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Moderate Lead Exposure Elicits Neurotrophic Effects in Cerebral Cortical Precursor Cells in Culture

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Lead (Pb) persists as an environmental toxicant despite aggressive environmental and occupational regulation. Neurotoxicological effects of acute Pb poisoning range from subtle cognitive deficits, to clumsiness and ataxia, to coma and seizures. In adult neurotoxicity, reductions of blood Pb levels are often associated with reversal of clinical signs. In children, however, the effects are more likely to endure, with even low levels of chronic Pb exposure correlating with decreasing IQ. These persistent effects likely result from neurodevelopmental insults, such as altered cell survival or maturation, although the mechanisms have not been fully defined. In the present study we define the effects of moderate-level Pb exposure on mammalian neurogenesis using a well-characterized cortical precursor model. Gestational day 14.5 rat cerebral cortical precursor cells were cultured in defined media and cell number, precursor proliferation, apoptosis, and neuritic process outgrowth were assessed following exposure to a range of Pb acetate concentrations. Surprisingly, whereas a concentration of 30 μg/ml Pb acetate was acutely toxic to neurons, concentrations between 1 and 10 μg/ml Pb acetate (~3 μM and 30 μM Pb, respectively) increased cell number: 10 times as many cells exposed to 10 μg/ml Pb were present on day 4 as compared to control. The increase in cell number was not a result of increased proliferation, however, as DNA synthesis did not increase. Rather, Pb exposure maintained the survival of cortical precursors, as the progressive apoptosis occurring under control conditions was markedly reduced by the metal. Additionally, neuritic process initiation and outgrowth increased in a concentration-dependent manner, with processes four times as abundant on day 1 and twice as long on day 2. These results suggest that brief exposure to lead during neurogenesis directly affects cell survival and process development, potentially altering cortical arrangement. Consequently, alterations in neural circuitry may underlie some of the neurological effects of Pb exposure during brain development.
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Key words: lead (Pb); neurogenesis; neural stem cell; neuronal process outgrowth; neurotrophism; cell survival; cerebral cortex

Despite legislation in the past several decades limiting distribution, lead (Pb) paint persists in many old homes (Leighton et al., 2003), Pb pipes still leech the metal into drinking water (Anonymous, 2004), and leaded gasoline is still combusted throughout the world (Falk, 2003). Neurological consequences of Pb exposure range from a tingling sensation in the extremities to seizures and coma. While effects in adults are apparently reversible, the effects of childhood Pb exposure are often insidious and persistent. Numerous studies have shown a correlation between increasing blood Pb concentration and decreasing cognitive function in children (Baghurst et al., 1992; Bellinger and Dietrich, 1994; Canfield et al., 2003). While acutely toxic effects of Pb are seen at blood concentrations above 70–80 μg/dl (Ford, 2001; NRC, 1993), cognitive effects are correlated with levels as low as 1 μg/dl (Canfield et al., 2003).

The cerebral cortex is responsible for many higher-order functions such as thought processing and language. Changes in the cerebral cortex at cellular and subcellular levels may evade a neuropathologist's detection, yet still produce consequent deficits. Specifically, moderate levels of Pb poisoning may alter developing neuron–target interactions or produce changes in cortical neuron number, changes not apparent on tissue section analysis. In our previous studies we demonstrated that a variety of extracellular signals in the embryo can alter cortical precursor proliferation and cell cycle withdrawal and differentiation (Lu and DiCicco-Bloom, 1997; Zhou et al., 2001; Carey

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et al., 2002), although the effects of neurotoxicants on primary cortical precursors remain unexplored.

A variety of previous in vitro studies have shown acutely toxic effects of Pb on numerous brain cell populations: cerebellar neurons (Oberto et al., 1996), mesencephalic neurons (Scortegagna and Hanbauer, 1997), rod and bipolar cells of the retina (He et al., 2000), peripheral neurons, glial tumors, hippocampal neurons, and mixed neuron/glia cultures from the cortex (Audesirk et al., 1991; Kern et al., 1993). A study of the effects of Pb directly on cortical neurons, however, may be informative, since glial cells appear to be critical targets and modulators of metal toxicity in neuronal populations (Tiffany-Castiglioni et al., 1989; Tiffany-Castiglioni and Qian, 2001; Lindahl et al., 1999).

We therefore used a well-characterized model to define the effects of Pb on cortical neurogenesis. Cerebral cortical precursors from embryonic day 14.5 (E14.5) rats were cultured at low density, allowing assessment of single cells (Lu and DiCicco-Bloom, 1997; Zhou et al., 2001), in defined media in the absence and presence of Pb. We studied a range of Pb concentrations that grossly correlate with that present in the whole blood of affected children, although relationships of Pb concentrations in culture models to those measured in the blood remain uncertain, as the metal binds to many cells and molecules, altering its availability (Tiffany-Castiglioni, 1993; Lidsky and Schneider, 2003). Specifically, a Pb acetate concentration of 0.3–1 μg/ml is equivalent to 1–3 μM of elemental Pb, which equals ~20–60 μg/dl in blood, a range associated with cognitive deficits. Unexpectedly, at these concentrations Pb exposure elicited increased cell survival and process outgrowth, raising the possibility of novel mechanisms by which the metal may impact early brain development.

