HISTOCHEMICAL DEMONSTRATION OF MERCURY IN HUMAN TISSUE CELLS OF MINAMATA DISEASE BY USE OF AUTORADIOGRAPHIC PROCEDURE¹

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Histochemical technique using autoradiographic procedure was presented which can be used to demonstrate non-labeled mercury in paraffin-embedded tissues of the central nervous system and other organs. Compared to other histochemical methods, this technique allows the use of formalin-embedded, stored, serial paraffin sections and appears more sensitive for the demonstration of intracellular mercury in Minamata disease (methylmercury poisoning). The technique forms reaction products of high quality which can easily be seen in cell bodies and does not disturb the structure of tissue cells during preparation.

Earlier works have presented four kinds of histochemical methods which have been employed to demonstrate mercury in tissue sections. The first one histochemically demonstrates mercury in the tissue cells as brownish black granules by use of hydrogen sulfate (Justus 1901, Almkvist 1903, Cambar 1940), or ammonium sulfide (Chang & Hartman 1972). The second is a histochemical technique in which mercury is detectable by colored precipitates in metal chelating mechanism using diphenylthiocarbazide (Brandino 1927, Okamoto, Seno and Okumura 1944), dithizone (Okamoto, Seno and Okumura 1944) and rhodizonate (Feigl and Suter 1942). The third is to make metal precipitates detectable by use of stannous chloride (Christeller & Sammartino 1928, Kuroda 1951). The fourth is a silver impregnation technique (Timm 1958, Voigt 1958, Shiraishi 1964, Silberberg *et al.* 1969). These techniques have been tested and improved by many investigators, but there are no suitable methods to effectively demonstrate mercury, because of unspecific reaction with destructions of structure or of incomplete sensitivity.

Autoradiographic technique using ²⁰³Hg has recently been applied experimentally (Lippman, Findle and Gillette 1951, Cassano *et al.* 1969, Takahashi *et al.* 1971, Sakai 1975). However, it can not be used in the stored paraffin sections of human autopsy materials, apart from the atomic irradiation procedure. During autoradiographic experiments, the procedure in this technique was found to demonstrate mercury, in spite of the fact that intracellular mercury is not labeled (Umeda *et al.* 1969). The precipitation mechanism is still unknown, but the present study

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demonstrates that intracellular mercury in organs from the autopsy materials can be easily and more sensitively detected by the autoradiographic procedure without labeling.

MATERIALS AND METHODS

Human autopsy materials containing a variety of total mercury in Minamata disease were fixed in unbuffered 10% formalin. These tissues were processed in the usual manner through ethanol, xylol and paraffin and were stored in the usual manner. In the face of preparation, deparaffinized sections cut 4 μ m thick were incubated in Kardasewitsch's mixture (70% ethanol; 28% ammonia; 100:1-5) for 4 hours at room temperature, when formalin pigments were present in tissues. After washing in water, the sections were stained with hematoxylin and eosin and washed clearly with distilled water. They were then covered with

FIG. 1. The metal precipitates for histochemical demonstration of mercury are shown in granular forms in the tissue of kidney in an acute case of Minamata disease (No. 3201). The granular precipitates appear more intensely in the epithelial cells of proximal convoluted tubules than the distal convoluted tubules, while the precipitates are not demonstrated in the glomerulus.

FIG. 2. Section through medulla of the kidney of the same case (No. 3201). The metal deposition is more abundantly illustrated in the epithelial cells of Henle loop than in the proximal convoluted tubules. The precipitates are not demonstrated in the collecting tubules.

Sakura NR-M₂ by the usual dipping method in the dark room, kept and dried in the refrigerator for about 4 hours at 4°C. They were developed with the stocked developer FD-111 for 4 min at 20°C and were fixed with the photographic fixative following washing in water. After complete washing in water, the sections were dehydrated with a graded ethanol series and mounted with resin Bioleit following xylol treatment.

The total mercury in organs was estimated with the atom absorption method and dithizone technique. Methylmercury was chemically detected by chromatographic technique. A relationship between mercury content and sensitivity of staining was observed by using kidney from autopsy materials. Cadmium, iron and zinc were differentiated by the individual method.

RESULTS

Mercury was histochemically demonstrated as silver impregnation granules *in*

FIG. 3. The metal precipitates are also detected in the epithelial cells of proximal and distal convoluted tubules in the kidney of another autopsy case (No. 3159). The precipitates do not appear in the glomerulus and in the collecting tubules.

FIG. 4. Section through medulla of the kidney in control case which contains 0.835 $\mu\text{g/g}$ (wet weight) of total mercury. The metal precipitates appear only in the epithelial cells of thinner limb in some Henle loop. No metal deposition is detected in other epithelial cells.

