Insufficient Metallothionein Synthesis in the Lung and Kidney in Human Acute Inorganic Mercury Poisoning

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It has been assumed that "smelter disease" is caused by sulfuric dioxide. A typical episode resulting in "smelter disease" occurred in Fukushima, Japan. Twenty-seven workers became ill and eventually three of them died. The concentration of mercury (Hg) was found to be higher in all tissues and blood of the three victims than in those of normal Japanese, although the concentrations of zinc, cadmium, copper and lead in all tissues examined were within the normal range. The clinical course after the incident and autopsy findings clarified the cause of death to be acute Hg fume poisoning. To determine the histological localization of Hg and metallothionein (MT), Hg staining by the photo-emulsion method and immunostaining using anti-MT antibody were carried out. Numerous Hg granules were observed in the epithelia of the proximal tubules of the renal cortex using the photo-emulsion histochemical method. The liver of victims contained a few Hg granules in the hepatic cellular cytoplasm and sinusoid. Immunostaining of the kidney showed a strong positive reaction with anti-MT in the proximal tubules outside the medulla. The presence of Hg-bound MT in the kidneys of the victims was confirmed by gel chromatography. This is the first evidence of Hg-MT in the tissues of humans with acute Hg fume poisoning. Mercury might induce the synthesis of MT in human tissues. In addition, fractionation of the supernatants on gel chromatography revealed that most of the Hg in the kidney and lung of the patient who had the most severe renal and lung damage and who was the first of the three victims to die was distributed in high molecular weight protein fractions (HMW) and a small portion of Hg was bound to MT. These findings suggest that the amount of synthesized MT in tissues was not sufficient for MT to bind to Hg. The amount of Hg absorbed into tissues may be too large for MT to protect tissues, and thereby Hg may be bound to HMW.

Key words —— mercury-metallothionein, mercury fume, human acute poisoning, subcellular distribution, immunohistochemical method

INTRODUCTION

In industrial plants producing sulfuric acid, the insulated pipes of a tubular heat exchanger must be replaced every ten years. "Smelter disease" has been reported in workers who were engaged in the replacement of such pipelines. This disease was assumed to be caused by sulfur dioxide (SO_2), since the pipe-cutting operation using gas burners generated fumes containing a high concentration of SO_2. However, these fumes also contain a variety of toxic metals, including mercury (Hg), cadmium (Cd) and lead (Pb). Therefore, it is not clear whether smelter disease is caused by SO_2 or by toxic metals. Koizumi *et al.*[1] suggested that smelter disease found in workers of a smeltery in Akita, Japan was caused by Hg poisoning, not SO_2 poisoning.

The dangerous effects of Hg fumes have long

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been recognized, and many cases of acute Hg poisoning due to fume inhalation have been reported to have occurred not only in industrial factories but also in the home.[2-5] One detailed case report of Hg poisoning describes the diagnosis, causes, therapy, pathology, and the presence of high concentrations of Hg in tissues.[3]

It is well known that mercury vapor (Hg^0) is rapidly oxidized to divalent mercury ions (Hg^{++}) in blood and distributed to tissues, including those of the kidney and lungs, where they accumulate.[6] In animal tissues, inorganic Hg induces synthesis of metallothionein (MT), which is a cystein-rich, low molecular weight, and metal-binding protein.[7,8] MT plays an important role in the detoxification of heavy metals such as Hg and Cd.[9,10] However, the relationship between Hg accumulation in human tissues and its toxicity has not been investigated.

A typical episode of smelter disease occurred in Fukushima, Japan. Twenty-seven workers who had been involved in the operation of replacing several thousand pipes of a tubular heat exchanger became ill, and three of them eventually died. In these three cases, we investigated the possibility of Hg poisoning as the cause of death. Our clinical findings together with the results of autopsy and toxicological analysis suggest that the acute poisoning in "smelter disease" is caused by exposure to Hg fumes. To determine the preventive role that MT plays in acute Hg toxicity, we also investigated the form of Hg in the subcellular fractions of tissues.

MATERIALS AND METHODS

Reagents —— All of the reagents used for the determination of metals were of a special grade for metal analysis. Anti-MT antibody and Streptavidin Biotin Complex for immunohistochemical studies were purchased from Zymed Laboratories, Inc. (CA, U.S.A.) and DAKO (CA, U.S.A.), respectively. Autoradiographic emulsion NR-M2 for Hg staining was purchased from Konica Corporation (Tokyo, Japan). All other reagents were of the highest commercially available grade.

Victims —— Work to replace pipes in a tubular heat exchanger with new ones in a sulfuric acid manufacturing plant of a zinc smeltery in Fukushima was started on July 7, 1993. Between July 19 and 27, 27 workers complained of feeling poorly, and 13 of them were hospitalized between July 27 and 30. Three of these 13 workers, who had been engaged in the cutting of pipes with gas burners at the same place during the morning of July 26, eventually died (case 1, 64-year-old male; case 2, 50-year-old male; case 3, 50-year-old male). These 3 workers had worked only half a day in the plant.

