Influence on the cellular organization in central nervous system micromass cultures

by *Erik Walum*[1], *Kathryn Jones*[2], *Gun Ekblad-Sekund*[1], *Inger Varnbo*[1] and *Cecilia Clemedson*[1]
[1] Unit of Neurochemistry and Neurotoxicology, Stockholm University, S-106 91, Stockholm, Sweden.
[2] ICI Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire SK10 4TJ, UK.

INTRODUCTION

Exposure of fetal and neonatal rodents to relatively low levels of inorganic lead results in changes in gross morphology of brain regions and in fine structure of neurons (for review see *Audesirk* 1985). At concentrations of about 0.3–0.5 μg/ml blood, lead has been demonstrated to induce neurobehavioral deficits in rodents. These effects appear to be independent of the developmental stage during which exposure occurs. Thus, neurobehavioral deficits have been attributed to lead exposure during prenatal, postnatal as well as postweaning periods. A possible explanation of these observations is that low-level lead exposure may affect cell-cell interaction and synapse formation (*Cookman et al.* 1987). In support of this notion lead has been found to affect synaptogenesis and more specifically inhibit the desialytation of neuronal cell adhesion molecule (N-CAM; *Cookman et al.* 1987). N-CAM is a complex of membrane bound glycoproteins that is developmentaly regulated and believed to be intimately involved in the orderly structuring of the CNS.

Embryonic midbrain micromass cultures are started from disaggregated, undifferentiated neural epithelium and grown as small discrete islands at very high cell densities (*Flint* 1983). Cell to cell interactions are thereby facilitated. No measures are taken to enrich the cultures in one type of cell, and both neuroblasts and glioblasts can thus proliferate and interact unrestrictedly. A high degree of differentiation is attained during the course of cultivation (*Flint* 1983) and the cellular development closely resembles that in vivo. Cells that are destined to differentiate as neurons actively segregate from the other cells in the island and aggregate together into foci, which eventually become interconnected by an extensive network of neurites. These cultures may be used as models for studies of teratogenic and subteratogenic effects of chemicals on neuronal development (*Flint* 1987, *Walum & Flint* 1990). We have used foci formation in micromass cultures to study the concentration dependent effects of lead on early cell interactions. Comparisons have been made with the effects of trimethyltin chloride, cadmium chloride and mercury chloride.

MATERIALS AND METHODS

Rat embryo midbrain micromass cultures

The method has been described previously (*Flint* 1983, *Flint & Orton* 1984, *Flint* 1987). Embryos (10–15 per animal) were removed from pregnant Alderley Park:APfSD (Wistar-derived) rats, 13 days postcoitum, into a 1:1 mixture of horse serum and Earle's balanced salt solution (EBSS) at 37°C. Mid-brains (CNS) were removed from embryos with 34–36 somites (Fig. 1) by microdissection and pooled. CNS tissues were then successively washed in calcium and magnesium free EBSS (CMF, 20 min, 37°C) and trypsin (1 % w/v in CMF, 20 min, 37°C), then resuspended in Ham's F12 culture medium and dissociated into a suspension of individual cells by repeated trituration through a Pasteur pipette (internal mouth diameter adjusted to approximately 0.7 mm). The cell suspension was passed through a 10 μm mesh nylon filter to remove undissociated clumps of two cells or more. The cell suspension was adjusted to

