Restorative effect of mineral-encaging zeolite on metabolism of organotin, TBTCl, -intoxicated *Euglena* cell

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Abstract

In our previous paper the authors reported the restoration promoting effect of physiologically functional materials processed water (functional water) on tributyltin chloride (TBTCl) -intoxicated *Euglena gracilis*. Among the 'functional waters' examined, water processed with some mineral-encaging zeolites gave remarkable restoration effect on the TBTCl-intoxicated *Euglena* cells. Our present study was to investigate the behavior of TBTCl in *Euglena* cells incubated with/without Fe-encaging zeolite processed water (Fe-zeolite-water). By subcellular fractionation of TBTCl-intoxicated *Euglena gracilis* Z cells, atomic absorption spectrophotometry, GLC and GC-MS, it was revealed that TBTCl was readily incorporated into the cell and localized in cytosol, nuclei and cell membrane fractions. After incubation with Fe-zeolite-water for 3 hrs, tin was found in the extracellular fraction, and intracellular distribution analysis revealed that tin was detected mainly in the microsomal fraction, which suggest participation of phase I biotransformation enzyme system. Whereas in the cells incubated without Fe-zeolite-water, tin still remained in the cytosol fraction. GC-MS study revealed that dibutyl tin dichloride was the major metabolite of TBTCl. Those results indicate that the Fe-zeolite-water promoted biotransformation of TBTCl directly or indirerctly.

Introduction

Trace minerals are important for various cellular functions such as maintenance of homeostasis, xenobiotic metabolism and intracellular signal transduction[1)-3)].

Using the phytoplankton *Euglena gracilis* Z as a model organism, we have been studying the effects of several water species which give some interesting physiologically active functionalities especially on the detoxification of tributyltin chloride (TBTCl) -intoxicated cells[4)-6)]. Previously, we reported that

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Fe, Zn and Mn-encaging zeolites-processed water species promoted restoration of cell motility of TBTCl-intoxicated *Euglena gracilis* Z[7]. To regenerate the flagellum and restore the cell motility, it is firstly necessary to remove or reduce TBTCl toxicity in the target organs, that is, to excrete TBTCl out of the cell until harmless level.

This paper describes how Fe-encaging zeolite-processed water works on the TBTCl-intoxicated *Euglena* cells from the view point of biotransformation. The intracellular and extracellular distribution of TBTCl and its metabolites were analyzed by cell fractionation technique, atomic absorption spectrophotometry, gas chromatography and mass spectrometry.

Materials and Methods

Mineral-encaging zeolites

Fe-encaging zeolite was prepared according to the previous reports[6),7)].

Model organism, TBTCl intoxication and detoxification

A wild strain of *Euglena gracilis* Z was cultured in a Koren-Hutner medium[8),9)], at 28℃ under illumination (2800 lx) with a 12-hour light-on-off interval for 7 days. The cells at the early stationary phase were used for the following experiments.

The *Euglena* cells were exposed to 100 μM TBTCl for 5min, then centrifuged at 1,000xg, followed by washing three times with 150mM phosphate buffer (pH 6.5). Cell suspensions were incubated for 180min in control incubation medium (distilled water) or Fe-encaging zeolite-processed water, and then sonicated 5 times for 10sec at 20kHz. Cell suspension without incubation was also provided for subcellular fractionation.

Cell fractionation

All the procedures for subcellular fractionation were carried out at 0～5℃. Subcellular fractionation of TBTCl-intoxicated *Euglena* cells and *Euglena* without any treatment (referred as the control cells) was carried out according to Nakano et al.[10)] with slight modification. The subcellular organella of *Euglena* cell was purified by discontinuous sucrose density gradient after sonication of whole cells. The disrupted cells were suspended in 5mM Tris-HCl buffer, pH 7.4, containing 0.25M sucrose and 0.5 mM $CaCl_2$, then centrifuged at 1,000xg for 10 min to precipitate cell debris (crude nuclei fraction). The cell membrane, pellicle, nuclei and undisrupted cells were contained in this precipitate. The supernatant was then centrifuged at 8,000xg for 10min to obtain crude mitochondria. The supernatant was centrifuged at 105,000xg for 60min to fractionate the microsomal fraction and supernatant fraction containing cytosol (soluble fraction). The crude nuclei fraction was resuspended in 5mM Tris-HCl buffer (pH 7.4), followed by centrifuged at 150xg for 10min. The sediment was obtained from the intact cells. The supernatant layered on density of 1.20, 1.18 and 1.16 of sucrose, followed by centrifugation at 90,000xg for 90min. The nuclei fraction was obtained from the sediment, and the cell membrane

fraction was collected from the boundary between density of 1.18 and 1.16.