Symptoms resembling those of a cold, such as fever, headache and nausea, first appeared after lunch on July 26. Lung and kidney functions continued to deteriorate over the next few days, causing dyspnea, cyanosis and anuria. The three workers died on the 12th, 17th and 19th day, respectively, after exposure to fumes. The cause of death was diagnosed as respiratory insufficiency and acute renal failure in each case.

Autopsies on cases 1 and 2 were performed in Iwaki Kyoritsu Hospital, where these patients had been admitted. The autopsy on case 3 was performed in the Department of Legal Medicine, Fukushima Medical University School of Medicine. Autopsies and pathohistological examinations revealed diffuse cortical necrosis in the kidneys of all three patients. Details of clinical and pathological findings will be described elsewhere.

Samples —— For the determination of concentrations of metals, tissues collected at autopsy were stored at −80℃ until analysis. For histochemical study, the tissues were fixed in a formalin solution, and paraffin sections 3 μm in thickness were prepared according to the usual method.

Determination of Concentrations of Metals in Blood and Tissue —— For the determination of concentrations of Zn, Cd and Cu, 0.2 g of tissue or blood was digested in 3 ml of an acid mixture (HNO_3 : $HClO_4$ = 19 : 1 v/v) at 120℃ for 3 h and then at 200℃ until the solution became clear. After digestion, the residues were diluted with ultra-pure water (Japan Millipore Ltd., Tokyo) to an appropriate volume, and metal analysis was carried out by atomic absorption using a Hitachi Z-6100 spectrometer (Hitachi Co., Tokyo).

To determine the concentration of Hg, 0.1 g of tissue or blood was digested in 0.5 ml of HNO_3 and 2 ml of H_2SO_4. The solutions were heated at 160℃ and then allowed to stand for 30 min at room temperature. The residues were diluted with ultra-pure water to 10 ml in a water bath. The Hg concentration was measured using an atomic absorption spectrometer equipped with a mercury detector (Nippon Instruments Co., Tokyo).

Gel Chromatography —— Tissues were homogen-

ized in cold 0.25 M sucrose–10 mM Tris–acetate buffer (pH 8.0) to make a 25% homogenate using a Polytron homogenizer (Kinematica, GmBH, Luzern, GDR) and then centrifuged at 105000 *g* for 60 min in a Beckman L8-80 ultracentrifuge (Beckman Co, Ltd. Tokyo). The supernatant was chromatographed on a HiLoad Superdex 75 (16 × 600 mm, Pharmacia Fine Chemicals, Sweden) and eluted with 10 mM Tris–acetate buffer (pH 8.0) at a flow rate of 1 ml/min. Fractions 2.5 ml in volume were collected, and 1.5 ml of each fraction was digested with 0.6 ml of acid mixture ($HNO_3 : HClO_4 = 4 : 1$ v/v) at 140℃ for 2 h. The digested samples were diluted with 5 ml of 0.02 N HCl, and metal analysis was performed using an atomic absorption spectrometer (Z-6000, Hitachi Ltd., Tokyo).

Histochemical Studies —— Immunohistochemical staining with anti-MT antibody was carried out according to the method of Suzuki *et al.*[11] except for the use of Streptavidin Biotin Complex. Mercury staining was carried out by the photo-emulsion histochemical method according to Tokunaga *et al.*[12]

RESULTS

Concentrations of Hg, Cd, Cu, Pb and Zn in Tissues and Blood

The concentrations of Hg in the tissues and blood of cases 1 and 3 were higher than those in normal Japanese[13] (Table 1). Particularly notable was the fact that the concentrations of Hg in the lung and kidney of the victims were over ten-times higher than those in normal Japanese. Hg levels in the brain were also higher in the victims than in normal Japanese (data not shown). The concentrations of Hg in the lung, liver and blood of case 1 were higher than those of case 3, whereas the renal concentration of Hg was higher in case 3 than in case 1. Tissue and blood samples for chemical analysis were not taken from case 2 at autopsy.

The liver Zn concentration was a little higher than normal in case 3, while the kidney Zn concentration was lower than normal in case 1 (Table 1). The Zn concentrations in other tissues of case 1 and case 3 were within normal ranges. The concentrations of Cd, Pb and Cu in the tissues and blood of cases 1 and 3 were also within normal ranges.

Table 1. Concentrations of Metals in Tissues and Blood

Metal	Tissue	Concentration of metals μg/g or μg/ml		
		Case 1	Case 3	Normal Japanese[a]
Mercury	Lung	3.31	0.8	0.015— 0.30
	Liver	4.54	2.84	0.16 — 1.3
	Kidney	25.8	60.04	0.18 — 2.6
	Blood	0.59	0.31	0.016— 0.11
Zinc	Lung	13.80	13.28	8.9 —25
	Liver	73.55	114.85	21 —82
	Kidney	19.14	87.74 (Cortex)	27 —95
			52.40 (Medulla)	
	Blood		8.4	7.8 —16
Cadmium	Lung	0.751	0.28	0.15 — 2.3
	Liver	6.748	1.156	1.1 —23
	Kidney	12.568	46.411 (Cortex)	10 —94
			21.190 (Medulla)	
	Blood		0.136	0.05 — 0.58
Copper	Lung	0.037	0.063	0.098— 0.81
	Liver	0.642	0.195	0.16 — 1.0
	Kidney	0.02	0.125 (Cortex)	0.16 — 1.2
			0.032 (Medulla)	
	Blood		0.166	0.10 — 0.53

a) Concentration of heavy metals in normal Japanese reported by Sumino *et al.*[13]

