CYTOTOXICITY OF METHYLMERCURIC CHLORIDE ATTENUATED BY SERUM PROTEINS AND IT'S RELEASE FROM THE TREATED CELLS *IN VITRO*

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Abstract.....Knowledges on the characteristics in toxicity of methylmercuric chloride (MMC), obtained in the present series of experiments *in vitro*, would be summarized as follows: (1) No appreciable cell-phase-specific age-response to MMC through the cell cycle of JTC-11 line of cells *in vitro* was shown. Cytolethal sensitivity of the mitotic cells to MMC was equal to that of the exponentially growing cells. (2) Culture media were prepared in graded concentrations of serum proteins and the various albumin/globulin ratios. Cytolethal toxicity of MMC in the graded treatment periods and concentrations was assayed in the prepared media. The apparent MMC toxicity was decreased both by albumin and by globulin. The both serum proteins appeared to be cytoprotective from MMC toxicity. In culture medium containing albumin, or inactivated bovine whole serum, the cytolethal toxicity of MMC was not observed in the MMC treatment concentrations below 0.5 $\mu\text{g/ml}$, but it was observed for any MMC treatment periods in the any concentrations above 0.5 $\mu\text{g/ml}$. Increases in the concentrations of either albumin or globulin, or the both, correlated with the decrease of MMC toxicity. (3) MMC uptake to the given number of cells was measured and decreased MMC uptake of cells in the medium containing serum protein, compared with that in the serum-protein-free medium, was obtained. Rapid transfer of MMC from the cells to the MMC free medium was observed during the first 30 min after the MMC treat-

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ment, which was followed by a slower exponential regression of MMC content of the cells during the successive incubation in the MMC free growth medium.

Key words : Methylmercuric chloride, serum proteins and decreased cytotoxicity.

INTRODUCTION

For better understanding of the mechanism of organic mercury intoxication (D'Itri 1972), a series of experiments was carried out based on our previous studies (Kano et al. 1976) in which we observed as follows : 1) there appeared a critical concentration in cytolethal action of methylmercuric chloride, MMC, which was not dependent on MMC treatment period so far assayed within 6 hrs, 2) inactivated bovine serum partially protected JTC-11 line of cells from the cytolethal action of MMC *in vitro*, 3) the apparent MMC toxicity in acute cytolethality was decreased with increase in the serum concentration but it nevertheless remained to some extent at the high concentrations of the serum and 4) the serum increased the critical concentration in the toxicity of MMC.

There appears still a paucity in the information and models with respect to transfer of MMC between cells and body fluid *in vivo*, or culture medium *in vitro*. In the present series of experiments, we attempted to elucidate 1) whether or not there was a cell phase dependent response to MMC (age response), 2) whether or not serum fraction(s) would contribute to attenuate the cell killing effect of MMC and 3) the profile on the MMC uptake and release of the cells *in vitro*.

MATERIALS AND METHODS

JTC-11 cell line *in vitro* was maintained stationarily in conventional CO₂ incubator. Colony forming ability of variously treated cells with MMC and other drugs was regarded as survivals from the treatments, as generally practiced. MMC was treated under various conditions such as the concentrations, treatment periods, cell culture media during the treatment and the phases on the cell cycle of the cells to be treated. To synchronize cells at the border between so-called gap-1 and DNA synthetic phases (G₁-S border) on the cell cycle, hydroxyurea (HU) was added because HU killed S phase cells and blocked cell progression at G₁-S border. Other hand to synchronize cells at metaphase of mitoses, cells were treated with colcemid with which cell phase progression was stopped and the cells were accumulated at the metaphase. The accumulated metaphase cells were collected by gentle agitation of culture plate by which only metaphase cells were easily suspended into the medium since the shape of only metaphase cells was spherical. Cells after the various treatments were fed with fresh enriched growth medium and finally incubated for colony formation.

MMC was extracted from the treated cells and the extracted MMC was measured by gas chromatograph.