MATERIALS AND METHODS

Cultures

Time-mated pregnant Sprague Dawley rats were obtained from Hilltop Labs (Philadelphia, PA). After sacrifice at E14.5, embryonic skull and meninges were removed and dorsolateral cerebral cortex was dissected, mechanically dissociated, and plated at 1×10^6 cells on poly-D-lysine (100 μg/ml)-coated 35-mm dishes or 24-multiwell plates in defined medium (insulin omitted), as described previously (Lu and DiCicco-Bloom, 1997). Culture medium was composed of a 50:50 (v/v) mixture of DMEM and F12 (Invitrogen, Grand Island, NY) containing penicillin (50 U/ml) and streptomycin (50 μg/ml) and supplemented with transferrin (100 μg/ml) (Calbiochem, La Jolla, CA), putrescine (100 μM), progesterone (20 nM), selenium (30 nM), glutamine (2 mM), glucose (6 mg/ml), and bovine serum albumin (10 mg/ml). Unless stated otherwise, components were obtained from Sigma (St. Louis, MO). Cultures were maintained in a 5% CO_2/air incubator.

Lead Concentrations

Lead Acetate [$Pb(C_2H_3O_2)_2$] (Sigma) was dissolved with warming in purified deionized water at a working solution concentration of 1 mg/ml. Pb acetate dilutions (0.3, 1, 3, 10, 30 μg/ml) were prepared in defined media and added to culture dishes prior to the cells. To preserve exposure conditions, culture media were not changed during the experiment incubation period. Purified deionized water was used as vehicle control for the concentration of 30 μg/ml.

Cell and Neurite Assessment

Living cells in three randomly selected 1-cm strips at 200× magnification [(3% of area)/dish, 3-4 dishes/group] were counted over 4 days under phase microscopy using previous methods (Lu and DiCicco-Bloom, 1997; Zhou et al., 2001). Different sets of parallel control and Pb-exposed dishes were counted on the different days so that the potential stress of counting did not impact later cell assessments. Using microscope stage micrometers, the counted regions were randomly positioned by setting the Y-axis at predetermined arbitrary locations (135, 140, 145) and all cells with intact cell membranes and morphology observed on the culture dish surface were counted as the stage was advanced from right to left over a 1-cm distance. These positions correlated roughly to one strip in the upper, middle, and lower thirds of the 35-mm dish. Routinely, cell numbers within these fields were within 10–15% of each other, indicating that initial cell plating was equally distributed. Zero-time cell counts were obtained 2 hr after plating and were found to be no different among different culture conditions (Control = 254 ± 21; Pb = 237 ± 24; mean cell number/strip ± SEM; n = 9 dishes), indicating that Pb addition did not alter initial cell attachment as a possible means of altering later cell counts. In previous studies characterizing these embryonic precursors after 2–3 days incubation, 85% of cells exhibited neuronal markers, MAP2 or beta-III tubulin, and 10% expressed precursors protein, nestin, whereas <1% displayed glial markers (Carey et al., 2002).

To assess neurites, cells bearing processes greater than 1 cell soma diameter at 8 hr and 2-cell soma at 48 hr were assessed in living cultures to prevent the loss of fragile processes that can occur during multiple solution changes associated with fixation and immunocytochemistry using previous methods (Lu and DiCicco-Bloom, 1997; Zhou et al., 2001). To improve visualization of thin processes for accurate length measurement, we enhanced neurite visibility by fixing cells at 48 hr and staining for neuron-specific enolase (NSE). Neurite length was measured under phase microscopy using an eyepiece micrometer. Length was assessed on every cell to appear sequentially in a counting strip chosen randomly as described above until 25 cells were counted. The radial distance from the center of the cell soma to the end of longest process was assessed, scoring 25 cells/dish and 3–4 dishes/group for each experiment. A subset of dishes were assessed independently by both investigators, yielding the same results.

Immunocytochemistry

After 4% paraformaldehyde fixation (20 min), immunocytochemistry was performed using polyclonal rabbit antibodies against NSE (Chemicon, Temecula, CA) and visualized using a Vectastain ABC Kit (Vector Laboratories, Burlingame,

CA) and DAB, using methods described previously (Lu and DiCicco-Bloom, 1997). Photographic images were obtained using a Zeiss Axiovert 35 microscope and processed using Adobe PhotoShop on a Macintosh computer.

DNA Synthesis

Incorporation of [^{3}H]thymidine ([^{3}H]dT) was used to assess DNA synthesis, indicating that cells were in S-phase of the mitotic cycle (DiCicco-Bloom et al., 2000). Cells were incubated with [^{3}H]dT (1 μCi/ml; Amersham, Arlington Heights, IL) during the final 2 hr of 24-hr cultures. Incorporation was assayed by beta scintillation spectroscopy. Unless stated otherwise, experiments were performed three or more times, with three to four samples/group/experiment.