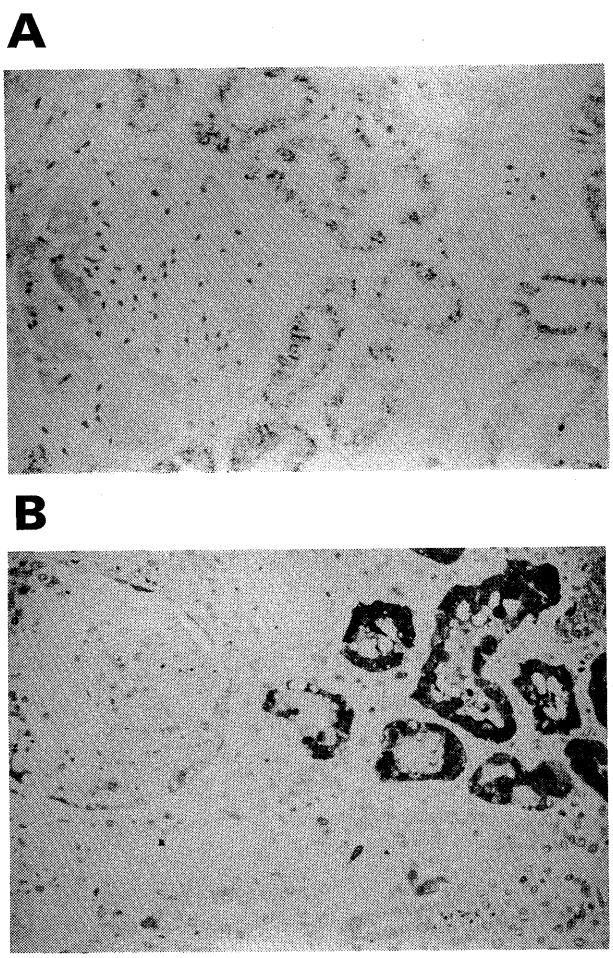

Fig. 1. Kidney of Case 1

A: Black Hg granules in the epithelium of a proximal tubule. Photo-emulsion method. Counter-stained with nuclear fast red. ×400. B: Strong positive reaction for anti-MT in the epithelium of a proximal tubule outside the medulla. Immunostaining. Counter-stained with hematoxylin. ×400.

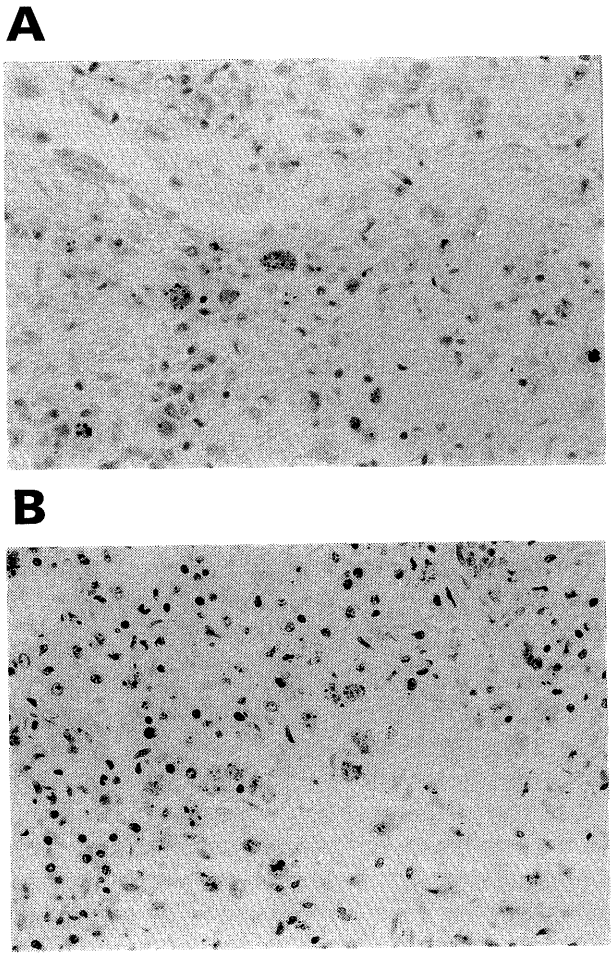

Fig. 2. Lung of Case 3

A: A small number of Hg granules in macrophages. Photo-emulsion method. Counter-stained with nuclear fast red. ×400. B: Slight positive reaction for anti-MT in macrophages. Immunostaining. Counter-stained with hematoxylin. ×400.