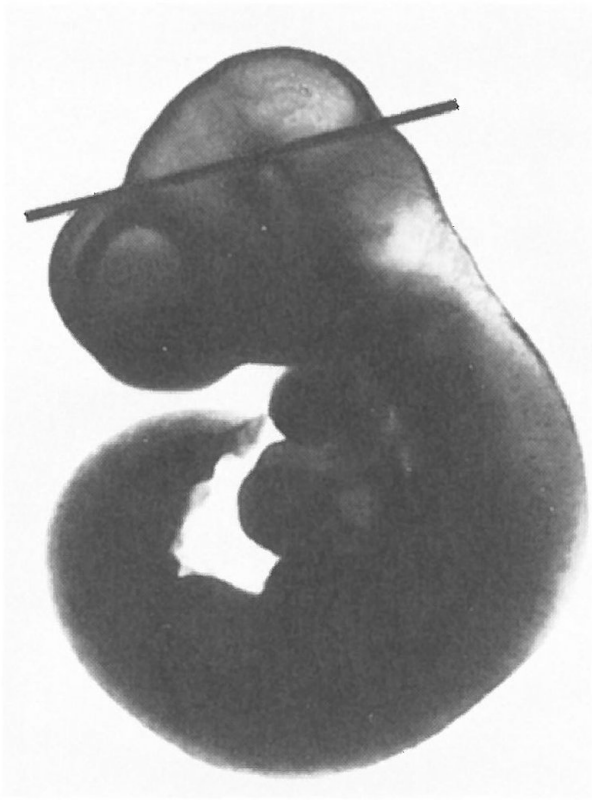

Figure 1. Rat embryo, 12,5 days of age. Line indicates the section used to remove midbrain.

give 5×10^4 CNS cells per 10 µl. Culture medium was supplemented with 10 % fetal bovine serum, and 585 mg L-glutamine, 1×10^6 I.U. penicillin and 100 mg streptomycin per litre. Aliquots (10 µl) of the cell suspension were delivered to 35 mm tissue culture dishes (Falcon 3801 Primaria) or to 96-well plates (Falcon 3872 Primaria). Dishes contained five separate CNS aliquots, and wells a single aliquots. Cultures were incubated at 37°C in 5 % CO_2 : 95 % air and 100 % humidity for 2 h. Test compound was added in culture medium in a final volume of 2 ml (dishes) or 200 µl (wells) and cultivation continued for 5 days.

Chicken embryo midbrain micromass cultures

The method used for chicken embryo midbrain micromass cultures was a slight modification of that described for rat brain cultures. Fertilized hen's eggs were delivered by Linköping Kontrollhönseri, Sweden. Chicken embryos were obtained from eggs incubated for 3.5 days. Thirty embryos were removed under sterile conditions and washed once in EBSS in a petri dish. From there on the two procedures were identical with the exception that no trypsin was used to dissaggregate the chicken cells.

Neuronal-glial cell line micromass cocultures

The continuous mouse neuroblastom cell line C1300, clone N1E115 (*Amano et al.* 1972) was recloned in our laboratory. This subclone, designated SUNE, was grown in 150 cm^2 tissue culture flasks in Ham's F10 medium supplemented with 4 % fetal calf serum, 9 % newborn calf serum, 50 units/ml of penicillin and 50 µg/ml of streptomycin. A finite cell line, Ap, of rat glial cells was established by subcultivation of a primary astrocyte culture from newborn rat brain hemispheres. Ap cells were grown in the same manner as described for SUNE cells. Both cell lines were subcultivated once and medium was changed twice a week. The cells were maintained at 37°C in a humidified atmosphere of 4 % CO_2 in air. Four hours before the addition of lead, cells were trypsinised (0.25 % trypsin in a Ca^{2+} and Mg^{2+} free phosphate buffered balanced salt solution), mixed in a concentration of 1.25×10^6 Ap cells and 0.75×10^6 SUNE cells and plated as 10 µl droplets in 35 mm Falcon Primaria tissue culture dishes, five aliquots per dish. Cells were allowed to settle for 4 h at 37°C, forming circular cell islands, then 2 ml of supplemented F10 medium containing various concentrations of lead was added to the dishes. These neuronal-glial cell line cocultures were then cultivated for 5 or 7 days at 37°C without any change of medium.

Fixation and staining

Dishes: Medium was removed from the dishes and replaced with 1 ml of 10 % formaldehyde per dish. The fixative was remo-

ved with running tap water after 20 min. Cells were then stained for 1–3 min with haematoxylin, followed by washing with tap water. The cultures were then air dried.

