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BINDING OF RADIOACTIVE METHYLMERCURIC CHLORIDE IN SUBCELLULAR FRACTIONS OF NEUROBLASTOMA AND GLIOMA CELLS IN CULTURE

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SUMMARY

The binding of radioactive methylmercuric chloride ($[^{203}\text{Hg}]\text{-CH}_3\text{HgCl}$) with various subcellular fractions of glioma (C-6) and neuroblastoma (NBP₂) cells was studied. Confluent cells were incubated in the presence of radioactive CH_3HgCl for a period of 5 h, and then various fractions were isolated. Most of the radioactivity was associated with the cytosol and particulate fractions. The cytosol and lipoprotein fractions of glioma cells incorporated more radioactive CH_3HgCl than those of NB cells. The specific radioactivities of the particulate, lipid and chromatin fractions were comparable in both cell types.

INTRODUCTION

It is now well established that methylmercuric chloride (CH_3HgCl) produces extensive damages to neurons, neuroglia and nerve fibers and causes neurological disorders which are referred to as Minamata disease [1–3]. CH_3HgCl accumulates in the central nervous system in rather large proportions after ingestion or after intravenous or intraperitoneal administration [4–7]. We are currently using cultures of rat glioma (C-6) and mouse NB cells as model systems to examine the cellular and molecular basis of CH_3HgCl -induced damages to the nervous tissue. We have shown that the glioma cells are much more sensitive to CH_3HgCl than NB cells on the criteria of inhibition of cell division and cell death [8]. In order to understand the cellular and molecular

Abbreviations: EGTA, ethyleneglycol tetraacetic acid; NB, neuroblastoma; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; TCA, trichloroacetic acid.

mechanisms of the effects of CH_3HgCl , knowledge of cellular localization and binding of CH_3HgCl with various subcellular fractions is important. In addition, a comprehensive study on the binding of CH_3HgCl to various fractions of glioma and NB cells may be important in explaining the reasons for the differential sensitivities of these cell types to CH_3HgCl . We report that the cytosol and lipoprotein fractions of glioma cells incorporate more radioactive CH_3HgCl than those of NB cells.

MATERIALS AND METHODS

Cell culture

Mouse neuroblastoma (clone NBP_2) [9], and rat glioma cells (clone C-6, passage 19–35) [10,11] were used in this study. Neuroblastoma cells are grown in F12 medium containing 10% agammaglobulin newborn calf serum, whereas glioma cells are grown in MEM containing 10% fetal calf serum [8]. The growth media also contain penicillin (100 U/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$). The cells are maintained at 37°C in a humidified atmosphere of 5% CO_2 . Cells ($2 \cdot 10^6$ glioma; $0.25 \cdot 10^6$ NB) were plated in Lux tissue culture dishes and growth medium (20 ml) was changed 2 and 4 days after plating. At day 4 medium was changed twice because of confluency. At 5 days after plating fresh growth medium (5 ml) was added 30 min before the addition of [^{203}Hg]- CH_3HgCl . Cells were incubated in the presence of radioactive [^{203}Hg]- CH_3HgCl (2 $\mu\text{Ci}/\text{ml}$) for a period of 5 h. After incubation glioma cells were washed twice with PBS, pH 7.0 and the cells were incubated in the presence of 0.25% trypsin solution for 35–40 min. Cells were removed from the dish and washed twice with growth medium and twice with PBS. NB cells were removed from dishes by incubating them in pancreatin solution (0.25%) for 15 min, and they were washed in the manner described for glioma cells.

Preparation of nuclei

Nuclei were isolated from NB and glioma cells according to the method of Hymer and Kuff [12] with subsequent modifications [13]. The cells were suspended in 4 vol. of 0.5% Triton X-100, 20 mM EDTA, and 80 mM NaCl, pH 7.4, and mixed gently but thoroughly by repeated expulsion from a transfer pipette. The nuclear suspension was centrifuged at 8000 rev./min for 10 min in an International Centrifuge. The supernatant was removed and saved, and the pellet was washed with 5 vol. of the detergent solution (0.5% Triton-X-100, 20 mM EDTA and 80 mM NaCl, pH 7.4). The detergent-cleaned nuclei were washed with 5 vol. of 0.5 M NaCl, 2 mM PMSF and 10 mM Tris-HCl, pH 8.0.