The content of tin in the cell membrane, nuclei, crude mitochondria, crude microsome and soluble fractions were applied to the following the extraction of tin compounds.

Extraction and purification of tin compounds from cell organella

After incubating the ***Euglena*** cell suspension with TBTCl, the tin compounds were extracted and analyzed by atomic absorption spectrophotometry. This procedure was carried out according to Stephenson et al[11].

For the quantitative analysis of tin compounds , samples were subjected to flameless atomic absorption spectrophotometer (HITACHI 180-30) equipped with graphite atomizer (HITACHI GA-2B).

Analysis of metabolites of TBTCl in *Euglena* cells

Authentic standards

Tri-n-butyltin chloride, di-n-butyltin dichloride (>97%) were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo). Di-n-butyl (3-hydroxybutyl) tin chloride, di-n-butyl (3-oxobutyl) tin chloride and di-n-butyl (4-hydroxybutyl) tin chloride were synthesized according to the method described by Fish et al.[12]. Di-n-butyl (3-carboxypropyl) tin chloride was synthesized according to the method described by Suzuki et al.[13].

Reagents

Methylmagnesium bromide (3M diethylether solution) was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo). Other chemicals and reagents were all guaranteed grade.

Gas Chromatography - Mass Spectrometry (GC - MS).

GC-MS was operated under the same condition as described by Matsuda et al.[14].

Sample preparation

The extraction and purification of organotin compounds from cells were carried out by the procedure[15] as follows: ***Euglena*** cell suspension (200ml) was centrifuged for 5min (1,000xg), then 10ml of 3 N HCl was added to the cell pellet, then the suspension was vigorously shaken. Diethyl ether (20ml) and NaCl (2g) were added, and the mixtures were shaken for 5min, followed by centrifuged for 5min at 1,000xg. This extraction procedure was repeated twice. The supernatant was evaporated under *vacuo*, and the residue was dissolved in n-hexane (5ml). The solution was transferred to a Sep-Pak silica gel cartridge (SUPELCO, USA). The cartridge was washed first with n-hexane (20ml), and then elution was performed with a mixture of n-hexane-ethylacetate (2 : 1, 50ml). The eluate was evaporated off under *vacuo*, and the residue was dissolved in diethyl-ether (2ml). Grignard reagent (methylmagnesium bromide 2ml) was carefully added and the solution was allowed to stand for 1 hr at 40℃. Ten ml of water was added in ice bath. After gentle mixing, 0.2g of anhydrous Na_2SO_4 and 5ml of NH_4Cl saturated solution were added and the solution was vigorously shaken. The supernatant was evaporated off under *vacuo* and dissolved in n-hexane (1ml) for GC-MS.

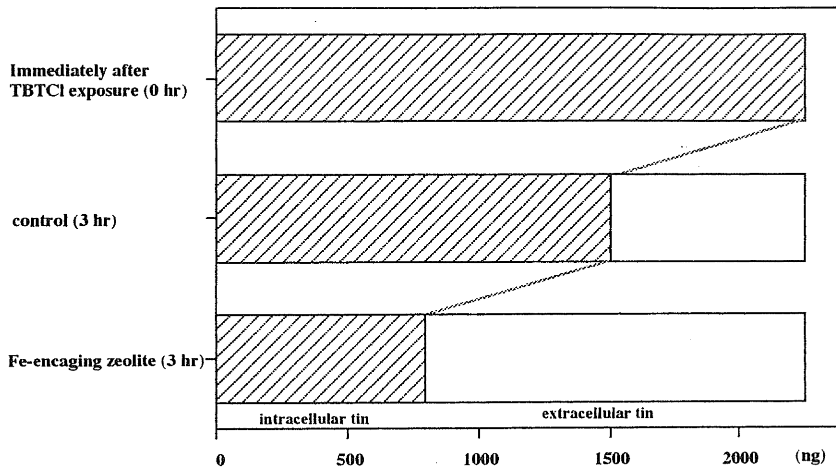

Fig. 1 Intracellular and extracellular amounts of tin compounds on *Euglena gracilis*