96-well plates: After removal of the medium from the plate, cells were fixed for 20 min with 4.5 % glutaraldehyde in water, and washed once with Dulbecco's phosphate buffered saline (PBS). The PBS was replaced with 200 µl neutral red stain (0.1 % w/v neutral red in PBS). Cells were stained for 60 min at room temperature, the stain removed and the cells washed twice with PBS. Stain was eluted from the cells into 200 µl acid alcohol (0.5 % v/v acetic acid in 50 % ethanol) per well for a minimum of 2 h.

Morphometric analysis

The number of (stained) individual foci within each micromass island (Fig. 4) was measured by an automated image analyser (AMS 40–10 colony counter, Analytical Measuring Systems, Saffron Walden, Essex, UK) attached to a dissecting microscope. The image analyser detects and counts areas of high contrast in the cultures, caused by selective staining of the differentiated cell foci. The concentration (IC50) at which each compound inhibits the formation of differentiated foci by 50 % of the control value was estimated graphically from the plotted concentration mean effect.

Determination of cell survival

Stain intensity (optical density) of the alcohol eluates of neutral red in each well was measured at 550nm with a Multiskan spectrophotometer (Flow Laboratories, Irvine, Scotland). Cell number is directly related to the absorbance of the eluted stain. The concentration (IC50) at which each compound reduced the cell number by 50 % of the control value was estimated as in the morphometric analysis.

In experiments with neuron-glial cell line micromass cocultures cell survival was estimated by determination of the diameter of the entire cell island using a measuring eyepiece in an inverted microscope.

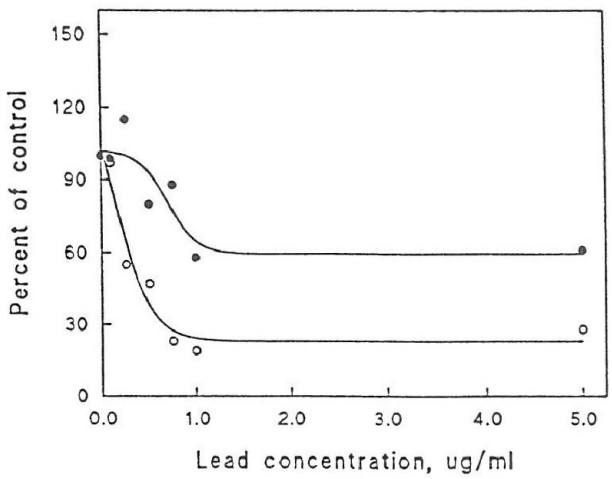

Figure 2. The effect of lead acetate on cell survival (●—●) and foci formation (○—○) in rat embryo midbrain micromass cultures. Each point represents the mean value of four measurements.

RESULTS

In rat embryo midbrain micromass cultures lead acetate was found to inhibit foci formation in a concentration dependent maner in the range of 0.25 to 1.0 µg/ml (Fig. 2). At these concentrations lead had a much less pronounced effect on cell survival (Fig. 2), and IC50 values for foci formation and cell survival were found to differ significantly ($p < 0.01$; Table 1). The other tested metal

Table 1. Effects of heavy metal compounds on foci formation and cell survival in rat embryo midbrain micromass cultures.

Compound	IC50 (µg/ml)	
	Foci formation	Cell survival
Trimethyltin chloride	0.28±0.05	0.33±0.05*
Lead acetate	0.48±0.23	0.86±0.16**
Cadmium chloride	6.6±2.1	7.0±2.9*
Mercury chloride	0.20±0.08	0.30±0.08*

The IC50 values were determined graphically from the plotted concentration mean responses of four independent quadruplet experiments (± S.E.M.).

* Difference between foci formation and cell survival values is not statistically significant.