Cell Death Assays

We assessed cell death using two different assays for apoptosis, including the Apoptosis Detection Kit (R&D Systems, Minneapolis, MN) and Hoechst 33342 (Molecular Probes, Eugene, OR) staining to detect nuclear condensation and fragmentation. Cells were incubated in control and Pb acetate containing media (3 μg/ml) and fixed at 2 or 3 days as above. The apoptosis assay was performed according to the manufacturer's instructions using Proteinase K at 1:200 for 15 min for permeabilization and HRP-DAB reaction for visualization; assessments were performed under brightfield and phase microscopy. For Hoechst 33342 staining, cells were incubated in 20 μg/ml for 15 min, rinsed three times in phosphate-buffered saline (PBS), and assessed under UV epifluorescence on a Leica inverted microscope. Cells in 10 randomly selected visual fields from three dishes each were assessed under 200× magnification for expression of apoptotic markers. Experiments were performed twice for each assay.

Statistics

Raw data from multiple experiments were grouped for statistical analysis by Student's *t*-test and ANOVA (KaleidaGraph 3.6) on a Macintosh computer. For presentation after statistical analysis, data from each experiment were normalized to the mean of its control and grouped data are expressed as percentage of control.

RESULTS

Effects of Pb on Cortical Precursor Cell Number

Numerous previous studies have shown that moderate to high concentrations of Pb can induce apoptosis of differentiated neurons (He et al., 2000; Oberto et al., 1996; Scortegagna and Hanbauer, 1997; Sharifi et al., 2002). To define the effects of Pb on developing primary cortical precursors, we assessed cell number following several days exposure to a range of Pb concentrations (Fig. 1a). In defined medium lacking trophic factors, control cell number decreased progressively over 4 days, a change that was associated with the accumulation of fragmented cell ghosts, as dead cells remained attached to the culture dish surface. Cells treated with 0.3 μg/ml Pb acetate (1 μM Pb) exhibited a decline in live cell number similar to control. Surprisingly, however, cul-

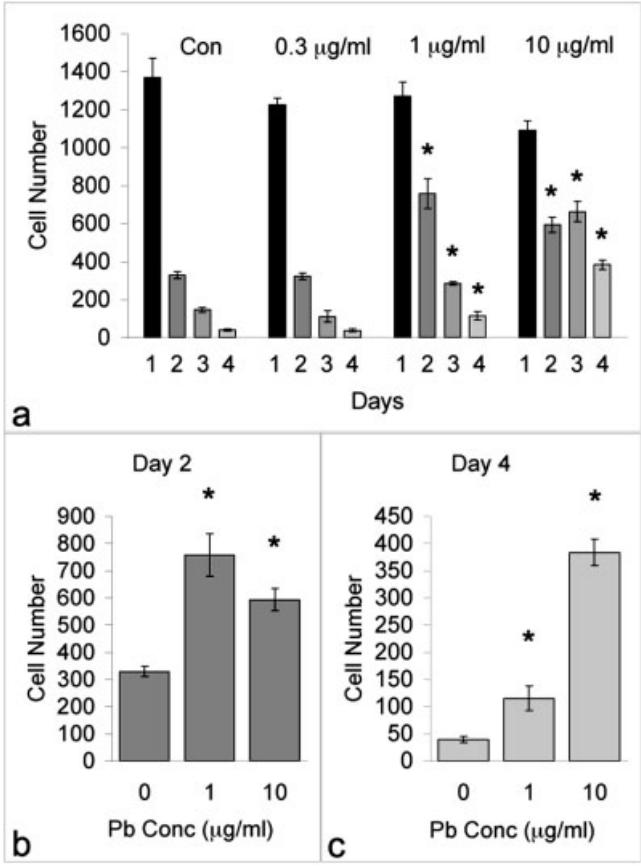

Fig. 1. Effect of Pb acetate exposure on cell number and cell death. **A:** All groups had similar cell numbers on day 1. While the cell number in control and 0.3 μg/ml Pb-treated groups decreased by two-thirds daily over the final 3 days, the 1 μg/ml Pb-treated group deceased by only one-half daily. Cells exposed to 10 μg/ml Pb decreased by one-half from day 1 to day 2, remained constant from day 2 to 3, and finally decreased by one-half on day 4. $^*P < 0.05$ compared to control daily cell number means. **B:** On day 2, twice as many cells were present in media containing 1 and 10 μg/ml Pb. **C:** By day 4, 10 times as many cells were present after exposure to 10 μg/ml Pb. Values are expressed as mean cell number per 3% of culture dish ± SEM. $^*P < 0.05$. Experimental groups consisted of 3–4 dishes and the experiment was performed more than 4 times. **D–G:** Cells were incubated 2 days in control (**D,F**) and 3 μg/ml Pb acetate (**E,G**) containing medium, and were fixed and processed for apoptotic nuclear labeling. Cells are presented by phase (**D,E**) and brightfield (**F,G**). Apoptotic cells (arrowheads) appear small in diameter under phase (**D,E**) and exhibit dark reaction product (**F,G**), and occur twice as often under control conditions. **H,I:** Cells incubated 3 days in control and Pb-containing medium were fixed and stained with Hoechst 33342 to reveal nuclear morphology. Nuclei that exhibit small diameter and increased intensity (arrowheads) were far more common in control (**H**) than Pb (**I**) containing media, whereas nuclei with normally dispersed chromatin (arrows) were more numerous in the Pb group (**I**), consistent with the metal providing a trophic signal to cultured cortical precursors. Scale bar = 25 μm. Experimental groups consisted of three dishes each and the experiment was performed twice. Figure can be viewed in color online via www.interscience.wiley.com.

