Cytotoxicity of methyl bromide
**EFFECT OF METHYL BROMIDE ON CULTURED
MAMMALIAN CELLS**

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Abstract.....Cytotoxicity of methyl bromide on cultured mammalian cells was examined. Lethal concentrations of methyl bromide on HeLa cells and primary cultured muscle, kidney and brain cells were similar (10 μ g/ml). Acute toxic effect was induced by methyl bromide itself. And its cytotoxicity was reduced in the presence of glutathione. The action of methyl bromide seemed to be nonspecific protoplasm-toxic.

Key words : Methyl bromide, cytotoxicity, mammalian cultured cells, HeLa cells, primary cultured cells, direct action

INTRODUCTION

Methyl bromide is used largely as a fumigant for disinfestation of wood and stored grains. It is odorless in dilute concentrations in air and perhaps for this reason there have been a number of cases of accidental human poisoning (Hine, 1969). The peculiarity of its toxic effect lies in the psychiatric depression of the central nervous system and neurologic disorders, which may persist after the symptoms of acute poisoning, such as pulmonary edema, convulsions and unconsciousness (Collins, 1965; Rathus et al., 1971). As for the mechanism of action of methyl bromide, the following two theories have been presented from the results in animal experiments and biochemical studies; the action results from an accumulation of the hydrolysis products, especially bromide (Miller et al., 1943), and the toxicity derived from its methylating action on proteins such as SH enzymes (Lewis, 1948) or histidine residue (Winteringham, 1955). Its direct action on mammalian cells, however, still remains obscure.

In this study the direct effect of methyl bromide and its hydrolysis products on cultured mammalian cells was examined. The toxicity of serum saturated with methyl bromide was also tested.

MATERIALS AND METHODS

Cell culture

HeLa cells and primary cultured cells from the skeletal muscle, kidney and brain of 1- to 3-day-old Sprague Dowley rats were used for toxicity experiments. HeLa cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 10% calf serum. Cells were dissociated from skeletal muscle and brain by treating with 0.25% trypsin. Cells from the kidney were dispersed by 0.05% collagenase treating of the minced organ. The primary cultured cells from tissue or these organs were cultivated in MEM plus 10% foetal bovine serum. One hundred penicillin and 100 $\mu\text{g/ml}$ streptomycin were added to the medium.

Chemicals

Liquified methyl bromide (b. p._{-76.0}, 4.5°C) packed in a can obtained from Sanko Chemical Co., LTD, Tokyo, was bubbled into 50 g of ice-cold ethyl alcohol until its weight increased to more than 51 g. The solution obtained was then diluted with ethyl alcohol to a concentration of 2% (w/v) on the basis of the weight and was kept at -30°C as a stock solution. For testing toxicity, dilution of the stock solution was made with ethyl alcohol and the diluents (at 200 times the concentration) were directly added to the medium in culture vessels. The concentration of methyl bromide tested were from 1 to 100 $\mu\text{g/ml}$. Final alcohol concentration in the medium was 0.5%, whereby little effect was noted on cell growth. As methyl bromide is extremely volatile, it was handled in ice-cold water. Glutathione (GSH) was obtained from Sigma Chemical Co. and it was dissolved directly in the medium just before use.

Serum treated with methyl bromide

Liquified methyl bromide packed in a can was bubbled into 40 ml of foetal bovine serum for 2 hrs at 37°C and then excess methyl bromide which had been in a gaseous state was eliminated under reduced pressure at 37°C. Analysis by gas chromatography revealed that there remained no free methyl bromide and methyl alcohol in the treated serum. The content of Br in the treated serum was analyzed by a method reported by Fishman and Skougstad (1963). The serum was used for toxicity assay by diluting with MEM.

Toxicity test on cultured cells

Cover-slip culture method was employed for the toxicity test. In the case of HeLa cells 1 ml of cell suspension at a concentration of 1×10^5 cells/ml was distributed into small square tubes (SST) with cover-slips and incubated for 1 day. Methyl bromide diluted in ethyl alcohol was applied to the culture medium for 2 to 72 hrs in silicone-stoppered SSTs. After 3-day cultivation including the period of the treatment, the cover-slip from each SST was fixed with Carnoy's fixative and stained with hematoxylin and eosin for cytotoxicity and cytomorphological study.

In the case of primary cultured cells, preliminary cultivation for one or two weeks was performed before the treatment with methyl bromide. The period of the applica-

tion was 3 days. Fixation and staining were the same as in the case of HeLa cells.