Distribution of Mercury in Tissues

The photo-emulsion histochemical technique was used for Hg staining of tissues. Many dark brown granules indicative of Hg were observed in the kidney of case 1. Most of the these were found in the renal cortex, especially in the epithelia of proximal tubules (Fig. 1A). Similar findings were obtained in case 2. Numerous small Hg granules were found in the epithelia of proximal tubules in case 3 (Fig. 3A), but only a few granules were observed in the medulla. The Hg granules were larger in size and fewer in number in cases 1 and 2 than in case 3.

A few Hg granules were present in the hepatic cellular cytoplasm and sinusoid of cases 1 and 3 (Fig. 4) but were scarely found in case 2. A few Hg granules were observed in macrophages in the lung of case 3 (Fig. 2A) but not in those of cases 1 and 2 (not shown).

Localization of Metallothionein in Tissues

A strong positive reaction with anti-MT antibody was observed in the renal proximal tubules outside the medulla of case 1 (Fig. 1B) and case 2 (not shown). The kidney of case 3 showed a strong positive reaction in the epithelia of proximal tubules but only a weak positive reaction in the medulla (Fig. 3B).

The liver showed a faint positive reaction in the hepatic cellular cytoplasm of case 3 (Fig. 4B), whereas the reaction was negative in cases 1 and 2. The reaction was slightly positive in the pulmonary macrophages of case 3 (Fig. 2B) but negative in those of cases 1 and 2 (not shown).

Form of Mercury in Subcellular Fractions

The distribution of Hg in subcellular fractions was determined. Figure 5 shows the percentage of Hg in the 105000 *g* supernatant and precipi-

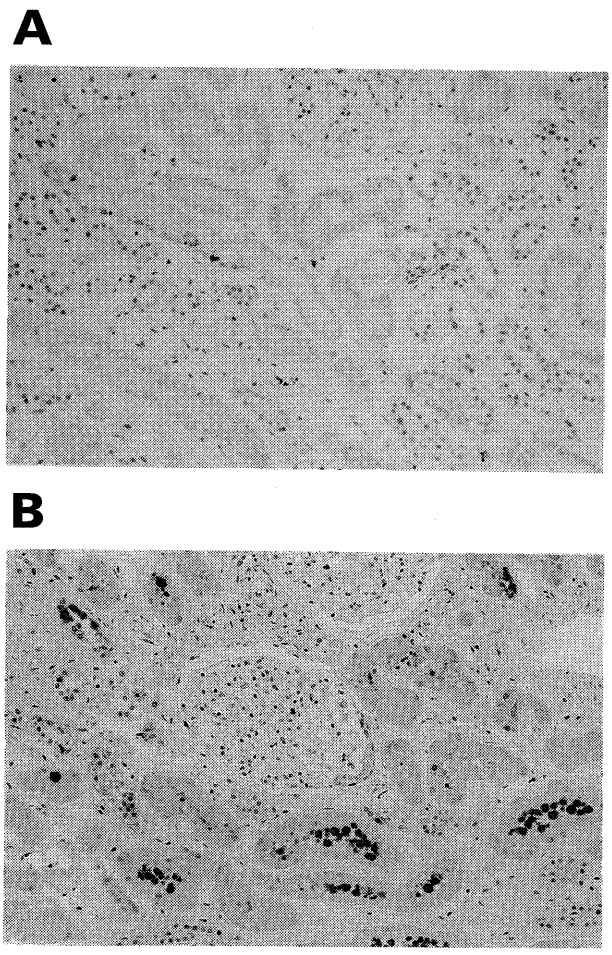

Fig. 3. Kidney of Case 3

A: Small black Hg granules in the epithelium of a proximal tubule. Photo-emulsion method. Counter-stained with nuclear fast red. ×400. B: Strong positive for anti-MT in the epithelium of a proximal tubule and intralumen. Immunostaining. Counter-stained with hematoxylin. ×200.

