Bull. Environm. Contam. Toxicol. 25, 441-447 (1980)

Long-Term Effects of Methylmercury (II) on the Viability of HeLa S3 Cells

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When studying the effect of methylmercury(II)[1] on the viability of HeLa S3 cells (GRUENWEDEL and FORDAN 1978), on their intracellular metabolic activities such as DNA, RNA, and protein synthesis (GRUENWEDEL and CRUIKSHANK 1979), and, as seen via scanning electron microscopy, also on the surface characteristics of their outer plasma membrane (GRUENWEDEL et al. 1979), we noted that there appears to exist a threshold of toxicant concentration in the growth medium below which the cells remain alive and, for instance, display the metabolic activity of control cells, but above which an abrupt decrease in intracellular synthetic activity takes place and rapid cell death occurs. The threshold was identified by us as having a value near 3 μM $CH_3Hg(II)$ (GRUENWEDEL et al. 1979).

The threshold value was obtained from experiments in which the cells had been exposed to methylmercury for 12 hr, equivalent to a generation number of about 0.6 (GRUENWEDEL et al. 1979), and the longest intoxication period ever studied by us did not exceed 36 hr or approximately 1.7 generation times (GRUENWEDEL and FORDAN 1978, GRUENWEDEL and CRUIKSHANK 1979). These periods are clearly too short to establish with confidence a threshold value. In fact, they are too short to demonstrate that a true threshold does indeed exist: for not only is it possible that a minimum time is needed for methylmercury to be taken up by the cells but it is also possible that a finite time is required for the cellular processes to come to a standstill, even if the binding of the toxicant by the cells should be a rapid event.

The present communication reports on experiments in which asynchronous HeLa S3 suspension-culture cells were exposed to methylmercury for periods lasting up to 432 hr or almost 21 generations. The cell response is expressed in terms of cell viability as given by the trypan blue dye exclusion test. Also presented are some preliminary data on the extent and rate of toxicant up-

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[1] Methylmercury(II), or simply methylmercury, denotes the chemical entity $CH_3Hg(II)$ without reference to any particular anion that might accompany this organomercurial in the complex growth medium.

0007-4861/80/0025-0441 $01.40

take by the cells using ^{203}Hg-labeled methylmercury as a marker.

MATERIALS AND METHODS

Stock Cell Culture

Information concerning the source and routine maintenance of the HeLa S3 cells used in this work can be found elsewhere (GRUENWEDEL and CRUIKSHANK 1979). Eagle's minimum essential medium (MEM-Joklik modified), supplemented with 10% fetal calf serum, was used throughout the investigation (Grand Island Biological Co.).

Chemicals

CH_3HgOH (97+%) was purchased from Alfa Products, Ventron Corp. $CH_3{}^{203}HgCl$, dissolved in 0.02 M Na_2CO_3 at a specific activity of 0.64 Ci/g, was a product of New England Nuclear. Further information concerning chemicals and other material is contained in GRUENWEDEL and CRUIKSHANK (1979) and GRUENWEDEL et al. (1979).

Intoxication

Intoxication experiments were performed in duplicate at 37°C. They involved, in brief, adding microliter aliquots of stock CH_3HgOH, dissolved in phosphate-buffered saline, to milliliter portions (40 ml) (ratio 1: 1000 v/v) of a cell suspension, each portion at a cell density of 0.3 X 10^6 cells/ml, and treating the various samples (at given toxicant concentrations) for different periods of time. Final methylmercury concentrations amounted to 0.1, 1.0, 2.0, 3.2, 10.0, and 32.0 µM, respectively. During the first 36 hr of incubation, cells were removed at 6, 12, 24, and 36 hr, respectively, for viability determinations. After that, viability determinations were performed in 24 hr intervals. Medium change occurred in 24 hr intervals also, with the viability determination preceding the medium change. All new media contained methylmercury at the requisite levels. The cell density was adjusted to 0.3 X 10^6 cells/ml at each medium change. Incubations were performed in water-saturated 95% air-5% CO_2. The New Brunswick Scientific Model G76 gyrotory water-shaker bath was used in the experiments.