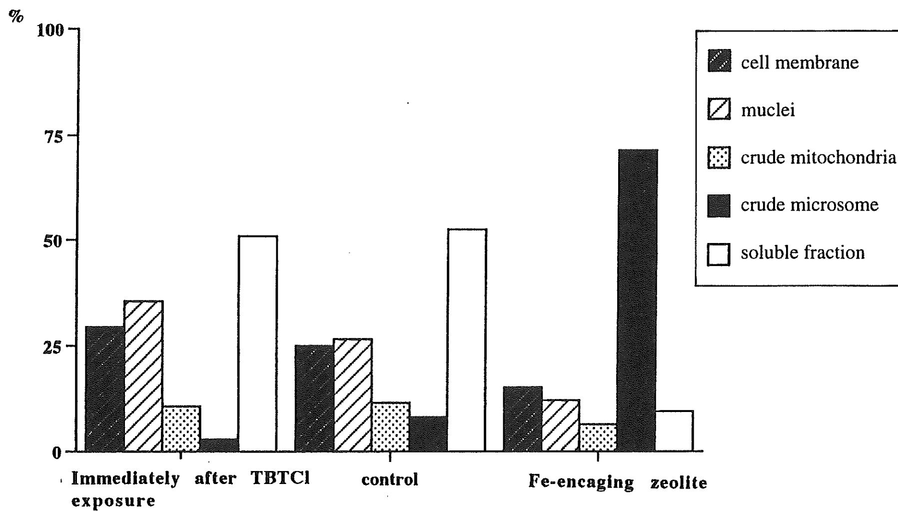

Fig. 2 Localization of tin in TBTCl-intoxicated *Euglena gracilis* Z

Results

Intracellular and extracellular analysis of tin by atomic adsorption

To confirm the effect of Fe-encaging zeolite-processed water on the excretion of tin compounds from *Euglena* cells, intracellular and extracellular amounts of tin were analyzed by atomic absorption spectrophotometry. Figure 1 shows the amounts of the incorporated tin and the excreted tin incubated after 0 and 3 hrs. The extracellular amount of tin in the incubated with Fe-encaging zeolite-processed water was significantly higher than that of control cells; i. e., 748.2ng of tin was detected in the extracellular fraction of the control, while 1458.1ng was in the extracellular fraction of Fe-encaging zeolite processed water.

Subcellular localization of tin in TBTCl-intoxicated *Euglena gracilis*

The intracellular distribution of tin at 0 hr and 3 hr after exposing *Euglena gracilis* to TBTCl was compared. Figure 2 shows the subcellular localization of tin in TBTCl-intoxicated *Euglena gracilis* cells. More than 39% of incorporated TBTCl at 0 hr localized in the cytosol fraction, and 27.4% was in the nuclei fraction, 22.7% was in the cell membrane fraction, and only 2.2% was in the crude microsomal fraction. In the TBTCl-intoxicated *Euglena* cell incubated for 3 hrs in the distilled water, more than 42% of TBTCl was localized in the cytosol fraction, 21.5% in the nuclei fraction, 20.2% in the cell membrane fraction, and 6.6% in the crude microsomal fraction. On the other hand, the incorporated TBTCl after incubation in Fe-encaging zeolite-processed water was found mostly in the microsomal fraction (62%), while 13.3% was found in the cell membrane fraction, and 10.6% in the nuclei fraction.

Analysis of metabolites of TBTCl in *Euglena gracilis*

The mass spectra of methylated metabolites of TBT in *Euglena* cells and standards are shown in Fig. 3. Metabolites were identified by comparing their retention time (t_R) on the chromatograms and mass fragments after their transformation to tetraalkyltin compounds by methylmagnesium bromide. The identification of metabolites by authentic standards was also confirmed by GC-MS. The major peak on the chromatogram was eluted at t_R equivalent to dibutyltin (DBT). By comparing the mass spectra with the methylated product of authentic standard of DBT (A') at t_R=17.80min, the extract of extracellular metabolites incubated in Fe-encaging zeolite-processed water (A), was confirmed to be DBT as a major metabolite of TBTCl in *Euglena gracilis*. On the other hand, unknown peaks were also detected at t_R=19.08 and 21.25min on the chromatogram of extracellular extract from the sample of Fe-encaging zeolite-processed water, and at t_R=18.14 and 18.90min on the chromatogram of intracellular extract from the control and Fe-encaging zeolite-processed water.

A confirmation of unknown peaks observed after Rt=16.02min has not been carried out yet, however, it is supposed to be more hydrophilic organotin compound.