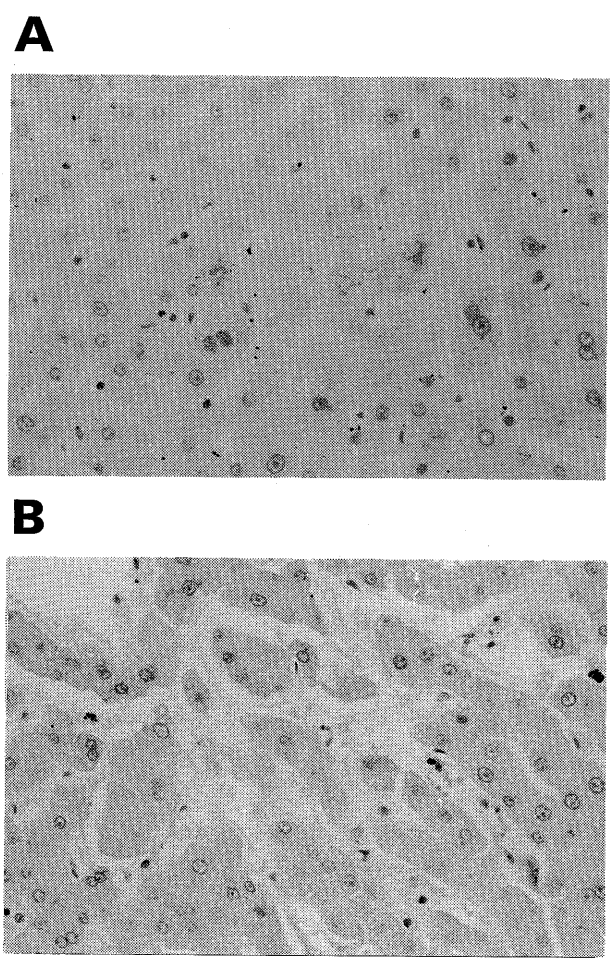

Fig. 4. Liver of Case 3

A: A small number of small Hg granules in the cytoplasm and the sinusoids. Photo-emulsion method. Counter-stained with nuclear fast red. ×400. B: Faint positive reaction in the cytoplasm of hepatic cell. Immunostaining. counter-stained with hematoxylin. ×400.

tate to the total amount of Hg. Most of the Hg was distributed in the 105000 *g* supernatants except for the kidney of case 1. In the kidney of case 1, the relative content of Hg in the supernatant to total Hg was only 20%, while it was about 70% in the kidney of case 3.

Fractionation of the supernatant from the kidney of case 3 on a HiLoad Superdex 75 revealed two peaks of fractions containing Hg (Fig. 6). The second peak corresponded to the elution volume of authentic rat liver MT, and most of the Hg (82% of the supernatant) was found in these fractions. Most of the Zn (70%), Cd (92%) and Cu (75%) was also found in the same fractions.

On the other hand, fractionation of the kidney supernatant of case 1 revealed three peaks containing Hg (Fig. 6). Only 13% of Hg in the supernatant was found in the third peak, corre-

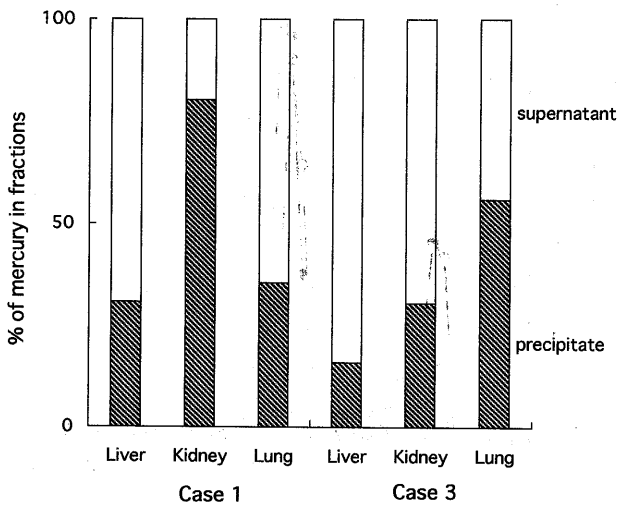

Fig. 5. Percentage of Mercury in 105000 × *g* Fraction Relative to Total Mercury in Tissue Homogenates