Labeling

The mode of incubation was the same as the one described above. ^{203}Hg-labeled methylmercury was employed at the constant level of 1 µM, or 5 µCi/sample, with cold methylmercury making up the remainder if the total toxicant concentration exceeded 1 µM. Organomercurial levels amounted to 1.0, 2.0, 3.2, 10.0, and 32.0 µM, respectively, during labeling, and label uptake was monitored at time points corresponding to 1, 6, 12, 18, and 24 hr of incubation, respectively. To determine the extent of methylmercury binding to the cells, a 2 ml aliquot of cell suspension was re-

moved from each sample and each was placed into one of five disposable plastic centrifuge tubes. While the remaining suspensions were returned to the water bath for continued incubation until the next time point was reached, the removed aliquots were subjected immediately to centrifugation for 4 min at 1,000 rpm. This treatment sufficed to sediment all cells to the bottom of the centrifuge tubes. Using a 1 ml Eppendorf pipet, and replacing the pipet tip each time with a new one to avoid contamination, precisely 1 ml portions of supernatant were removed from each centrifuge tube immediately after centrifugation. Both the 1 ml supernatant portions of each aliquot and the corresponding 1 ml remains, containing the labeled cells, were assayed for radioactivity. From the radioactivity found in the lower 1 ml portion of an aliquot and the activity measured in the 1 ml supernatant the degree of methylmercury binding was determined. It was verified that this mode of label separation produced results that were indistinguishable from those obtained by first filtering off the cells from the labeling medium and then washing them with phosphate-buffered saline a few times. Further details will be given elsewhere (GRUENWEDEL and GLASER, publication in preparation). Radioactivity measurements were performed in the Beckman Model 250 liquid scintillation counter.

RESULTS

Intoxication

Fig. 1 contains the results of incubating HeLa S3 cells in the presence of varied concentrations of methylmercury for prolonged periods of time. Plotted is the viability of the cells, given as percent live cells, against the number of cell generations n whereby

$$n = t/T_g \qquad (1)$$

In Eq. (1), t is the time at which cells were removed from the medium for viability determinations and T_g is the generation time of the cells. T_g was found to be 21 hr under our experimental conditions (GRUENWEDEL and CRUIKSHANK 1979).

Since control cells and cells that had been incubated in the presence of 0.1 and 1.0 μM $CH_3Hg(II)$, respectively, displayed only random fluctuations in their number of live cells, their viability data were combined. They are represented by the solid line in the figure, corresponding to a cell viability of 93 ± 2% (± SD). The open-circled data points denote cells that had been incubated in the presence of 2.0 μM $CH_3Hg(II)$. No acute toxic effects are noted under these circumstances for about 5-6 generations, or 105-126 hr of incubation, since the individual data meander about the 93% line within the standard deviation given. Some effects on cell viability, however, are noted beyond 6 generations although the cells, in general, still maintain a high degree of viability (75% < viability < 90%). Definite toxic effects are noted with

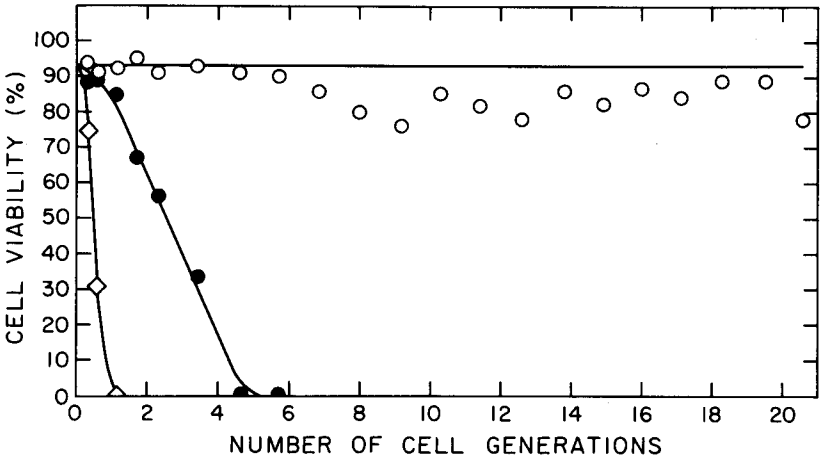

Figure 1. Changes in HeLa S3 cell viability as a function of incubation time and methylmercury concentration in the growth medium. Control cells and cells that had been exposed to 0.1 and 1.0 μM $CH_3Hg(II)$, respectively (—); cells in presence of 2.0 μM $CH_3Hg(II)$ (○); cells in presence of 3.2 μM $CH_3Hg(II)$ (●); cells in presence of 10.0 μM $CH_3Hg(II)$ (◇). For further details see text.