** Difference between foci formation and cell survival values is statistically significant ($p < 0.01$).

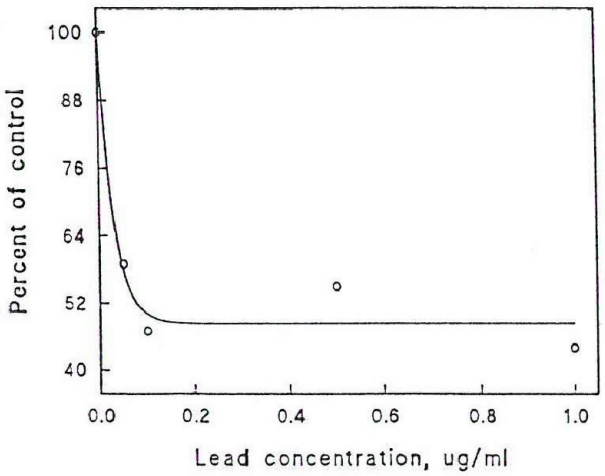

Figure 3. The effect of lead acetate on foci formation in chicken embryo midbrain micromass cultures. Each point represent the mean of four measurements.

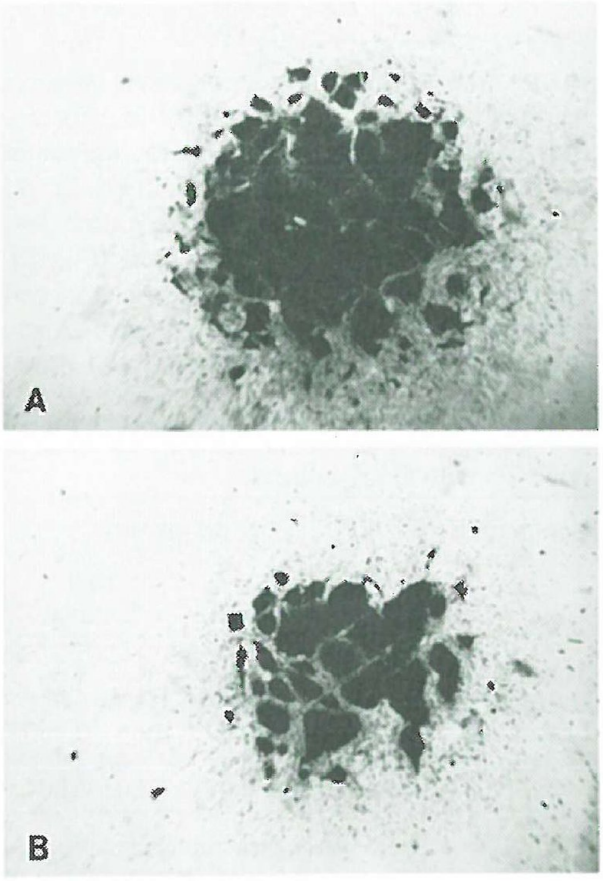

Figure 4. The effect of lead acetate on foci formation in chicken embryo midbrain micromass cultures. The cultures have been stained and photographed to emphasise the neuronal foci. A: control culture, B: culture treated from 0 to day 5 with 0.5 µg lead acetate per ml of medium. Enlargement: 20 ×.

compounds, trimethyltin, cadmium and mercury, were found to be highly toxic to the embryonic midbrain cells but did not produce a selective effect on foci formation (Table 1).

Foci formation in chicken embryo midbrain micromass cultures was sensitive to lead exposure in a concentration range of 0.05 to 0.5 µg/ml (Fig. 3). The reduction in foci number at 0.5 µg/ml is illustrated in Fig. 4.

When SUNE and Ap cells were mixed and plated in the same way as the embryonic cells the cell lines formed micromass cultures, which were structurally very similar to the midbrain cultures. The neuronal cells thus actively segregated from the glial cells in the island and aggregated into foci. However, this foci formation was not sensitive to lead. No reduction in foci number could be detected at lead concentrations up to 5.0 µg/ml, nor did lead produce any marked cytotoxicity in these cultures after 5 days incubation (Fig. 5). Incubation had to be extended to 7 days for lead to exert a cytotoxic effect at 5.0 µg/ml in these cocultures (Fig. 5).