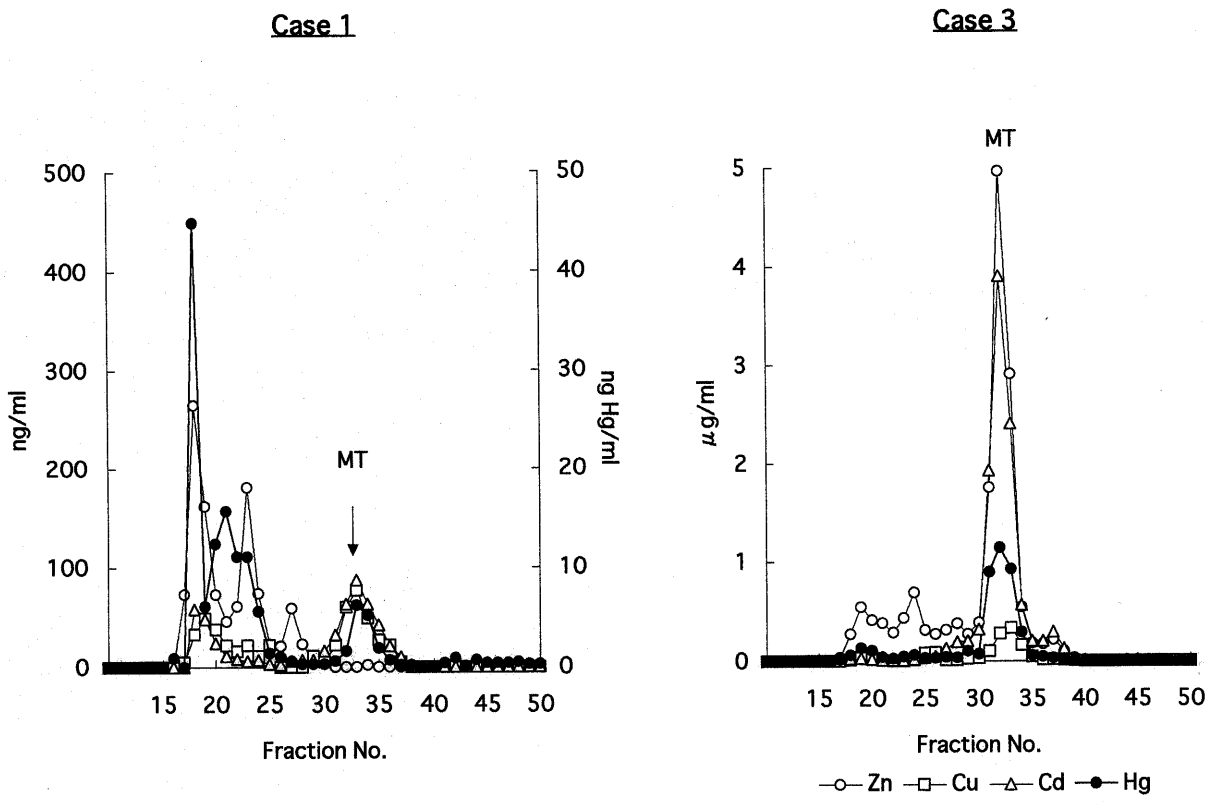

Fig. 6. Elution Profiles of the Kidney on a Hiload Superdex 75

The kidney homogenate was centrifuged at 105000 × *g* for 60 min. The supernatant was applied to a HiLoad Superdex 75 and eluted with 10 mM Tris-acetate buffer (pH 8.0). Fractions 2.5 ml in volume were collected and analyzed by atomic absorption spectrometry.

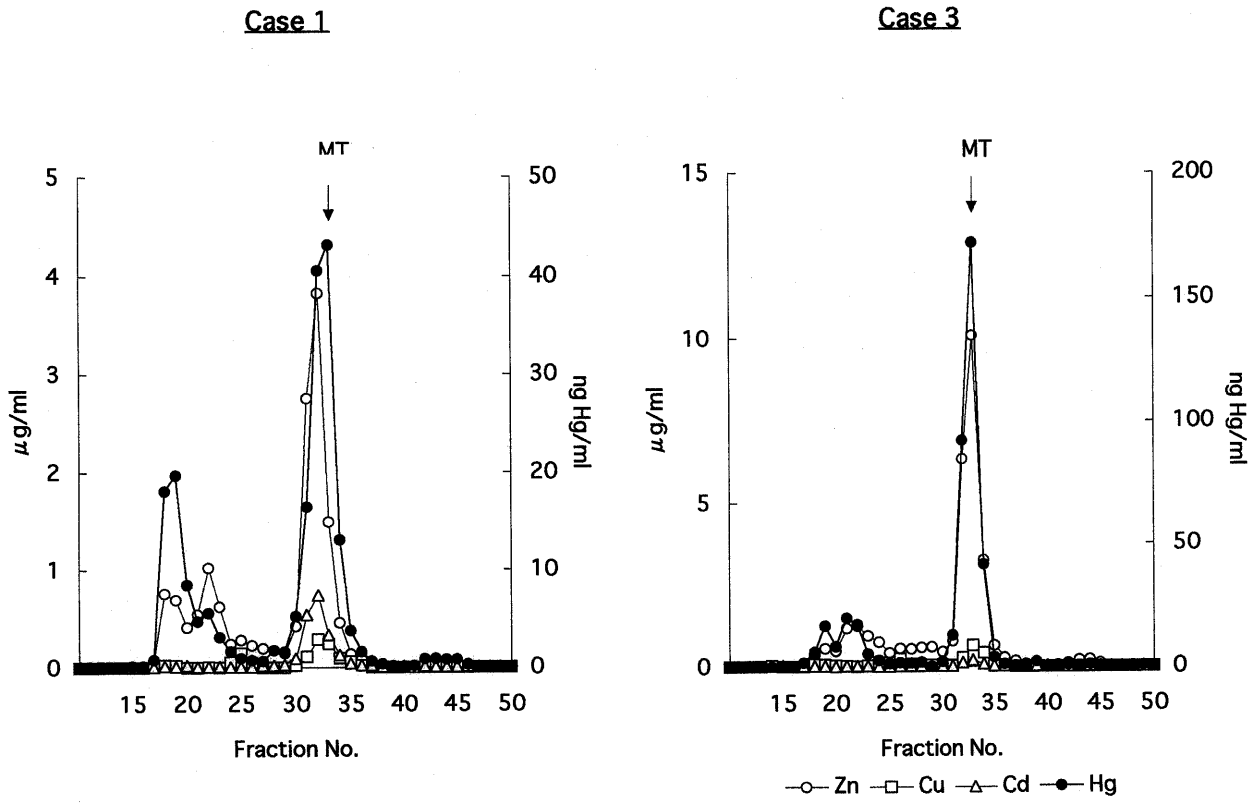

Fig. 7. Elution Profiles of the Liver on a Hiload Superdex 75

The column conditions are the same as those stated in the legend to Fig. 6.