The degree of cytotoxicity was estimated by microscopic observation on a scale ranging from "0" (no cellular damage) to "4" (complete cell degeneration or cytolysis) according to Toplin (Toplin, 1965; Saito, et al., 1971).

RESULTS

Toxicity of serum treated with methyl bromide on HeLa cells

Lewis (1948) has reported that methyl bromide acts as a powerful methylating agent by demonstrating S-methyl cysteine in a protein solution treated with methyl bromide. Thus, the toxicity of methyl bromide may be attributed to the toxicity of products such as methylated protein in serum reacted with methyl bromide. So the toxicity of serum treated with methyl bromide by thorough bubbling was examined. The serum contained $488 \mu\text{g/ml}$ of Br ($5.1 \times 10^{-3}\text{M}$), but did not contain methyl bromide. So the contents of Br were equivalent to those of methyl group reacted with serum components. The serum obtained was added to MEM at 10% volume to make $5.1 \times 10^{-4}\text{M}$ Br concentration. When the toxicity of the medium was tested on HeLa cells, no cellular damage was observed after a 3-day incubation. The result suggests that the reaction products between methyl bromide and serum components do not cause any acute toxicity under our experimental conditions.

Vehicles in cytotoxicity test of methyl bromide

As mentioned above, serum treated with methyl bromide had no effect on HeLa cells. Methyl bromide, however, may be decomposed by contact with organic compounds in serum. Therefore, cytotoxicity of methyl bromide in medium was compared with that in Hanks' balanced salt solution (BSS). As methyl bromide is extremely volatile, cytotoxicity was tested in a closed culture system using silicone-stoppered SST.

After a 1-day preliminary cultivation the cultures were washed with BSS and 1 ml of ice-cold medium or BSS was added. One-two hundredth of methyl bromide solution was then quickly added to the vehicles. The final concentration of methyl bromide was 100, 32, 10 and $3.2 \mu\text{g/ml}$.

After a 2-hr incubation at 37°C in a condition sealed with silicone stoppers, cells were washed with BSS 3 times, reincubated in 1 ml of control medium for 3 days and then fixed and stained. The degree of cytotoxicity is shown in Table 1. Direct treatment

Table 1 Cell Injuries of HeLa Cells after Treatment with Methyl Bromide for 2 Hours in Hanks' BSS and Medium

Vehicles	Volume (ml)	Concentration ($\mu\text{g/ml}$)			
		100	32	10	3.2
Hanks' BSS	1	4*	4	0	0
Medium	1	4	4	0	0

*The degree of cytotoxicity is measured from 0 (no cellular damage) to 4 (complete cytolysis or degeneration) after 3-day cultivation including the period of treatment.

Table 2 Cytotoxicity of Methyl Bromide on HeLa Cells in Various Volume of Medium in SST after 72-Hour Cultivation

Volume of medium (ml)	Concentration ($\mu\text{g/ml}$)				
	100	32	10	3.2	1
1	-	-	4*	0	0
10	-	-	4	3.5	0
13.5			4	4	0
1**	3	0	0	-	-

* As to the degree of cytotoxicity, see the footnote of Table 1.

**Test was performed in SST with morton caps instead of silicone stoppers.

with methyl bromide on cells clearly induced cytotoxicity. Contrary to our expectation, however, no difference was found between two vehicles under the condition employed, denoting that there was little correlation between the cytotoxicity of methyl bromide Table 1. and the presence of organic compounds in the medium.

Effect of the volume of medium in SST on cytotoxicity test

As methyl bromide is gaseous at 37°C, the atmospheric area in SST would affect the solubility of methyl bromide in the medium. Relation between the volume of the medium and the degree of cytotoxicity was examined. As shown in Table 2 all cells were dead at the concentration of 10 $\mu\text{g/ml}$ after 3-day treatment. At 3.2 $\mu\text{g/ml}$ the degree of cytotoxicity was 0, 3.5 and 4 in the culture at 1, 10 and 13.5 ml of the medium, respectively. When the cytotoxicity test was performed at an open culture system (covered with morton caps instead of silicone stoppers), the degree of cytotoxicity at 100 $\mu\text{g/ml}$ was only 3. From the result, 10 ml of medium in SST was thereafter employed for cytotoxicity test.

Effect of treatment period on the toxicity of methyl bromide

The effect of the treatment period on the toxicity of methyl bromide on HeLa cells was examined using SSTs with 10 ml of the medium. The result is shown in Fig. 1. The degree of cytotoxicity is measured after a 72-hr cultivation including the period of treatment with methyl bromide. Cytotoxicity increased with the increasing period of treatment up to 24 hrs. Cell injuries after incubation for 24, 48 and 72 hrs with methyl bromide were similar. Complete degradation of methyl bromide may occur within 24 hrs. It would seem necessary to examine the chemical stability of methyl bromide during the cultivation period.