Each item is interpreted in detail as follows :

1) Cells ; The JTC-11 cell line, registered by the Japan Tissue Culture Association, was

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colonially cloned and used throughout the present series of experiments *in vitro* which was adopted as one of the lines exponentially proliferated *in vitro*. This cell line has malignant character *in vivo* of mice.

2) Culture media ; JTC-11 cells were maintained in growth medium, MLN-15, 1 liter of which contained 730 ml of Eagle's MEM (Nissui Seiyaku Co., Ltd., Tokyo) solution, 20 ml of 2.5 w/v % lactalbumin hydrolysate (Difco Lab., Detroit, MI.) solution, 100 ml of NCTC-135 (Difco) solution, 150 ml of inactivated bovine serum and antibiotics. Numerals in succession after MLN-, or MEM-, indicate the serum volume percentages. The bovine serum was beforehand heated at 56°C for 30 min in water bath, to inactivate heat-labile components of the complement of serum.

3) Methylmercuric chloride, MMC, treatment ; The original MMC solution, 1 mg CH_3HgCl /1 ml ethanol (Tokyo Kasei Kogyo Co. Ltd.), was diluted ten times in water, which had been distilled twice and filtered, to obtain the appropriate 10X solution, 100 $\mu\text{g/ml}$. One ml of the thus diluted MMC 10X was added to the cell culture plates, which had been re-fed with 9 ml of fresh MEM-15 or MEM-0 prior to the MMC treatment as scheduled, to obtain the final concentration, 1X, of 10 $\mu\text{g/ml}$. The plates under the treatment were incubated at 37°C for the scheduled periods of 0.25, 0.5, 1, 2, 3, 4.5 and 6 hours. In a part of the experiments, cells were synchronized with hydroxyurea prior to the MMC treatment at the various phases of cell cycle.

4) Hydroxyurea, HU, treatment ; The solution containing 30 or 50 mM hydroxyurea (Sigma Chemical Co., St. Louis, Mo.) in MEM solution, was filtered and added to the plates under the exponential growth of the cells, which had been re-fed with fresh MEM-15 prior to the HU treatment, at the final concentrations of 3 mM in order to synchronize the cells at the G_1 -S border or 5mM to kill the cells in the S phase. To synchronize the cells in exponential growth, the cells were treated with HU in a final concentration of 3 mM for 6 hours at 37°C. The treated cells were rinsed with MEM, re-fed with MLN-15 and then incubated at 37°C for the scheduled periods to observe progression in the cell cycle of the surviving cells from the first HU treatment. The second HU treatment was given at final concentration of 5 mM for 2 hours at 37°C. The cells after the second treatment were rinsed with MEM and re-fed with MLN-15 for the final colony forming incubation. The second HU was treated to monitor the phase progression of the cells during the interval incubation time between the first and second HU treatments, while the replicate cell plates were treated with MMC (sequence of HU-MMC) to observe the age response profile to MMC.

5) Colcemid treatment ; The solution containing 0.25 $\mu\text{g/ml}$ of colcemid (Kanto Chemicals Co., Tokyo) in MEM solution, was filtered and added to the cell culture at the final concentration of 0.025 $\mu\text{g/ml}$ to synchronize the cells at the metaphase in the mitosis. After the colcemid treatment at 37°C for 5 hours, mitotic cells were suspended by gentle agitation of the culture plates since only mitotic cells were spherical and therefore easier to be suspended than the cells in the other phases. The prepared cell suspension was centrifuged twice. The thus prepared mitotic cells were exposed to MMC in suspension and the MMC sensitivity of the mitotic cells was observed by colony

formation of the survived cells.

6) Measurement of serum proteins ; Two inactivated sera obtained from two different bovines were used for the measurement by Buret method in which electrophoretic assay was performed on cellulose acetate membrane at pH 8.6 by barbital buffer. The membrane was stained by ponceau 3R. Total protein content, fractions of serum proteins and A/G ratio were tabulated in the average value of those obtained from the two different inactivated sera as shown in Table 1.

Table 1. Quantitation of inactivated bovine serum proteins.