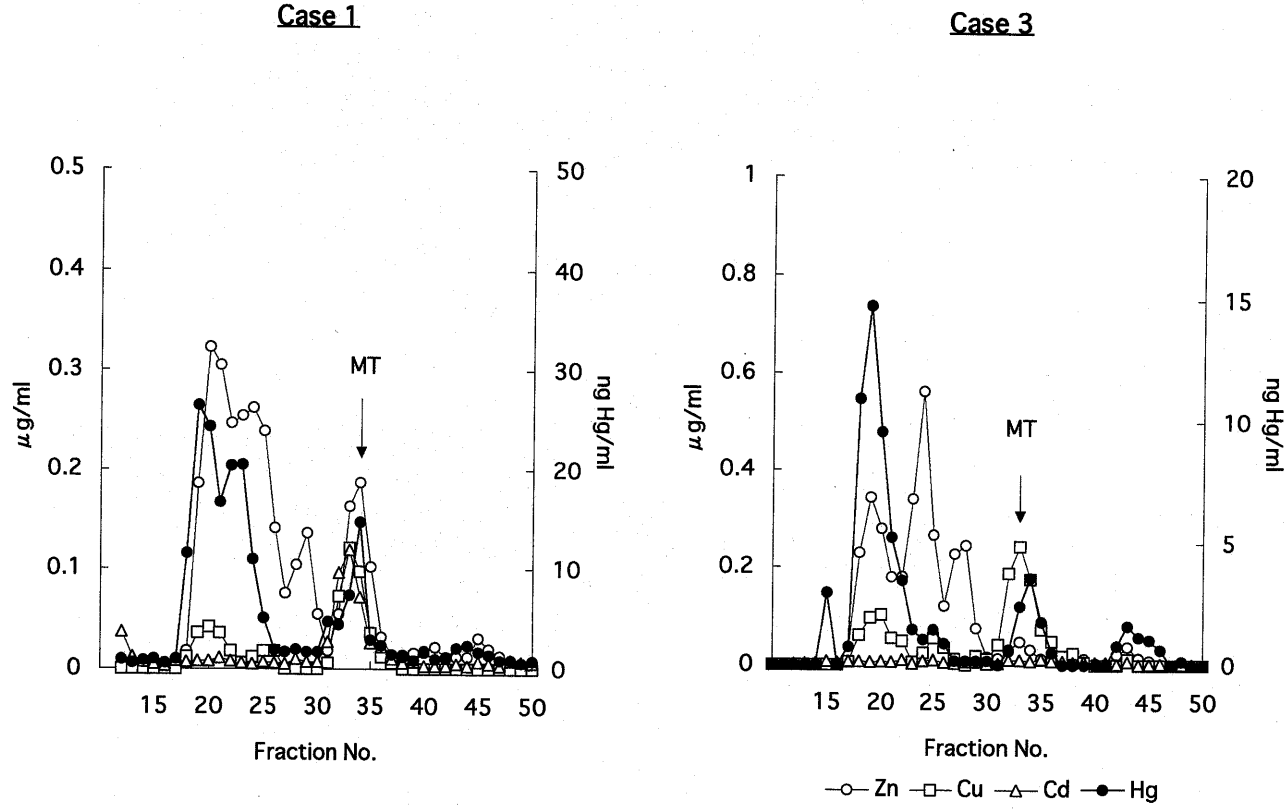

Fig. 8. Elution Profiles of the Lung on a Hiload Superdex 75
The column conditions are the same as those stated in the legend to Fig. 6.

sponding to the elution volume of MT. The third peak also coincided with peaks of Cd (64%) and Cu (54%); Zn was not detected in the third peak (MT). Most of the Hg was distributed in the first and second peaks of fractions (high molecular weight fraction: HMW), indicating non-specific binding of Hg to proteins.

Figure 7 shows chromatographical patterns of the liver samples of cases 1 and 3. Hg was distributed over three peaks, and most of it (63%) was detected in the third peak (MT fractions). Most of the Zn (62%), Cd (88%) and Cu (73%) was present in the same fractions. The chromatographical pattern of the liver sample of case 3 also showed 79% recovery of Hg in the MT fractions. Over 60% of Zn and Cu in the supernatant was also present in the MT fractions.

The elution profiles for the lung samples of cases 1 and 3 demonstrate that only a small portion of Hg (19% in case 1 and 13% in case 3) was bound to MT, whereas most of the Hg was found in the HMW fractions (Fig. 8).

DISCUSSION

This paper describes three fatal cases of a typical smelter disease. At first, it was thought that the severe respiratory and renal failures had been caused by SO_2 poisoning. However, autopsies revealed severe damage to the lung and kidney, that is, diffuse organized pneumonitis and renal tubular necrosis. Moreover, the patients died 12 days to 19 days after the incident, which is too long for SO_2 poisoning.

The sludge adhering to the inside of insulated pipes contained high amounts of mercuric sulphate, iron, aluminum, Cu, Zn, Pb, and Cd. Since tissue concentrations of Pb, Cd and Zn were within normal ranges, it is likely that these metals did not contribute to the development of severe tissue damage. A simulation study of the work process confirmed the generation of SO_2 and Hg fumes at the accident site. The concentration of Hg was estimated to be 43.4 mg/m^3, about 800-times higher than the maximal tolerance level (0.05 mg/m^3).[6] The estimated concentration of SO_2 was 2 to 5 ppm, a level that can cause irritation of conjunctiva and nasal mucosa[14] but does not exceed the maximal tolerance level (5

ppm)[15] and is therefore not lethal.