In animal experiments Miller and Haggard (1943) have suggested that there is the delayed action of methyl bromide due to intracellular bromidism. In Table 3 cytotoxicity was scored on cells reincubated for various periods in the control medium after treatment with methyl bromide for 6 hrs. There was little increase in cytotoxicity, that is, no delayed action on cultured HeLa cells was observed.

Cytotoxicity of methyl bromide

Fig. 1 Effect of treatment period on the toxicity of methyl bromide.
As to the degree of cytotoxicity, see the footnote of Table 1.

Table 3 Cototoxicity Observed in the Cells Reincubated for Various Periods in Control Medium after Treatment with Methyl Bromide for 6 Hours

Incubation period in control medium (hrs)	Concentration ($\mu\text{g/ml}$)		
	32	10	3.2
0	3.5*	1	0
12	3.5	1	0
18	4	2	0
72	4	2	0

*As to the degree of cytotoxicity, see the footnote of Table 1.

Morphological changes of HeLa cells treated with methyl bromide

Cytomorphological changes of HeLa cells induced by alkylating agents such as mitomycin C, nitromine and so on have been characterized by enlargement of cytoplasm and nuclei as a result of inhibition of DNA synthesis by the interaction between the chemicals and DNA (Saito et al., 1974). Because methyl bromide is known chemically to be a methylating agent, morphological changes induced on HeLa cells were

Photo. 1. HeLa cells, control culture.
HE staining, 20 x 10

Photo. 2. HeLa cells treated with 3.2 µg/
ml methyl bromide for 3 days.
HE staining, 20 x 10

examined. The result demonstrated pyknotic change in some parts of the cells and no characteristic enlargement of the cells was observed (Photo 1, 2).

Cytotoxicity of methyl alcohol and sodium bromide

The cytotoxic effect of methyl alcohol and sodium bromide, both which are hydrolysis products of methyl bromide, was tested. Examination of their toxicity after 3-day treatment revealed that 100% lethal concentration of methyl alcohol and sodium bromide on HeLa cells was 2.5 M and 10^{-1} M, respectively. Their concentrations were extremely high as compared with that of methyl bromide by which the complete cell damage occurred at ca. 10^{-4} M (10 µg/ml) in the experiment performed under the same condition. Thus, our result does not support a concept that the acute action of methyl bromide on HeLa cells results from an accumulation of the hydrolysis products.

Cytotoxicity of methyl bromide in the presence of glutathione

It has been reported that toxicity of methyl bromide is reduced by dosing SH compounds such as 2, 3-dimercapto-1-propanol (Murphy, 1975). So cytotoxicity of methyl bromide in the presence of GSH was examined using HeLa cells. Toxicity induced on treatment for 3 days was reduced by the simultaneous addition of 10^{-3} M GSH in the medium (Table 4). Thus, there is a possibility that methyl bromide reacts with cellular SH compounds easily.

Table 4 Cytotoxicity of Methyl Bromide on HeLa Cells in the Presence of Glutathione

Concentration of glutathione (M)	Concentration of Methyl Bromide (µg/ml)			
	32	10	3.2	1
0		4*	0	0
10^{-3}	2	1	0	0

*As to the degree of cytotoxicity, see the footnote of Table 1.

Toxicity test on primary cultured cells

One method for demonstrating the toxicity of organotropic chemicals by tissue culture technique is to use cells with the organ-specific function. Primary cultured cells are considered to be suitable for this purpose, because they maintain some of the original functions of organs from which they are derived (Umeda, 1977). Methyl bromide is known to induce convulsions and to impair neurologic and psychiatric functions of the central nervous system and have a toxic action on the kidney (Prain et al., 1952; Hine, 1969). Thus, the cytotoxicity of methyl bromide on primary cultured cells from the muscle, kidney and brain of newborn rats was examined. In the muscle culture fibroblastic cells and spindle-shaped myoblasts grew. The latter fused to form multinuclear myoblasts after 2 or more days of cultivation. In the kidney culture,

Photo. 3. Primary cultured cells from rat brain, control culture. HE staining, 20 x 10. 3 types of cells are observed.

- (I) Large cells forming cell sheets.
- (II) Medium-sized cells embedded in fibers.
- (III) Smaller dispersed cells with fibrous protrusions like neurogenic cells.