cells that had become exposed to 3.2 μM $CH_3Hg(II)$ beyond 24 hr of incubation, and only dead cells were found in the medium near $\underline{n}$ values of 5. Lastly, rapid cell death is encountered at methylmercury levels of 10.0 μM and above: while the cell viability decreases in about 10-11 hr of incubation below the 50% mark in presence of 10.0 μM $CH_3Hg(II)$ it decreases in about 4-5 hr of incubation below the 50% level in the presence of 32.0 μM $CH_3Hg(II)$ (the latter data have been omitted from the figure).

Labeling

Results of the labeling experiments are displayed in Table I. The numbers listed with the several periods of incubation are the fractions of methylmercury found associated with the cells at the given levels of organomercurial in the growth medium. ^{203}Hg-labeled methylmercury was used as a marker. By plotting the data versus incubation time it can be readily verified that methylmercury binding progresses linearly with time at 1.0 and 2.0 μM $CH_3Hg(II)$, respectively, while a slight deviation from linearity can be observed beyond 12 hr of incubation at 3.2 μM $CH_3Hg(II)$. Dramatic inhibition of methylmercury binding can be noted with cells that had been in contact with 10.0 μM $CH_3Hg(II)$ for more

TABLE I

Methylmercury Binding by HeLa S3 Cells

$[CH_3Hg(II)]_{added}$	Fraction of Added Methylmercury Bound				
[μM]	Incubation Time [hr]				
	1	6	12	18	24
1.0	0.07	0.12	0.21	0.30	0.37
2.0	0.05	0.13	0.21	0.31	0.38
3.2	0.08	0.18	0.26	0.33	0.38
10.0	0.13	0.29	0.35	0.34	0.28
32.0	0.23	0.27	0.28	0.24	0.13

than 12 hr, and there is little continued uptake of toxicant, if any at all, beyond the first hour of incubation in a medium that is 32.0 μM in $CH_3Hg(II)$.

DISCUSSION

The results shown in Fig. 1 demonstrate that there exists indeed a concentration range of methylmercury in the growth medium in which HeLa S3 suspension-culture cells do not experience adverse effects over a prolonged period of time as far as cell viability is concerned. Since the experiments consisted of incubating the cells continuously for 3 weeks at the methylmercury levels listed, we are certain that "delayed" cell responses can be excluded.

The range includes with certainty the concentration level of 1.0 μM $CH_3Hg(II)$: at this level, neither DNA, RNA or protein syntheses are inhibited to any degree for incubation periods up to 12 hr (GRUENWEDEL and CRUIKSHANK 1979, GRUENWEDEL et al. 1979) nor do the cells assume under the scanning electron microscope an appearance that differs from the one displayed by control cells (GRUENWEDEL et al. 1979). Hence, from what has been found in this study, we would conclude that these situations continue to exist also beyond a 12 hr incubation period as the cells, treated for 432 hr at 1.0 μM $CH_3Hg(II)$, display the viability of control cells.

Disturbances in cell viability become noticeable at 2.0 μM $CH_3Hg(II)$ only after incubation periods exceeding 105 hr. It is,

therefore, not surprising to see that under these conditions the rate of toxicant uptake does not differ from the one shown by cells in the presence of 1.0 μM $CH_3Hg(II)$ since label binding was not followed for more than 24 hr. We identify tentatively the concentration level of 2.0 μM $CH_3Hg(II)$ as the threshold below which the cells do not experience persistent damage in terms of metabolic activity and cell viability but above which damages must be expected.