Preparation of cytoplasmic components

The detergent-containing supernatants and subsequent washings of the nuclear pellets saved during isolation of nuclei, were centrifuged using a Type

40 Beckman rotor at $100\,000 \times g$ for 90 min at 4°C .

Total lipids. The top layers of total lipids were removed by a gentle aspiration. The total lipids were mixed with 5 vol. of acetone, allowed to stand overnight at 4°C and centrifuged at 10 000 rev./min in a Sorvall-2B Centrifuge for 10 min. The lipoproteins were obtained as acetone precipitates whereas the lipids were obtained as acetone solutions.

Cytosol. The clear supernatants from the appropriate individual extracts were combined to obtain the soluble cytoplasmic protein fractions.

Particulate. The sediments comprised the microsomal and the mitochondrial fractions.

Preparation of chromatin

The purified nuclei were allowed to swell in 2 vol. of distilled water at 0°C for 30 min and lysed by homogenization using a Dounce tissue grinder. The chromatin material was mixed with an equal volume of 0.4 mM EGTA, 4 mM PMSF, and 20 mM Tris-HCl, pH 8.0, and centrifuged through 1.7 M sucrose in an SW 27.1 Spinco rotor at $115\,000 \times g$ for 90 min at 4°C . The purified chromatin pellet was suspended in a minimal volume of 0.4 mM EGTA, 4 mM PMSF, and 20 mM Tris-HCl, pH 8.0, homogenized to obtain a uniform suspension, and stored at 4°C .

RESULTS AND DISCUSSION

Most of the radioactivity (about 70% of homogenate radioactivity) was associated with the cytosol and particulate fractions of neuroblastoma and glioma cells (Table I). This is consistent with an earlier observation on brain cortical slices in which most of the mercury was present in the cytosol, mitochondrial and microsomal fractions [14]. TCA-insoluble fractions of glioma and NB cells contained 66% and 52% of their total radioactivities, respectively (Table II). The purified chromatin fractions of glioma and NB contained 1.9% and 3.4% of their homogenate radioactivities, respectively, whereas the corresponding lipid fractions contained only 0.5% and 1.0% of their homogenate radioactivities (Table I). Several recent studies using the techniques of radioautography and ultracentrifugation on animal brain tissues have indicated that the nuclear fraction contained only a very small proportion of the administered organic mercury [15]. CH_3HgCl has been reported to bind primarily with the nuclear membrane and only a very little amount was found within the nucleus [15-19]. In our study, only 2-3% of homogenate activity was found to be in purified chromatin fraction. However, this amount could be sufficient to alter some of the functions of chromatin. Indeed, we have observed (Ramanujam and Prasad, unpublished observation) that the relative amounts of specific proteins and their phosphorylation activities in the crude nuclear, cytosol and particulate fractions of glioma cells were markedly increased and decreased after chronic treatment (40 days) of these cells with lower concentrations of CH_3HgCl (0.05 and $0.1\ \mu\text{M}$). Thus,

TABLE I

DISTRIBUTION OF [^{203}Hg]- CH_3HgCl IN VARIOUS SUBCELLULAR FRACTIONS OF GLIOMA AND NEUROBLASTOMA CELLS IN CULTURE

The confluent cells which were collected by centrifugation were lysed in 4 vol. of 0.5% Triton X-100, 20 mM EDTA and 80 mM NaCl, pH 7.4. The various fractions were prepared according to the procedure described in the MATERIALS AND METHODS. The values in parentheses represent % of homogenate radioactivity present in each fraction. The experiments were repeated twice and each value represents an average of six samples.

Fractions	Specific radioactivity dpm/mg proteins $\cdot 10^4$	
	Glioma	Neuroblastoma
Homogenate	176 \pm 3 ^a	125 \pm 3
Cytosol, 100 000 $\times g$	164 \pm 10 (50%)	119 \pm 6 (40%)
Cytosol, 100 000 $\times g$ (protein-bound)	81 \pm 4	69 \pm 6
Particulate, 100 000 $\times g$	128 \pm 5 (19%)	124 \pm 4 (29%)
Chromatin	18 \pm 0.6 (1.9%)	21 \pm 1 (3.4%)
Lipids	(0.5%)	(1.0%)
Lipoproteins	433 \pm 9 (7%)	156 \pm 3 (6%)

^a S.D.