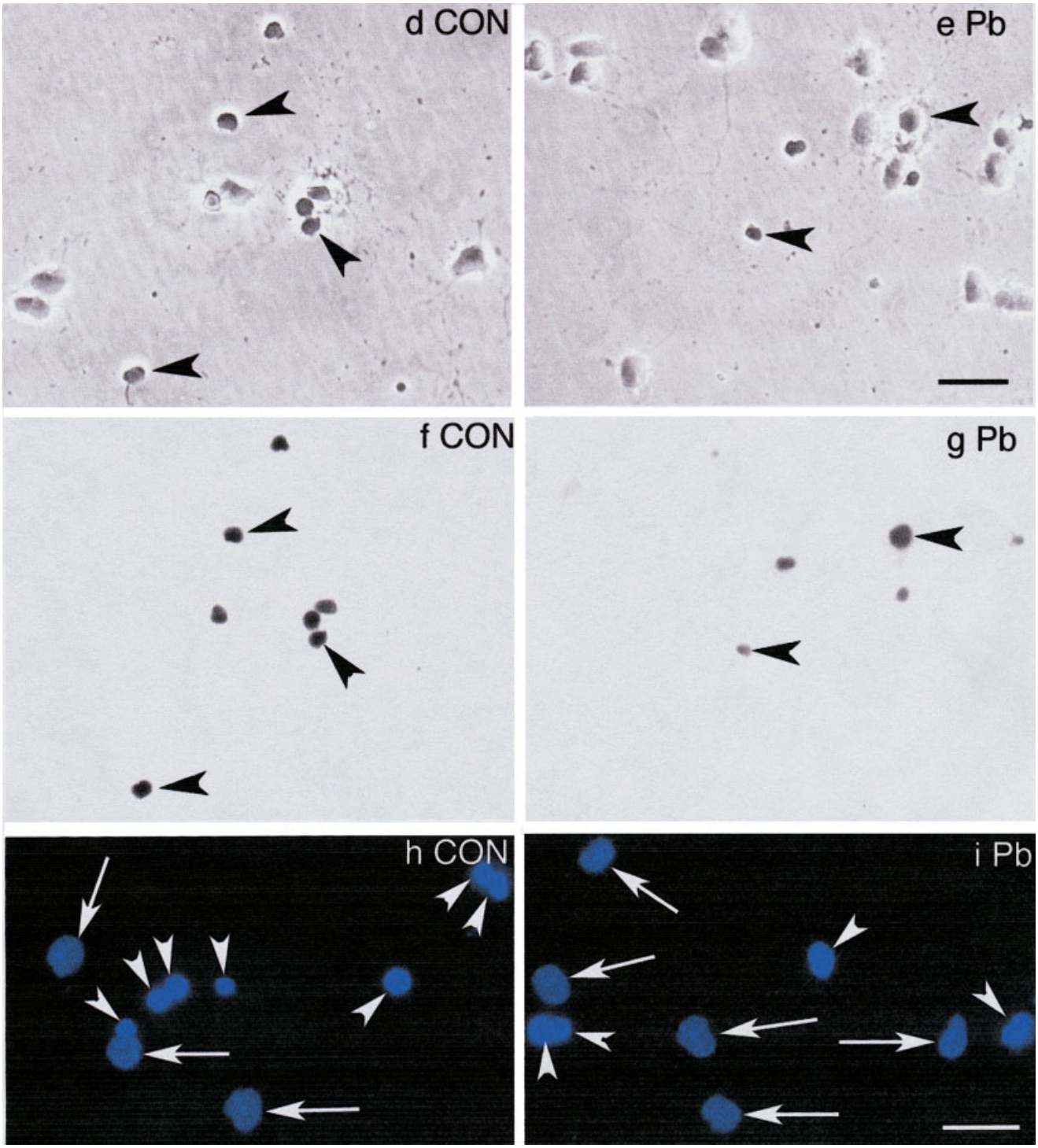

Figure 1. (Continued.)

tures exposed to 1 μg/ml Pb acetate (3 μM Pb) exhibited the same number of cells on day 1 as controls, but lost far fewer cells over the next 24 hr, suggesting the metal elicited a possible trophic effect. Furthermore, at a 10-fold higher Pb acetate concentration, 10 μg/ml, an apparent cell survival-promoting affect was sustained: cell number did not change from day 2 to 3. In contrast, when cells were treated with the highest concentration, 30 μg/ml Pb, there was a rapid 60% decrease in cell number on day 1 compared to control, followed by a

less robust decrease on successive days (data not shown). Thus, concentrations below 30 μg/ml Pb acetate were studied further.