Total protein	Albumin	α -globulin	β -globulin	γ -globulin	A/G ratio
8.5 g/dl	46.3 %	17.8 %	18.2 %	20.3 %	0.865

7) Substitution of serum in culture medium with albumin-globulin mixtures ; Serum albumin was obtained commercially and solved in the neutral and isotonic buffer salt solution, while globulin obtained commercially hardly solved unless surface active agent was added. Since it is considered that contamination of surface active agent to solve globulin may result in some accessory effect on the colony forming ability of the cells treated, this type of contamination should be avoided. In the present experiments, bovine serum albumin solution and inactivated bovine whole serum were mixed in the graded ratios to prepare the various mixtures in different A/G ratios. Protective effects of these mixtures against MMC toxicity were compared. Eight grams of albumin (Armour Pharmaceutical Co., I11.) was dissolved in 100 ml of the twice distilled water, and the solution was filtered. Protein solution in graded albumin-globulin ratios were prepared by mixing the albumin solution to inactivated bovine serum, A/G ratio of which had been assayed beforehand. The cells were treated with MMC in the media containing the various albumin-globulin mixtures instead of that containing the conventionally inactivated whole bovine serum in given concentrations.

8) Culture conditions ; (a) The cells were seeded in appropriate numbers per glass plate to yield the pertinent number of colonies as the surviving fractions. About 100 colonies for a plate, 6 cm in diameter, were suitable for the present culture system. (b) The plates were preincubated over-night in water saturated air with 5 % CO₂ at 37°C. (c) The control cell growth was followed during the experiment on the successive days after the cell seeding. (d) The cells were treated with MMC, or with HU followed by MMC, in various concentrations for variously scheduled periods. The treatments of the cells with HU-HU, and with HU-MMC, were carried out according to the scheduled sequences and interval times. (e) After the variously scheduled treatments, the plates were rinsed twice with MEM solution. (f) For the colony formation assay, the plates after the treatment were fed with 12 ml of MLN-15 and placed in the conventional CO₂ incubator and observed for colony formation of surviving cells. The surviving fraction for every treatment group was estimated from the number of colonies out of number of

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cells seeded and the survival curves were drawn. (g) For the MMC extraction, exponentially growing cells in the culture plates were treated with (i) 1.0 μg MMC/ml of MEM-0 for 1 hour, (ii) 1.0 μg MMC/ml of MEM-15 for 1, 2, 4 and 6 hours, (iii) 2.0 μg MMC/ml of MEM-15 for 6 hours and (iv) 1.0 μg MMC/ml of MEM-0 supplemented with albumin in the equivalent content to that of MEM-15 for 1, 2, 4 and 6 hours. The cells immediately after the treatment were rinsed twice with MEM-0 and trypsinized in 0.3 g trypsin/1000 ml of isotonic buffer. The trypsinized cells were centrifuged twice for the rinse and the cell collection.

9) MMC extraction; After the twice centrifugations, 1500 rpm for 10 min, the supernatants were separated for MMC measurements. The sediment after the first centrifugation was resuspended in MEM-0 for the second centrifugation and that after the second centrifugation was homogenated for the MMC extraction and measurement.

10) MMC measurement; The gas chromatograph, Model GC-6AE (Shimazu, Japan), was used for this assay. An electron capture detector was used to measure the MMC content. The glass column in length of 0.5 m and an internal diameter of 3 mm contained DEGS-HG, 20 % on Chromosorb-W (60-80 mesh). The column temperature was maintained at 120°C and the nitrogen flow rate was 60 ml/min. Methods of MMC extraction and measurement are illustrated as follows.

RESULTS

1) Cell age response to MMC :

The cells were synchronized at the G_1 -S border by HU treatment, or at the metaphase in the mitosis by colcemid treatment. After HU or colcemid was released, the surviving cells progressed forward synchronously on the cell cycle which was monitored by the second treatment with HU, since HU did not only synchronize the cells at the G_1 -S border but killed the cells which had remained in the S phase.

Thus synchronized cells were exposed to MMC in $5 \mu\text{g/ml}$ for 2 hours in the MEM-15 at the various phases on the cell cycle to calculate the surviving fractions. The age-response profiles to MMC and to HU are shown in Fig. 1. Apparent age-response to MMC was not observed, while that to HU in unimodal curve showed that the cells 5 hours in the HU-free growth medium at 37°C after the first HU treatment were in their S phase.