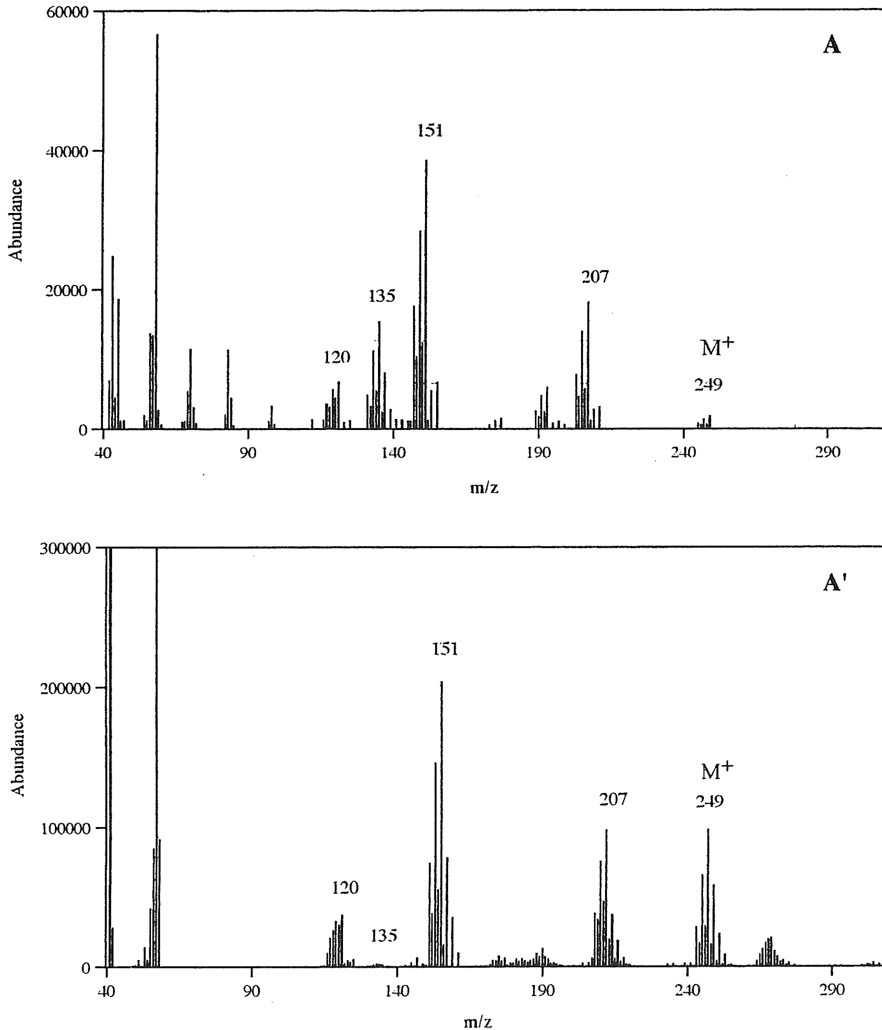

Fig. 3 GC-MS spectra of methylated metabolites in the extract and standards

A : extracellular extract incubated in Fe-encaging zeolite treated water

A' : authentic standard of DBT

Discussion

To reveal how Fe-encaging zeolite-processed water works on the detoxification of TBTCl-intoxicated ***Euglena*** cells was investigated from the biotransformational point of view. Subcellular localization of tin in TBTCl-exposed ***Euglena*** cell showed that TBTCl was rapidly incorporated into the cell. Comparing intracellular and extracellular distribution of metabolites of TBTCl in the Fe-encaging zeolite-processed water and the control, metabolite were mainly detected in the microsomal fraction of the Fe-encaging zeolite-processed water treated ***Euglena*** cells. The result clearly indicates that Fe-

encaging zeolite-processed water promoted metabolism and excretion of TBTCl. Concerning the fact that the tin mostly localized in the microsomal fraction, the metabolism of TBTCl relates to biotransformation reactions involving the phase I and may be phase II reaction, either, it is well known that iron is important as the catalytic site of the phase-I biotransformation enzyme, cytochrome P-450[15]. Parton et al. reported that Fe-encaging zeolite complex exerted an efficient mimic of cytochrome P-450 function[16]. Iron-encaging zeolite would participate in detoxification of the TBTCl in the ***Euglena*** cell *in vivo*.

On the other hand, it has been known that TBTCl can easily be metabolized in mammals, and the metabolites of TBTCl are resulted from hydroxylation and carboxylation at the butyl group of TBT or DBT compounds[17]. In ***Euglena gracilis***, the presence of a cytochrome P-450 mediated microsomal ethanol-oxidizing system, similar to that of hepatic cells, has been reported[17,18]. Interestingly, the major metabolite of TBTCl in ***Euglena*** is similar to that recognized in marine organisms[19]. Although the detailed mechanism on the effective excretion of TBTCl from cell by Fe-encaging zeolite-processed water has not yet been revealed, it must be because of enhanced metabolism of intracellular TBTCl to dealkylated metabolite such as DBT or more hydrophilic metabolites.

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