To further characterize dose–response relationships, we present the effects of the two most effective concentrations of Pb acetate, 1 (3 μM Pb) and 10 μg/ml (30 μM Pb) on days 2 and 4. Twice as many cells were alive by day 2 in the 1 and 10 μg/ml Pb exposure groups compared to control (Fig. 1b). Most significantly, by day 4 there were 3 times as many cells present after 1 μg/ml Pb acetate exposure, and 10 times more cells present in the 10 μg/ml group compared to control (Fig. 1c).

Based on the progressive loss of cells in the control group, one possible model for Pb effects was that the metal prevented ongoing cell apoptosis. To begin addressing this issue, we examined cultures at 2 days, when a robust difference in cell numbers was apparent in control compared to Pb acetate exposed cultures (Fig. 1b). For these studies of cell death, we compared cells incubated 2 days in control medium or exposed to an intermediate Pb acetate concentration (3 μg/ml) and analyzed apoptosis using a commercial kit. Pb acetate exposure reduced ongoing cell death more than 2-fold (Fig. 1d–g; Con = 47 ± 6.5; Pb = 21 ± 3.2; mean percent apoptotic nuclei/field ± SEM; $P < 0.002$). Furthermore, when we examined the number of contracted or fragmented cell nuclei using Hoechst 33342 staining on cells after 3 days incubation, the accumulation of dead cells in control conditions was easily apparent compared to Pb-exposed cultures (Fig. 1h,i). In controls, 90 ± 6% of cells exhibited contracted/fragmented nuclei, whereas only 47 ± 3% appeared apoptotic in the Pb-exposed group (mean percent/field ± SEM; n = 6; $P < 0.001$), indicating that the metal prevented cell apoptosis and suggesting that Pb elicited a trophic response in this model system.

Effects of Pb on Precursor Proliferation

The increase in cell number elicited by Pb may not only reflect rescue from cell death, but also an increase in cell proliferation, or a combination. However, the observed increase in cell number by day 2 was not a result of increased proliferation. Specifically, there was no increase in DNA synthesis at 24 hr over a range of Pb acetate concentrations including 1 μg/ml Pb, a concentration eliciting the greatest effect on cell number by day 2. Rather, there appears to be a trend of decreasing DNA synthesis with increasing Pb exposure (Fig. 2). These observations suggest that increased cell number observed following Pb exposure (1 and 10 μg/ml) was due primarily to enhanced cell survival, and not increased cell division.

Effect of Pb on Process Length

In addition to an increase in cell number, we also observed a change in process outgrowth (Fig. 3a–d). By day 2, twice as many cells exposed to 1 and 10 μg/ml Pb had a process as compared to controls (Fig. 3e). Additionally, of those cells with processes, the process length was 1.5 times greater for cells treated with 1 μg/ml Pb, and 2 times greater for cells treated with 10 μg/ml Pb (Fig. 3f). As a possible indicator of the rate of process growth, we measured the distance to the first process branch point. This measurement increased as well, with a 4-fold greater distance for cells exposed to 1 μg/ml Pb, and a 7-fold increase for those exposed to 10 μg/ml Pb.

A number of cellular mechanisms may underlie increased process growth observed at 2 days, especially since there are twice as many cells present in the Pb-exposed cultures at this time (Fig. 1b). To begin examining this issue, we assessed process initiation at 8 hr, a time when cell numbers among groups were the same. Cells exposed to 10 μg/ml of Pb for 8 hr were 4 times as likely to have grown a neurite than controls, indicating that the toxicant increased process initiation (Fig. 4a–c). Thus, Pb exposure increased process elaboration through enhanced neurite initiation and growth rate, events independent of its survival-promoting activity.

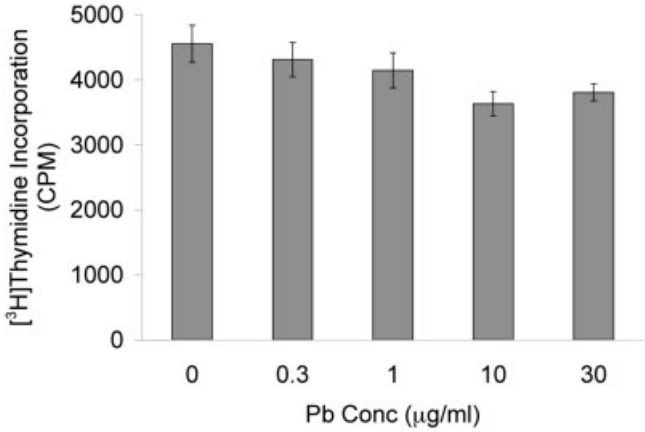

Fig. 2. Effect of Pb on cortical precursor cell DNA synthesis. Pb (0.3–30 μg/ml) does not stimulate [^{3}H]dT incorporation in 24-hr cultures, indicating that DNA synthesis and cell proliferation were not increased in the Pb-treated cells. Values are expressed as mean counts per minute ± SEM. Experimental groups consisted of four wells and the experiment was performed twice with similar results.

