclusion, BGM and VERO cell lines have demonstrated their usefulness in the study of the toxic effects of lead. The presence of the metal ion in the culture medium allows that its rapid uptake by the cell, while the phagolysosomic mechanism fails to digest it. This, then, can be considered as a good *in vitro* model for future studies of lead toxicity.

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References

Bonucci E., Barckhaus R.H., Silvestrini G., Ballanti P. and Di Lorenzo G. (1983). Osteoclast changes induced by lead poisoning (saturnism). Appl. Pathol. 1, 241-250.

Borenfreund E. and Puerner J.A. (1984). A simple quantitative procedure using monolayer cultures for cytotoxicity assays (HTD/MR-90). J. Tiss. Cult. Meth. 9, 7-9.

Boulahdour H. and Berry J.P. (1996). Intranuclear dense bodies after metal intoxication: a review of ultrastructural and microanalytical findings. Cell. Moll. Biol. 42, 421-429.

Cernochova D. and Kamarad V. (1994). Ultrastructure of mice Leydig cells at chronic lead intoxication. Acta Univ. Palacki. Olomuc. Fac. Med. 137, 31-33.

Cherian M.G. (1985). Rat kidney epithelial cell culture for metal toxicity studies. In Vitro Cell. Dev. Biol. 21, 505-508.

Choie D. and Richter G.W. (1972). Lead poisoning: rapid formation of intranuclear inclusions. Science 177, 1194-1195.

Dahling D.R. and Wright B.A. (1986). Optimization of the BGM cell line culture and viral assay procedures for monitoring viruses in the environment. Appl. Environ. Microbiol. 51, 790-812.

Fowler B.A. (1978). General subcellular effects of lead, mercury, cadmium, and arsenic. Environ. Health. Perspect. 22, 37-41.

Goyer R.A. (1968). The renal tubule in lead poisoning. I. Mitochondrial swelling and aminoaciduria. Lab. Invest. 19, 71-77.

Goyer R.A. (1983). Intracellular sites of toxic metals. Neurotoxicology 4, 147-156.

Goyer R.A. (2001). Toxic effects of metals. In: Casarett and Doull´s Toxicology. The Basic Science of Poisons. 6th ed. Amdur M.O., Doull J. and Klaasen C.D. (eds). Pergamon Press. New York. pp 691-736.

Goyer R.A., Krall A. and Kimball J.P. (1968). The renal tubule in lead poisoning. II. In vitro studies of mitochondrial structure and function. Lab. Invest. 19, 78-83.

Horn J. (1970). Isolierung und untersuchung von einschlubkörperchen in der ratten-niere nach chronischer bleivergiftung. Virchows Arch. (B) Zellpath 6, 313-317.

Kendall R.J., Veit H.P. and Scanlon P.F. (1981). Histological effects and lead concentrations in tissues of adult male ringed turtle doves that ingested lead shot. J. Toxicol. Environ. Health 8, 649-658.

Kendall R.J., Scanlon P.F. and Veit H.P. (1983). Histologic and ultrastructural lesions of mourning doves (*Zenaida macroura*) poisoned by lead shot. Poult. Sci. 62, 952-956.

Klaassen C.D. (2001). Casarett and Doull's Toxicology: The Basic Science of Poisons. 6th ed. McGraw Hill, New York.

Laszczyca P. (1989). The activity of mitochondrial enzymes in the muscles of rats subjected to physical training and subchronical intoxication with lead and zinc. Acta Physiol. Pol. 40, 544-551.

Legare M.E., Barhoumi R., Burghardt R.C. and Tiffany-Castiglioni (1993). Low-level lead exposure in cultured astroglia: identification cellular targets with vital fluorescent probes. Neurotoxicology 14, 267-272.

Mahaffey K.R., Capar S.G., Gladen B.C. and Fowler B.A. (1981). Concurrent exposure to lead, cadmium and arsenic. Effects on toxicity and tissue metal concentrations in the rat. J. Lab. Clin. Med. 98, 463-481.

McLaughlin J.R., Blackman D.M. and Martin W.G. (1980). Formation of lead-induced inclusion bodies in primary rat kidney epithelial cell cultures: effect of actinomycin D and cicloheximide. Toxicol. Appl. Pharm. 56, 418-431.

Nilsson J.R. (1979). Intracellular distribution of lead in Tetrahymena during continuous exposure to the metal. J. Cell. Sci. 39, 383-396.

Prosi F. and Dallinger R. (1988). Heavy metals in the terrestrial isopod Porcellio scaber Latreille. I. Histochemical and ultrastructural characterization of metal-containing lysosomes. Cell. Biol. Toxicol. 4, 81-96.

Romero D., Gómez-Zapata M, Luna A. and García-Fernández A.J. (2003). Morphological characterisation of BGM (Buffalo Green Monkey) cell line exposed to low doses of cadmium chloride.

Toxicol. In vitro 17, 293-299.

Sternlieb I. and Goldfischer S. (1976). Heavy metals and lysosomes. Front Biol. 45, 185-200.

Stiller L.D. and Friedrich H.J. (1983). Ultrastructural and ultrahistochemical investigations of lead-induced intranuclear inclusion bodies in rat kidney. Exp. Pathol. 12, 133-141.

Tang H.W., Yan L.H., Hu X.H., Liang Y.X. and Shen X.Y. (1996). Lead cytotoxicity in primary cultured rat astrocytes and Schwann cells. J. Appl. Toxicol. 16, 187-196.

Tiffany-Castiglioni E. (1993). Cell culture models for lead toxicity in neuronal and glial cells. Neurotoxicology 14, 513-536.

Vicente-Ortega V., Martínez-García A.F., Cremades-Campos A., Rodríguez-Vicente J., Calderón-Rubiales F. and Martínez-Díaz F. (1996). Ultrastructural investigation of lead-induced intranuclear inclusion bodies in mice. Ultrastruct. Pathol. 20, 263-273.

Waymouth C. (1970). Osmolarity of mammalian blood and media for culture of mammalian cells. In vitro 6, 109-127.

Wielgus-Serafieska E., Zawadka A. and Falkus B. (1980). The effect of lead acetate on rat liver mitochondria. Acta Physiol. Pol. 31, 659-668.

Wilson P.D. (1986). Use of cultured renal tubular cells in the study of cell injury. Miner. Electrolyte Metab. 12, 71-84.

Zimmerhackl L.B., Momm F., Wiegele G. and Brandis M. (1998). Cadmium is more toxic to LLC-PK1 cells than to MDCK cells acting on the cadherin-catenin complex. Am. J. Physiol. 275, 143-153.

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