Fig. 1: Age-responses of JTC-11 cells, synchronized by Hydroxy Urea (HU), to Methylmercuric Chloride (MMC) and to HU.

abscissa ; Hours after first treatment with HU.

ordinate ; Surviving fractions in colony forming unit (c.f.u.).

Cells in MEM-15 were treated with HU in 3 mM for 6 hours and incubated with MMC-free growth medium for various intervals, then treated again with HU in 5 mM for 2 hours (open circles) or with MMC in $5 \mu\text{g/ml}$ for one hour (closed circles).

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2) Protective effects of inactivated whole serum, serum albumin and serum globulin on MMC cell killing :

Survivals from the MMC treatments were assayed in various prepared media as follows; [1] MEM-0, [2] MEM-15, which contained 15 v/v % of inactivated whole serum, [3] MEM-0 supplemented with various concentrations of serum albumin,

[4] MEM-0 supplemented with constant concentrations of albumin and various v/v percentages of inactivated whole serum, or [5] MEM-0 supplemented with constant v/v percentages of inactivated whole serum and various concentrations of albumin. MMC treatment was performed in the final concentration of $2\mu\text{g/ml}$ for graded periods.

Difference between the lower two curves (2) and (3) in fig. 2 was resulted from the protective effect of albumin while that between the upper two curves (1) and (2) was resulted from the protective effect of serum protein other than the albumin. Dependence of the MMC toxicity on the concentration was assayed comparatively in the various culture media, as measured by colony forming ability of the cells in Fig. 3. MMC treatment conditions and parameters of MMC concentration-survival curves in

Fig. 2: MMC treatment time-survival curves in various serum protein conditions.

abscissa; Treatment periods with MMC in $2.0\mu\text{g/ml}$.

ordinate; Surviving fractions in c. f. u..

MMC treatments were performed in culture media with variously conditioned serum proteins as followed :

(1) open circles : MEM+inactivated whole serum in 15 v/v%

(2) open triangles : MEM+albumin in the equivalent content to that of 15 v/v% inactivated whole serum

(3) open squares : MEM alone.

Fig. 3: MMC treatment concentration-survival curves in graded MMC treatment periods and various serum protein conditionings of the media.

abscissa ; Treatment concentrations of MMC in $\mu\text{g/ml}$.

ordinate ; Surviving fractions in c. f. u..

[1] open squares : 30 minutes in MEM-0

[2] closed squares : 1 hour in MEM-0

[3] open circles : 6 hours in MEM-15 (MEM-0+15 v/v% inactivated whole serum)

[4] open triangles : 6 hours in MEM+albumin in the equivalent content to that of MEM-15

Fig. 3 were tabulated as shown in Table 2. The critically cytolethal concentration of MMC observed may depend on the protective effect of serum proteins, both albumin and globulin, against MMC in the lower concentrations as shown in Fig. 3.

To compare the roles of albumin and globulin for the protective effect, the cell survivals from given MMC treatments were assayed in various media which were composed of both a given globulin concentration and graded albumin concentrations. The larger was the total serum protein contents, the larger was the surviving fraction of the cells as shown in Figs. 4 and 5. The surviving fractions then reached a plateau at about 8 gram/1000 ml of total protein contents in the media, regardless of the prepared albumin-globulin mixing ratios. When the concentration of either albumin or globulin was increased, an improved protection against cytolethal damage of MMC was obtained which correlated with the increments of serum proteins in the medium.

3) Measurement of MMC uptake by the cells :

The cells were treated with MMC in $1.0 \mu\text{g/ml}$ for graded periods in (a) MEM-0, (b) MEM-0 supplemented with albumin in the equivalent content to that of MEM-15 and

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Table 2. Methyl Mercuric Chloride (MMC) treatment conditions and the survival parameters.