DISCUSSION

Our observations indicate that Pb exposure increased the number of developing cortical neurons in culture. The increase in neuron number was a consequence of enhanced cell survival (trophism) through reduced cell death, rather than increased precursor cell division. These results were unexpected, as the preponderance of data suggests that Pb exposure is detrimental to cell survival when it is affected. Studies of Pb effects on cells in the nervous system, including retinal receptors (Fox et al., 1997; He et al., 2000), peripheral neural tumors, hippocampal and cortical neurons (Audesirk et al., 1991; Kern et al., 1993), and oligodendrocyte progenitors (Deng and Poretz, 2002), as well as those out-

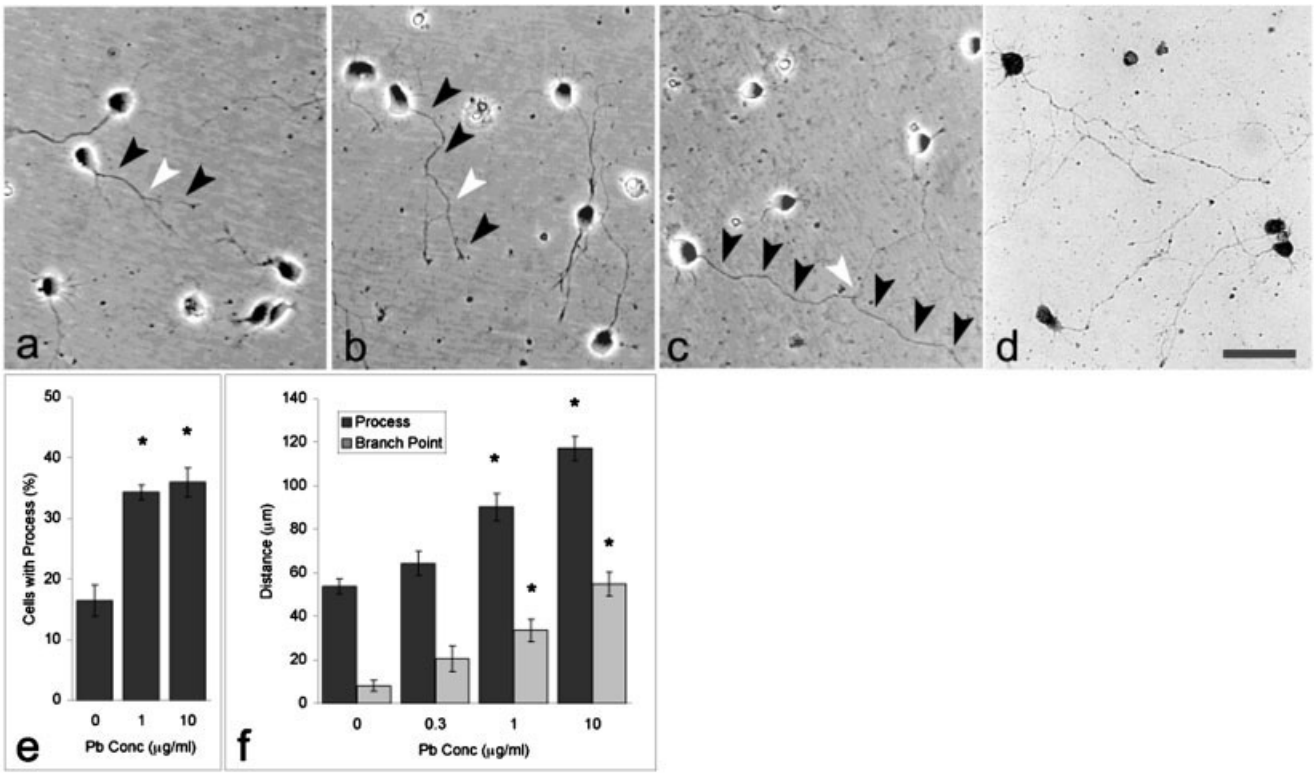

Fig. 3. Effects of Pb on process length and branching. **A–C:** Living cells viewed under phase microscopy. At 2 days, process length (black arrowheads) was shortest in control (**A**) and grew successively longer in cells exposed to 1 μg/ml Pb (**B**) and 10 μg/ml Pb (**C**). Distance to the first branch point (white arrowhead) was also increased, suggesting that Pb enhanced process growth rate. **D:** To enhance the detection of processes, cells were assessed after immunocytochemical staining for NSE. **E:** Twice as many cells exposed to 1 and 10 μg/ml Pb bore processes compared to controls. **F:** Of cells bearing a process, the length was 68% greater for those exposed to 1 μg/ml Pb and over 2 times as long for 10 μg/ml Pb. The distance to the first branch point also increased concurrently. Data are expressed as mean ± SEM (scale bar = 30 μm). $*P < 0.05$. Experimental groups consisted of 3–4 dishes and the experiment was performed more than 4 times.

side the CNS, such as alveolar macrophages (Shabani and Rabbani, 2000) and fibroblasts (Iavicoli et al., 2001), indicate that metal exposure increased cell death over the concentration range employed herein. However, our studies also demonstrated enhanced cell death at higher concentrations (30 μg/ml). On the other hand, a prior study of hippocampal cells and peripheral tumors (Audesirk et al., 1991) and another in hepatocytes (Iavicoli et al., 2002) suggested survival-promoting effects may be induced by Pb exposure.