In all experiments poor concentration-effect relations were obtained for lead concentra-

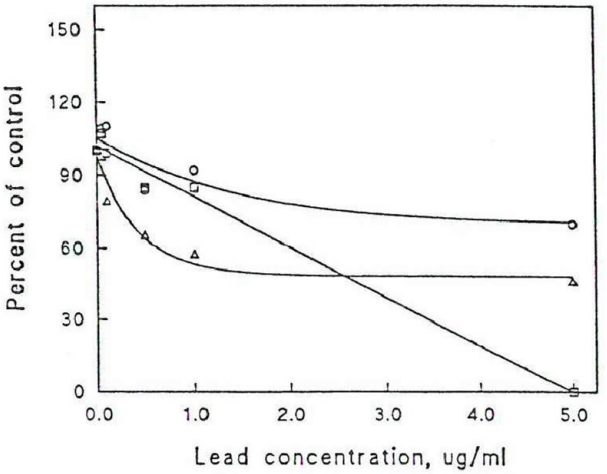

Figure 5. The effect of lead nitrate on foci formation in rat embryo midbrain micromass cultures (△——△) after 5 days exposure and on cell survival in celline micromass cocultures after 5 (○——○) and 7 (□——□) days exposure. Each point represents the mean of two determinations.

tions exceeding 1.0 μg/ml. This is most likely due to an accentuated interaction between lead and serum proteins in the culture medium at high lead concentrations.

DISCUSSION

Using inhibition of foci formation and reduction in cell number as endpoints for differentiation and cytotoxicity respectively, the rat embryo mid-brain micromass cultures are currently employed together with rat embryo limb bud micromass cultures as a model system for prediction of teratogenic effects (*Flint* 1987). Validation studies have shown that this in vitro assay has an accuracy of prediction of greater than 90 % (*Flint & Orton* 1984).

Since the cultured embryonic mid-brain (CNS) cells undergo differentiation processes closely resembling those taking place in vivo, it should also be possible to use these cultures in studies of subteratogenic effects of chemicals on brain development (i.e. causation of functional, but not necessarily morphological, anomalies). Exploration of this possibility has given promising results (*Walum & Flint* 1988, 1990, 1991). In this study we found that lead caused disturbances in the orderly structuring and development of rat and chicken embryo midbrain micromass cultures. These effects occurred at concentrations lower than those that produced general reductions in cell growth and survival in the cultures. They were, however, comparable to those blood concentrations that have been shown to be functionally neuroteratogenic in the rat. A time-lapse video micrographic analysis of cell movements in unexposed rat embryo midbrain micromass cultures showed that neuroblast migration was highly directed towards aggregate formation, whereas in lead exposed cultures neuroblasts appeared to move more randomly (unpublished observations).

The ability of lead to induce impairment of cellular organization in the rat midbrain cultures was compared to that of three other heavy metal compounds. None of these caused a selective effect on foci formation. Consequently, lead seems to exert a specific effect. This notion was supported by the finding that neuronal cell aggregation could not be selectively inhibited in cell line micromass cocultures. Therefore, lead most likely caused the structural effects through interference with a true embryonic property expressed only in the embryonic cultures.

We conclude from these results that embryonic midbrain micromass cultures may serve as valuable models for further studies of the cellular specificity and selectivity of lead induced structural disturbances in the developing brain and of the underlying molecular mechanisms.

Acknowledgements

This study was supported by the Swedish Fund for Scientific Research Without Animal Experiments, the National Swedish Environmental Protection Board and a grant from ICI (Pharmaceuticals Division)plc. Thanks are due to Mrs W. Davies for performing the image analysis on chicken embryo cultures.