No. & symbol in fig. 3.	Culture medium at MMC treatment. (Supplement MEM-0 with)	Period of MMC treatment (hr)	Concavity of survival curve	Critically cytolethal concentration (μg MMC/ml)
(1) Open square	Nothing	1/2	Upward	Not appreciable
(2) Closed square	Nothing	1	Upward	Not appreciable
(3) Open circle	15 v/v % inactivated whole bovine serum	6	Downward	Circa 0.5-1.0
(4) Open triangle	Equivalent albumin content to that of MEM-15	6	Downward	Circa 0.5-1.0

Fig. 4: Protective effect of albumin in presence of constant globulin concentrations
abscissa ; Albumin contents of medium in gram/liter.
ordinate ; Surviving fractions in c. f. u..
Cells were incubated with $2.0 \mu\text{g}$ MMC/ml for 6 hours in variously conditioned media.

Symbols represent globulin contents present in medium.

- (1) open circles : 0 gram/liter
- (2) closed circles : 1 gram/liter
- (3) open triangles : 2 gram/liter
- (4) closed triangles : 3 gram/liter

Fig. 5 : Protective effect of globulin in presence of constant albumin concentrations
 abscissa ; Globulin contents of medium in gram/liter.
 ordinate ; Surviving fractions in c. f. u..

Cells were incubated with $2.0 \mu\text{g}$ MMC/ml for 6 hours in variously conditioned media.

Symbols represent albumin contents present in medium.

- (1) open circles : 2 gram/liter
- (2) closed circles : 3 gram/liter
- (3) open triangles : 4 gram/liter
- (4) closed triangles : 5 gram/liter
- (5) open squares : 6 gram/liter

(c) MEM-15. The MMC uptakes of the 10^6 cells in the 1 hour treatment in the prepared media (a), (b) and (c) were $0.358 \mu\text{g}$ in (a), $0.0812 \mu\text{g}$ in (b) and $0.0176 \mu\text{g}$ in (c), respectively. The uptakes in (b) and (c) were then saturated so far as assayed for 6 hours as shown in Fig. 6. In the further experiment of this assay, cells were treated with $2.0 \mu\text{g}/\text{ml}$ for 6 hours in MEM-15 and showed an uptake of $0.0173 \mu\text{g}$ for 10^6 cells. The cells after the treatment with MMC were rinsed, re-fed with fresh MEM-15 and further incubated at 37°C for the graded periods. The cell proliferation was examined by cell number counting for 3 hours after the MMC treatment. No cell proliferation was observed after the MMC treatments with $1.0 \mu\text{g}/\text{ml}$ for 1 hour in MEM-0 and with $2.0 \mu\text{g}/\text{ml}$ for 6 hours in MEM-15, respectively. MMC incorporated, or adhering, to the cells was released into the MMC-free culture medium within 30 min of the post-treatment incubation at 37°C . With further post-treatment incubation than the first 30min, MMC contents of cells were decreased exponentially as shown in Fig. 7.

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Fig. 6: MMC uptake of cells.

abscissa ; Incubation periods in hour with $1.0 \mu\text{g}$ MMC/ml.

ordinate ; MMC contents in $\mu\text{g}/10^6$ JTC-11 cells.

MMC was treated in (a) MEM-0 (closed circle), (b) MEM-0+albumin in the equivalent content to that of MEM-15 (open circles) and (c) MEM-15 (open triangles).

Fig. 7: MMC contents of the cells in the MMC-free growth medium. Decrease of the MMC contents were observed after the MMC removal from the culture medium, MLN-15.

abscissa ; Hours after the MMC removal in MMC-free growth medium, MLN-15.

ordinate ; MMC contents in $\mu\text{g}/10^6$ JTC-11 cells.

Cells were treated with MMC in $1.0 \mu\text{g}/\text{ml}$ in MEM-0 for 1 hour (open circles) or in $2.0 \mu\text{g}/\text{ml}$ in MEM-15 for 6 hours (open triangles).

DISCUSSIONS

1) It is considered that an aspect of biological mechanism of action of MMC can be understood by age-response experiments in which MMC-sensitive, and -resistant, phases on the cell cycle are to be specified, if any.