An additional trophic effect induced by Pb was increased process outgrowth. A greater percentage of Pb-exposed cells grew processes, and of those cells with processes, neurite length was significantly increased. The increase, however, was not due to selective survival, as even at a time when cell number was equal across groups, more cells in the Pb-exposed group had a process. Furthermore, the changes in process initiation were observed as early as those elicited by classical trophic factors, such as vasoactive intestinal polypeptide (VIP) and nerve growth factor (NGF) in neurons (Pincus et al., 1990, 1994). Since the first branch point of the process was farther from the cell body in Pb-exposed cells, these observations suggest that the metal induced both increased process initiation as well as enhanced rate of growth, a parameter that future time-lapse studies may address. In marked contrast to our results, a number of studies report inhibitory effects of Pb on neurite initiation or outgrowth in hippocampal, mesencephalic, and retinal ganglion neurons over a broad range of concentrations, including those we employed (Cline et al., 1996; Kern and Audesirk, 1995; Schneider et al., 2003; Scortegagna et al., 1998). However, positive neuritogenic effects have been observed in mixed hippocampal cultures and neural tumors (Audesirk et al., 1991; Crumpton et al., 2001), where neuron–glial cell interactions may play a modulatory role (Tiffany-Castiglioni et al., 1989; Tiffany-Castiglioni and Qian, 2001; Lindahl et al., 1999). However, in the current studies the stimulation of neurite outgrowth by Pb occurs directly and not through glia, as previous characterization indicates that 85% of cells exhibited neuronal markers, MAP2 or

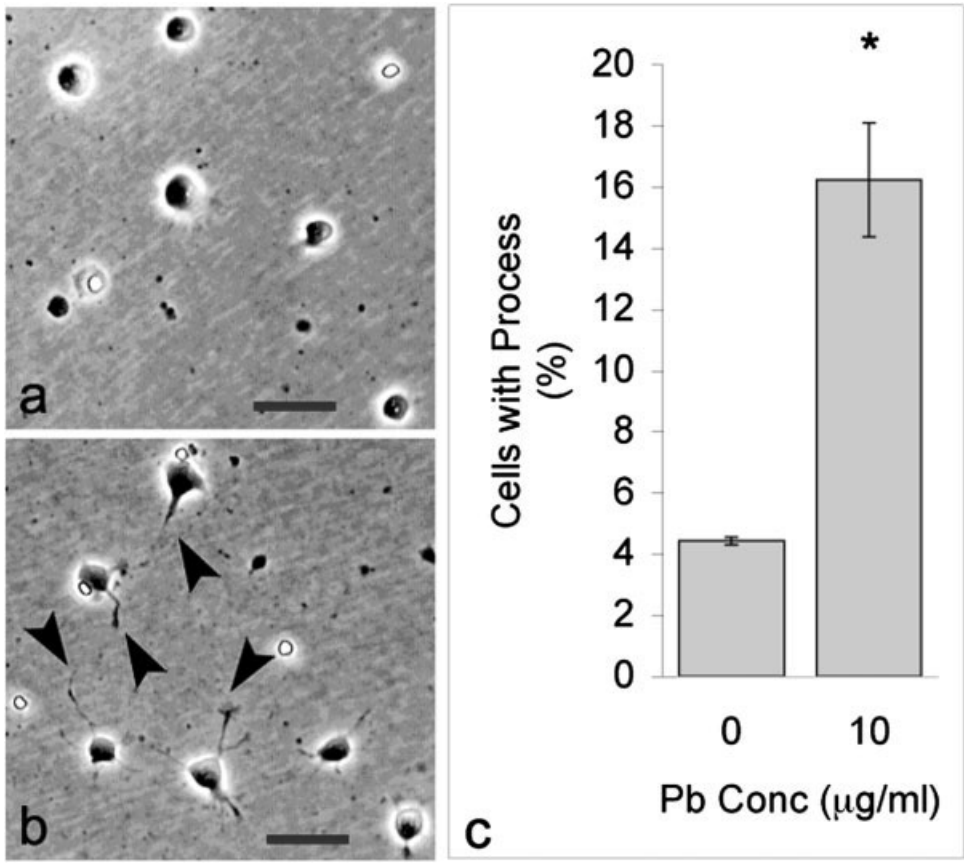

Fig. 4. Effect of Pb on process outgrowth. **A,B:** Living cells viewed under phase microscopy. Arrowheads indicate process-bearing cells. At 8 hr, control cells (**A**) exhibit far fewer processes than cells exposed to 10 μg/ml Pb (**B**). **C:** Four times as many cells have a process after Pb exposure. The proportion of cells bearing a process is expressed as mean percent ± SEM of cells exhibiting a process at least 1 cell soma in length. $*P < 0.05$. Experimental groups consisted of four dishes and the experiment was performed twice.

beta-III tubulin, and 10% expressed precursor protein, nestin, whereas <1% displayed glial markers (Carey et al., 2002), and neurite-bearing cells rarely make contact with other cells.