Photo. 4. Primary cultured cells from rat brain treated with 8 µg/ml of methyl bromide for 72 hrs. HE staining, 20 x 10. Disappearance of large cells (I) and the disorder of fibers produced with medium-sized cells (II) are characteristic.

Table 5 Cytotoxicity of Methyl Bromide on Primary Cultured Cells from Rat Brain

Types of cells	Concentration (µg/ml)			
	10	8	6	4
I	4*	3	2	0
II	4	1	0	0
III	4	2	1	0

*As to the degree of cytotoxicity, see the footnote of Table 1.

several types of epithelial cells and fibroblastic cells grew. After 3-day cultivation with methyl bromide using SSTs with 10 ml medium, 100% lethal concentration of methyl bromide on the muscle and kidney culture was 10 $\mu\text{g/ml}$, that is, the same concentration as in the case of HeLa cells. Moreover, there was little difference in cytotoxicity, the degree of which was 2 at 3.2 $\mu\text{g/ml}$, on the different types of cells grown in both primary cultured cells.

In the brain culture, at least 3 morphologically different types of cells grew after 2-week cultivation (Photo 3); large cells which formed faintly stained cell sheets and had clear nucleoli (type I), medium-sized eosinophilic cells which grew embedded in the fiber (type II), and smaller eosinophilic dispersed cells which had scanty cytoplasm with fibrous protrusions like neurogenic cells. Cytotoxicity of methyl bromide on the culture is shown in Table 5. At 3.2 $\mu\text{g/ml}$ no cellular damage was observed. At 8 $\mu\text{g/ml}$ the degree of cytotoxicity on type I, II and III cells was 3, 1 and 2, respectively. Susceptibility to methyl bromide seemed to be slightly different among 3 types of cells. Disappearance of type I large cells and disorder of fiber produced with type II medium-sized cells were characteristic.

DISCUSSION

In our present study it was demonstrated that the acute action of methyl bromide on cultured mammalian cells was not due to its hydrolysis products or a result of its reaction with organic compounds in the medium. Methyl bromide itself seemed to induce acute toxic effect. As the cytotoxicity of methyl bromide was reduced extremely in the presence of GSH, it might react with protein and other components of cells. Its reaction with DNA, however, was not like chemotherapeutic alkylating agents, such as mitomycin C and nitromine. Thus, the action of methyl bromide seemed to be non-specific protoplasm-toxic. Lethal concentration of methyl bromide on HeLa cells and primary cultured cells from muscle, kidney and brain was similar. In a detailed study, however, a slight difference in the susceptibility among the cell types from brain was observed.

The most notable feature of methyl bromide toxicity are the delayed appearance of symptoms after the exposure. It was difficult to elucidate this delayed action of methyl bromide in our cell culture experiments. Cell lines in culture have rapid cycles in metabolism and cell generation. When a chemical with delayed action is applied to rapidly growing cells, a possibility exists that the chemical incorporated in the cells might be diluted away after repeated cell duplication. Therefore, in the toxicity test on such chemicals it would necessary to use slowly growing cells or essentially non-growing cells; namely, methods for subacute or chronic toxicity test should be designed in tissue culture study. In this sense, the use of stationary culture of the brain may be an interesting experimental tool for future analysis of the action of neurotoxic substances. Functional examination such as action potential of nerve cells maintained in culture for a relatively long period can be examined after the application of such substances.

Bromide produces neurologic and psychiatric symptoms similar to methyl bromide

(Collins et al., 1965). Its toxic effect on HeLa cells was low. It would also seem necessary to examine the subacute or chronic effects of bromide.

There are many tissue culture techniques. Examinations by cell counts or formation of colonies are quantitative ones. In the present study, a semi-quantitative and mainly qualitative method was adopted. The obtained results, however, offer important informations on the toxicity of methyl bromide, denoting the method to be very useful.

SUMMARY

Cytotoxicity of methyl bromide on cultured mammalian cells was examined. Acute toxic action was induced by methyl bromide itself and its cytotoxicity was reduced in the presence of glutathione. Its hydrolysis products or its reaction products with organic compounds in the medium was extremely low in toxicity. Although methyl bromide is known to be a methylating agent, morphological changes induced on HeLa cells were different from the changes characteristic of chemotherapeutic alkylating agents. Lethal concentration of methyl bromide on HeLa cells and primary cultured muscle, kidney and brain cells was similar ($10 \mu\text{g/ml}$). In a detailed survey, however, there was a slight difference in the susceptibility among the cells from primary culture of brain.

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