Cells were first treated with HU which synchronized cells at border between gap₁ and DNA synthetic phases (G₁-S border). The synchronized cells were treated, after the various interval periods, with 5.0 μ g MMC/ml of MEM-15 for 1 hour at various phases on cell cycle. Cell age specific response to MMC was not appreciably observed in the surviving fractions. Synchronized cells in the metaphase of mitosis by colcemid and immediately treated by MMC showed no appreciable difference from that of the exponential cells regarding the surviving fractions. Thus, the action of MMC may not be related to the age specific property of cells. Thrasher (1973) reported that MMC treatment of tetrahymena in doses of 0.07 to 0.104 μ g/ml may alter the DNA polymerase activity or purine and/or pyrimidine metabolism resulting in inhibition of DNA synthesis and elongated cell cycle time. Canda and Cherian (1973) reported that the insoluble non-histone protein from kidneys of rats, previously given MMC intravenously, bound 35 to 45 times more mercury than the other cell components and, since this protein bound with DNA, it was suggested that MMC may manifest its damage at the stage of mitosis. Umeda et al. (1969) and Fiskesjoe (1970) reported that MMC treatment showed inhibitory effects on mitosis of cultured human HeLa cells and primary human lymphocytes in vitro, respectively. These paper reported that inhibitory action(s) of MMC were observed in M and S phases, and that elongation in cell cycle was observed through the phases of the cell cycle. Present results showed an extent of cell killing throughout the cell cycle but no appreciable age response in cell killing examined by colony forming ability of JTC-11 line of cells.

2) Whether or not specifically affinitive serum fraction(s) for MMC does exist, is worth elucidating. Methylmercury preferably binds sulfhydryl and produces mercaptide. MMC transferred into hen eggs is found in the white and most of the MMC is contained in the albumin fraction (Irukayama and Kuwahara 1971). We found that the cytolethal toxicity of MMC was reduced both by albumin and globulin as shown in Fig. 3. Both these serum proteins may protect against toxicity of MMC. The critically cytolethal concentration of MMC, but not the treatment time, was observed in the MEM culture medium supplemented with albumin, in an equivalent dose to that of 15 v/v% inactivated whole serum. Both concentrations of albumin and globulin appeared independently to correlate to the protective effect against MMC. Thus, findings obtained from the present experiments on cell level may be consistent with those on the level of organic chemistry, such as mercaptide production by MMC and serum proteins.

3) It was reported that lower alkyl mercury incorporated into erythrocytes mainly binds with sulfhydryl of haemoglobin (Takeda et al. 1968; Garcia et al. 1974). Electron-microscopic studies on rats showed that the intracellular MMC was localized on mitochondria, microsomes, endoplasmic reticulum and nuclear membrane with little

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in the nucleus (Chang and Hartmann 1972). These reports suggest that organic mercury can be incorporated into cultured cells and the apparently adsorbed mercury on the cell surface would be on the process of incorporation. As shown in Fig. 6 MMC mixed beforehand with MEM supplemented by (b) the equivalent content of albumin to that of MEM-15 and by (c) 15 v/v% of inactivated whole serum was less incorporated to cells than the MMC in the serum-free medium (a). MMC in (b) was incorporated more than that in (c). Amount of MMC incorporated to the cells was decreased by supplementing either serum albumin (b) or inactivated whole serum (c). It means that serum protein other than the albumin, i.e. globulin, also decreased the MMC incorporation to the cells, suggesting that the cell membrane and serum proteins may compete each other in binding with MMC, and that the apparent MMC toxicity was attenuated *in vitro* by serum protein.

When the cells after the MMC treatment were incubated in the fresh and MMC-free growth medium, MMC in the cells was released into the medium. It remains to be elucidated whether or not this phenomenon is simply due to the gradient in the MMC concentration. The releasing process of the MMC from the cells appeared to be biphasic as shown in Fig. 7. The first stage of the release processed rapidly, the second more slowly, both exponentially. In the former, MMC which presumably adsorbed on the cell surface, was released rapidly into the MMC-free growth medium, while in the latter, the release of MMC which presumably had been incorporated in the cells was slow and remained partly in the cells.

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