Inhalation of Hg fumes can cause acute interstitial pneumonitis and bronchiolitis. Renal abnormalities can also occur in acute Hg fume poisoning.[2-5] On the other hand, acute severe airway obstruction is the most prominent lesion after exposure to a high concentration of SO_2.[14,16] Our autopsy findings coincided with the features of Hg poisoning rather than those of SO_2 poisoning. In addition, toxicological analysis disclosed high concentrations of Hg in tissues and blood. The tissue Hg contents were 3- to 20-times higher in the victims than in normal Japanese. We therefore concluded that the main cause of death was Hg poisoning, although SO_2 might have accelerated tissue damage in the early stage of poisoning.

This supports the findings of Koizumi *et al.*,[1] who investigated a similar incident in an industrial plant producing sulfuric acid. They detected high concentrations of Hg in blood, urine and hair of workers 3 to 4 weeks after the incident and therefore concluded that "smelter disease" was caused by Hg, not by SO_2. The workers had been wearing mask respirators with cartridges capable of complete absorption of SO_2 but not Hg.

Matthes *et al.*[3] reported two fatal cases of acute Hg vapor poisoning. The victims died 3 and 5 days, respectively, after the accident, and the Hg contents were 8.9 and 9.4 mg/kg in the liver, 6.3 and 9.3 mg/kg in the lung, and 79.1 and 60.0 mg/kg in the kidney, respectively. Although the tissue Hg contents were lower in the present cases than in those two cases, the tissue Hg contents in our cases were still 3- to 20-times higher than the normal range.[13] The biological half-life of elemental Hg is 18 hours in the lung and 64 days in the kidney.[18] In blood and plasma, a rapid absorption phase of Hg followed by a biexponential decline of the curves was seen.[19] These findings suggest that the tissue Hg contents in our cases were very high soon after the incident.

The subcellular distribution of Hg in the liver and kidney demonstrates that most of the Hg in the kidney of case 1 existed in the precipitate, not in the supernatant, suggesting that there was non-specific binding to cellular components in the precipitates and/or deposition of polymerization of Hg–MT. Furthermore, the Hg elution profile of the supernatant revealed that a relatively large portion of Hg was in HMW fractions, not in MT fractions, suggesting that biosynthesis of MT was insufficient for binding to Hg incorporated in tissues. Histological examination of the kidney of case 1 showed the disappearance of nuclei in renal cells, indicating severe renal tubular necrosis. Thus, most of the Hg was unusually bound to HMW in the kidney and lung of case 1. This patient was the first of the three to die. These findings suggest that the amount of Hg accumulated in the kidney and lung of case 1 might have been too much to protect the Hg toxicity by MT and might have caused the destruction of cells.

In the kidney of case 3, Hg-bound MT was also confirmed by histochemical observation. Photo-emulsion technique revealed numerous small Hg granules in the epithelia of proximal tubules outside the medulla in the kidney. Investigation of MT by the immunohistochemical method using a specific antibody showed strongly positive materials at the same site in the kidney. The number and size of Hg granules were similar to those of anti-MT positive materials. These results suggest that Hg and MT were distributed in the same site in the kidney, indicating that Hg was bound to MT. The Hg elution profile of the kidney sample of case 3 showed that most of the Hg was bound to MT. Histologically, however, severe renal tubular necrosis was not observed.

In animal experiments, induction of MT synthesis following exposure to Hg vapor has been reported. According to Cherian and Clarkson,[8] the exposure to Hg vapor for up to 8 days resulted in an increase in MT-like protein in the kidney but not in the liver of rats. Yoshida *et al.*[7] also reported enhanced MT synthesis in the kidneys of the neonatal guinea pigs after Hg vapor exposure within 12 hours after parturition. In the case of humans, Pulido *et al.*[19] isolated MT containing a small amount of Hg from the kidney of a patient who ingested mercurials for a long period. However, there has been no study on the presence of Hg–MT in human tissues after acute Hg poisoning.

There are two possible explanations for the detection of MT containing Hg in tissues. One is the induction of MT synthesis after exposure to Hg fumes, and the other is the exchange of Hg for Zn bound to MT, because Hg has a stronger affinity for apo-MT than Zn.[20] Considering that a

large amount of MT containing Hg was detected, it is more likely that Hg induces MT synthesis.

In conclusion, the cause of death in each of our three cases was believed to be acute Hg fume poisoning. Investigation of the subcellular fractions of Hg revealed the presence of Hg-MT in the kidney. The synthesis of MT might have been induced to protect the toxicity of Hg after exposure to Hg fumes, but the amount of Hg absorbed into tissues might have been too large for MT to protect tissues in the present cases. As Koizumi *et al.* stated,[1] the wearing of fully encapsulated suits with a supplier-air system that will shut off all possible routes of exposure is required to prevent workers from Hg fume poisoning. A new respirator mask with cartridges that can absorb both SO_2 and Hg is also desirable.

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