Methylmercury concentrations of 3.2 μM are lethal to the cells as can be seen from Fig. 1. The decline in cell viability starts at about 24 hr of incubation, and this is noted, too, by a slight decrease in toxicant binding around the same time (cf., Table I). Cells fall below the 50% viability levels at 10.0 and 32.0 μM of $CH_3Hg(II)$ in 10-11 and 4-5 hr of incubation, respectively, and this is mirrored, in the case of labeling, by the concomitant decline of methylmercury binding (cf., Table I). We would thus expect a major breakdown of label binding to the cells around $\underline{n}$ values of 2.5 (or 53 hr) if they are kept in a medium of 3.2 μM $CH_3Hg(II)$ (cf., Fig. 1, 50% viability mark).

The data given in Table I show that large quantities of toxicant are taken up by the cells, amounting to almost 40% of its medium concentration in the range 1-3.2 μM and at 24 hr of incubation. The data show further that methylmercury uptake by the cells is a rapid process: almost 25% of its concentration in the medium is found associated with the cells after only one hour of incubation if intoxication is performed in the presence of 32 μM $CH_3Hg(II)$. Thus, neither "delayed" cell responses nor a "slow" uptake of toxicant can be cited as evidence that a true threshold does not exist. Since we did not follow methylmercury binding beyond the 24 hr time point we cannot say anything about long-term toxicant accumulation. However, since HeLa S3 cells tolerate methylmercury up to levels of 1.0 μM for at least 20 generations, we would assume that an internal "steady-state" concentration is reached within a relatively short period of time and that accumulative effects can be discounted, even if only at the cell viability response level.

It is intriguing to express our binding data in more quantitative terms, say as "moles $CH_3Hg(II)$ bound per cell". Since our cell counts, using the hemocytometer, are not too reliable as far as statistical significance is concerned, we are prepared to offer only estimates at this point in time. We are in the process of extending our experiments to include cell counting and cell sizing with a high degree of precision (electric sensing zone measurements with the help of the Coulter Counter) and will report on this elsewhere (GRUENWEDEL and GLASER, publication in preparation). Under our experimental conditions, we know that HeLa S3 cells grow exponentially for at least 24 hr in the presence of 1.0 and 2.0 μM $CH_3Hg(II)$. Thus, by applying the familiar relation

$$N = N_o \times 2^n \qquad (2)$$

with N being the number of cells at the value n of the number of generations (cf., Eq. (1)) and N_o the cell number at $n = 0$ ($N_o = 3 \times 10^5$ cells/ml), it is readily shown, in combination with the data contained in Table I, that the levels of bound methylmercury range from 0.23 (1 hr of incubation) to 0.56 (24 hr of incubation) fmoles/cell (f ≡ femto = 10^{-15}) in presence of 1.0 μM $CH_3Hg(II)$ and from 0.32 (1 hr) to 1.15 (24 hr) fmoles/cell in the presence of 2.0 μM $CH_3Hg(II)$. At 3.2 μM $CH_3Hg(II)$, the range can be calculated as 0.83 fmoles/cell (1 hr) and 1.84 fmoles/cell (24 hr) assuming that exponential growth still exists. As noted above, cell viability starts to decline at 3.2 μM $CH_3Hg(II)$ beyond 24 hr of incubation. Hence, threshold > 1.8 fmoles/cell. At 10.0 μM $CH_3Hg(II)$, cell viability is 75% after 6 hr of incubation and methylmercury bound = 7.9 fmoles/cell (assuming still exponential growth) or 9.7 fmoles/cell should N_o have remained invariant. We tentatively postulate 2 < threshold < 10 fmoles of bound methylmercury per cell but wish to point out again that many approximations and assumptions are contained in the calculation.

In conclusion, HeLa S3 suspension-culture cells are uniquely suited to study methylmercury intoxication over prolonged periods of time since the cells do not possess a finite life span. Since they can be kept in suspension, they can be exposed to methylmercury in a most uniform and accurate fashion thereby becoming amenable to quantitative analysis.

ACKNOWLEDGMENTS

This work was supported by USPHS Grant ES 01115. The authors express their gratitude to Mr. J. F. Glaser for the help and advice rendered in the course of the investigation. They also wish to thank Professors C. Meares and C. Schmid, Department of Chemistry, UCD, for permission to use their liquid scintillation counting equipment to the fullest extent possible.

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