TABLE II

DISTRIBUTION OF RADIOACTIVE METHYLMERCURIC CHLORIDE [^{203}Hg]- CH_3HgCl IN TCA FRACTIONS OF GLIOMA AND NEUROBLASTOMA CELLS

The values in parentheses represent percentages of homogenate radioactivity.

Fractions	Radioactivity dpm $\cdot 10^6$	
	Glioma	Neuroblastoma
TCA insoluble (dpm/mg protein)	69 \pm 0.3 ^a (66%)	42 \pm 0.4 (52%)
TCA soluble (dpm/ml)	23 \pm 0.1 (22%)	14 \pm 0.1 (18%)

^a S.D.

small concentrations of CH_3HgCl can produce an extensive modification in gene expression.

The binding of radioactive CH_3HgCl with various subcellular fractions may involve some uncertainties. For example, it is not fully certain, if all the radioactivities of various fractions were incorporated during the incubation of intact cells or if portions or all of radioactivities could be attributed

to redistribution of CH_3HgCl among the subcellular fractions during homogenization and subsequent fractionation. Indeed, such a possibility has been suggested by Yoshino et al. [14]. The instability of CH_3HgCl -protein complexes in solution makes it exceedingly difficult to derive conclusive information on the binding and subcellular distribution of ^{203}Hg - CH_3HgCl . However, these data do provide some estimation on the relative distribution of CH_3HgCl in the subcellular fractions of these two cell types.

Table I shows that only about 50--60% of the cytosol radioactivities were protein-bound in both glioma and NB cells; however, the protein-bound radioactivity in glioma cells was higher than that in NB cells. The total homogenate, lipoproteins and cytosol of glioma cells also accumulated more ^{203}Hg - CH_3HgCl than the corresponding fractions of NB cells. Thus, the greater sensitivity of glioma cells to CH_3HgCl may in part be due to the fact that they accumulate more CH_3HgCl than NB cells. The specific radioactivity of the lipoprotein fraction of glioma cells was particularly high in comparison to that of NB cells. This would indicate that the binding affinity of CH_3HgCl for lipoprotein is greater in glioma cells than in NB cells. The specific radioactivities of the particulate, lipid and chromatin fractions were comparable in both cell types. The reasons for a higher uptake of radioactive CH_3HgCl in glioma cells in comparison to NB cells are unknown. However, a similar observation has been made in brain tissue of human victims of methylmercury toxicity. It has been observed [1, 20] that most of the mercury was located in the glial cells. This is in contrast to studies in rats in which neurons accumulated more radioactive methylmercury than glial cells [21, 22]. The reasons for the discrepancy in cellular accumulation of methylmercury compounds in cell culture model and human brain on one hand, and rat brain on the other, are unknown. However, the following possibilities can be mentioned: (a) species difference; (b) difference in experimental conditions, and (c) time interval between injection of methylmercury compounds and assay of radioactivity. The last factor was found to be very important in cell culture model. When the uptake and subcellular distribution of radioactive CH_3HgCl were assayed 5 h after incubation (time required for the uptake value to reach plateau was 4--6 h of incubation in the presence of radioactive CH_3HgCl), glioma cells accumulated more radioactivity than NB cells; however, if the cells were incubated in the presence of radioactive methylmercuric chloride for only 15 min, the reverse was true [8]. Hence, the monolayer cultures of glioma and NB cells behaved similar to human brain tissue with respect to accumulation of CH_3HgCl in neurons and glial cells, provided the study was performed at the time the cellular uptake of CH_3HgCl had reached equilibrium. It should be pointed out that the difference in the uptake of radioactive CH_3HgCl in glioma and NB may be due to the following: (a) difference in cell type; (b) difference in species (NB cells are from mice and glioma cells are from rats); (c) difference in growth media; and (d) different time intervals of incubation with different proteolytic enzymes. The difference in growth media may not be an important factor in the differential

uptake of CH_3HgCl , because glioma cells exhibited a greater degree of sensitivity to CH_3HgCl than NB cells even when they were grown in the medium (F12 with 10% agammaglobulin newborn calf serum) for NB cells. The effects of different time intervals of incubation with different proteases were difficult to evaluate, since they are inherent with the technique of preparing a single-cell preparation.

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