Summary

The effects of lead on cellular neuronal development and organization have been studied. Rat embryo midbrain micromass cultures were exposed to lead acetate (0.1–5.0 μg/ml) for five days. Differentiation was indicated by the formation of neuronal foci in the cultures. Effects on cell growth and survival were estimated using a neutral red staining method. Lead was found to inhibit foci formation at concentrations (0.25 and 0.5 μg/ml) which did not affect cell survival. This selective effect on neuronal development was not found for three other heavy metal compounds: trimethyltin chloride, cadmium chloride and mercury chloride. In chicken embryo midbrain micromass cultures, foci formation was inhibited by even lower concentrations of lead (0.05–0.5 μg/ml), whereas neuronal cell aggregation in cell line micromass cocultures was unaffected by lead in concentrations up to 5.0 μg/ml. It is concluded that lead causes a disturbance in the neuronal development in embryonic central nervous system micromass cultures by a specific and selective effect on a property only expressed in embryonic cells.

Sammandrag

Effekterna av bly på den cellulära neuronala utvecklingen och organisationen har undersökts.

Mikromasskulturer preparerade av mitthjärnan från råttembryon exponerades för blyacetat (0,1–5,0 µg/ml) i 5 dagar. Utvecklingsprocessen studerades genom en kvantifiering av uppkomsten av neuronala foci i kulturerna. Effekter på celltillväxt och -överlevnad mättes med hjälp av en neutralröttfärgningsmetod. Bly inhiberade focibildningen vid koncentrationer (0,25 och 0,5 µg/ml), som inte hade någon effekt på cellöverlevnaden. Denna selektiva effekt på den neuronala utvecklingen kunde inte iakttagas för tre andra tungmetallföreningar: trimetyltennklorid, kadmiumklorid och kvicksilverklorid. I mikromasskulturer av mitthjärnan från kycklingembryon hämnade bly focibildningen vid ännu lägre koncentrationer (0,05–0,5 µg/ml), medan nervcellsaggregationen i samodlade mikromasskulturer av neuroblastoma- och gliacellinjer inte påverkades av bly i koncentrationer upp till 5,0 µg/ml. Vi har, med utgångspunkt från de erhållna resultaten, dragit slutsatsen att bly i låga koncentrationer förorsaker störninger i den neuronala utvecklingen i embryonala mikromasskulturer från centrala nervsystemet genom att specifikt och selektivt påverka egenskaper som endast uttrycks i embryonala celler.

References

Amano, T. E. Richelson & M. Nirenberg: Neurotransmitter synyhesis in neuroblastoma clones. Proc. Nat. Acad. Sci. USA 1972, *69,* 258–263.

Audesirk, G.: Effects of lead exposure on the physiology of neurons. Prog. Neurobiol. 1985, *24,* 199–231.

Cookman, G. R., W. King & C. M. Regan: Chronic low-level lead exposure impairs embryonic to adult conversion of neuronal cell adhession molecule. J. Neurochem. 1987, *49,* 399–403.

Flint, O. P.: A micromass culture method for rat embryonic neural cells. J. Cell Sci. 1983, *61,* 247–262.

Flint, O. P. & T. C. Orton: An in vitro assay for teratogens with cultures of rat embryo midbrain and limb bud cells. Toxicol. appl. Pharmacol. 1984, *76,* 383–395.

Flint, O. P.: An in vitro test for teratogens using cultures of rat embryo cells. In: Vitro Methods In Toxicology, Atterwill, C. K., and Steel, C. E. eds. Cambridge University Press, Cambridge 1987, pp 340–363.

Walum, E. & O. P. Flint: Acrylamide, 2,5-hexanedione and β-aminopropionitrile toxicity tested in rat embryo mid-brain cell cultures. ATLA 1988, *15,* 238–244.

Walum, E. & O. P. Flint: Midbrain micromass cultures: a model for studies of teratogenic and sub-teratogenic effects on CNS development. Acta Physiol. Scand. 1990, *140, suppl. 592,* 61–72.

Walum, E. & O. P. Flint: Selective effects of acrylamide, metylene bisacrylamide and haloperi dol on neuronal development in rat embryo midbrain micromass cultures. Submitted for publication.