The effects of Pb we observe here in culture naturally raise questions about the possible relevance to metal exposure in developing children. The trophic effects in primary cortical precursors occurred at Pb levels that correlate with subtoxic events in children (20–60 μg/dl). These children are not obviously ill, yet they suffer subclinical deficits. No direct correlation exists between the Pb concentration in neuronal culture and that found in the blood of symptomatic children. In culture models, some studies have found the free ionic Pb concentration to be 100–1,000-fold lower than the original concentration (Audesirk et al., 1989). In human samples, Pb concentration in plasma is over 100-fold lower (0.27–0.70%) than that of whole blood (Hernandez-Avila et al., 1998). If the only active forms of the metal are the free ionic Pb in culture and the plasma Pb in blood, then the Pb concentrations may be approximately equivalent. Otherwise, they may be over 100-fold different (Audesirk et al., 1989; Tiffany-Castiglioni, 1993; Lidsky and Schneider, 2003). Furthermore, the relatively high concentrations required to elicit trophic effects may reflect regional-specificity of Pb effects. A selective effect of Pb on neuronal populations was recently demonstrated by Huang and Schneider (2004). In their work, relatively low concentrations of Pb, 0.03 μg/ml, decreased proliferation in neural stem cells from the striatum and ventral mesencephalon, while only concentrations above 3 μg/ml affected neural stem cells from the cortex: over 100 times the Pb concentration affecting the other neuronal populations, raising the possibility that forebrain neurons may be relatively resistant to the metal. The authors did observe, however, a uniform decrease in cell survival across all three neuronal populations with concentrations greater than 15 μg/ml Pb (>50 μM), similar to the response in our studies.

Thus, in aggregate, our observations suggest that Pb can elicit several trophic effects in developing cortical neurons. Neurons grew longer and more varied processes, and survived longer, responses typical of classical neurotrophic factors, including VIP/PACAP, NGF, and neurotrophin-3 (Pincus et al., 1990, 1994; DiCicco-Bloom et al., 1993, 2000). Further, these trophic effects varied with concentration. While a Pb concentration of 1 μg/ml elicited the greatest effect on cell survival on day 2, the 10 μg/ml concentration stimulated the longest process outgrowth on this day, suggesting differential effects of the different doses. Furthermore, by day 3 the greatest cell survival was maintained by 10 μg/ml, which elicited twice the cell number as 1 μg/ml, suggesting that either cellular response mechanisms or available

metal may be changing with time. Nonetheless, these separate and distinct effects of different concentrations of Pb on cell survival and process outgrowth suggest that the metal alters independent pathways as a function of concentration, an issue for future study.

Possible targets through which Pb may elicit its effects are numerous. While Pb is known to interfere with calcium sequestration and signaling, these interactions are usually detrimental (Ferguson et al., 2000; Lasley et al., 1999; Peng et al., 2002; Zhang et al., 2002). However, Pb has also been shown to directly interact with and enhance the activity of calmodulin (Habermann et al., 1983; Kern et al., 2000), which has been independently shown to increase cell survival (Egea et al., 2001; Perez-Garcia et al., 2004). Novel Pb targets that enhance neuronal survival may include pathways involving proteins in the Bcl-2 family. The possible roles of signaling pathways may be best examined through gene profiling approaches, studies that are currently under way.

Activation of various isoforms of protein kinase C (PKC) have been shown to enhance neurite outgrowth (Brodie et al., 1999; Hundle et al., 1995; Newton, 1995). Pb has been shown to interact with PKC, which may subsequently affect a variety of downstream signaling pathways (Chen et al., 1999; Cremin and Smith, 2002; Deng and Poretz, 2002; Jadhav et al., 2000). Additional targets that affect process initiation and outgrowth include the MAP kinase pathways and potentially interactions with actin and myosin and their regulatory proteins (Williams et al., 2000; see Discussion, Schneider et al., 2003).

In sum, our observations indicate that Pb induces enhanced neuronal cell survival and process outgrowth in primary neurons of the developing cerebral cortex in culture. Our studies, performed on virtually pure cortical precursors, as previously characterized (Carey et al., 2002), raise questions regarding Pb's effects in the complex environment of the developing brain in vivo. The occurrence of increased cell number and process elaboration may interfere with proper neuron–target interactions, especially since regressive events, including reductions in neuron numbers and the ''pruning'' of neuronal processes, are normally involved in brain maturation. In turn, alterations in neural circuitry may potentially contribute to the subclinical cognitive deficits observed in children exposed to Pb during brain development.

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