TABLE 1. *Mercury contents in kidney and distribution of mercury in kidney tissues in Minamata disease*

autopsy number	clinical course	total Hg $\mu\text{g/g}$ atom absorption method	dithizone method (wet weight)	methylmercury $\mu\text{g/g}$	kidney proximal convolution	Henle loops thick limb	thin limb	distal convolution	collecting tubule
3159	19(d)	147.9	144.0	8.62	++	##	++	+	—
3201	60	207.5	106.0	1.16	##	##	+	++	—
3388	90	150.3	49.8	9.32	++	##	++	++	—
2776	53	112.9	40.5	4.99	++	##	++	+	—
3355	96	62.4	22.6	9.69	++	##	++	+	—
3018	1.6(y)	26.4	37.4	0.364	++	##	++	—	—
4292	4.3	17.2	17.6	0.441	++	++	+	—	—
3216	2.9	—	12.8	—	+	##	++	++	—
3794	6.4	—	11.3	—	++	##	+	+	—
3567	2.5	—	9.4	—	+	++	+	—	—
3298	4.0	10.0	5.9	0.497	+	++	+	—	—
5539	13(*)	—	5.5	—	+	++	##	+	—
6077	7.0	8.8	3.2	0.821	+	+	—	—	—
5663	8.0	12.7	1.0	0.564	+	+	+	—	—
5360	11.7	5.1	—	0.352	—	+	—	—	—
6266	4-5(?)	3.6	—	0.121	—	++	+	—	—
6215	8.9	1.5	—	0.126	—	+	—	—	—
6027	(control)	0.835	—	0.097	—	±	—	—	—
6120	(control)	0.513	—	0.017	—	—	—	—	—
6195	(control)	0.141	—	0.042	—	—	—	—	—

d: day y: year

Control cases were also picked up from Kumamoto area.

situ in tissue cells (Figs. 1–8). The impregnation mechanism is not known yet, but it seems to be similar to the so-called, “Entwicklungsmechanismus” proposed by Timm and Voigt, because of non-labeling of intracellular mercury.

FIG. 5. Section through cerebellar cortex of a severely prolonged case of Minamata disease (No. 6383). The metal precipitates are clearly and more densely detected in the Purkinje cell layer and granular layer than in the molecular layer. However, no or little depositions are demonstrated in Purkinje cells. Macrophages contain metal granules in the cortex as well as in the meninx.

FIG. 6. High magnification of another portion in the same section of cerebellar cortex as Fig. 5. The fine granules of metal precipitates are clearly revealed in the cell body of a Purkinje cell illustrated. The precipitates also appear in the preserved granule cells as well as in Bergmann glial cells or phagocytes.

FIG. 7. Section through occipital cortex of the cerebrum obtained from the same case (No. 6383). High magnification of the third layer of brain cortex illustrates the metal precipitation in nerve cells preserved and in a microglial cell or phagocyte.

FIG. 8. High magnification of pons in the same case. The metal precipitates are clearly and abundantly demonstrated in the cell bodies of nerve cells in some nucleus of pons.

The sensitivity of this demonstration was tested by the use of successive materials containing varied amounts of mercury in the human kidney of Minamata disease (Table 1). The mercury contents in tissues assayed were considerably different between the new atom absorption method and dithizone technique, because the former was recently carried out at the same time by using formalin-stored materials, and the latter was appropriately performed at the time of each autopsy. Mercury at the high level of over 20 $\mu\text{g/g}$ wet weight was easily demonstrated in epithelial cells in kidney tubules, extending over a wide range of whole tubules with the exception of collecting tubules (Figs. 1, 2, 3). Mercury contents of about 10 $\mu\text{g/g}$ could also be clearly detected. One $\mu\text{g/g}$ of mercury could be demonstrated only in epithelial cells of Henle loops (Fig. 4).

Distribution of mercury in nervous tissues was examined in the new materials obtained from an autopsy case of severely prolonged patient who died 18 years after a typical onset of Minamata disease. Mercury content in tissues was still detectable by the atom absorption method in total mercury and by chromatography in methylmercury, although the distribution pattern was different from that in acute cases (Table 2).

The metal granules were histochemically demonstrated in nerve cells as well as phagocytes and astrocytes in the brain (Figs. 5–8). They were found mainly in cytoplasm. Almost all nerve cells of the cerebrum contained the metal granules in various numbers. The large nerve cells in the nuclei of the brain stem contained the metal granules in greater numbers than the nerve cells in cerebral cortex, in spite of being undisturbed (Fig. 8). The small nerve cells preserved in whole cerebral cortex were also loaded with various numbers of metal granules, but in general the granules were smaller in number in these cells. The metal granules were more predominantly demonstrable in the phagocytes, astrocytes and adventitial cells in the brain. In the cerebellum, the metal granules were predominantly demonstrable in the phagocytes and Bergmann glial cells (Figs. 5, 6). No granules were found in a large number of Purkinje cells, but they could be detected in some and appeared as fine granular particles in the cytoplasm. The granule cells preserved contained

TABLE 2. *Mercury contents in tissues of a severely prolonged case of Minamata disease.*

Tissues	Hg- content	Total Mercury $\mu\text{g/g}$ wet weight (Atom absorption method)			Methylmercury $\mu\text{g/g}$	
		present chron. case 18 yrs.	*control acute 19 days	*control acute 96 days	present chron. case 18 yrs.	*control acute 96 days
Cerebral Cortex		2.785	13.11	7.36	0.035	2.34
Medulla		1.762	13.64	—	0.043	—
Cerebellum		4.237	15.81	8.78	0.063	2.95
Liver		2.399	155.8	84.3	0.055	6.80
Kidney		5.585	147.9	62.4	0.064	9.67
Brain/Liver $\times 100$		122	8.6	9.5	86	38.8
Brain/Kidney $\times 100$		52	9.0	12.9	73	27.5

* Results of acute cases obtained from the formalin-fixed materials of the autopsys performed 18 years ago.

a small number of granules. These granule cells were more easily destroyed in Minamata disease. The nerve cells in dentate nuclei were still able to be preserved in spite of their containing a great number of the metal granules. There was no close relationship between distribution of mercury and localization of disturbance.

The specificity of this reaction for the demonstration of mercury was identified by control procedures. The iron substances were histochemically not demonstrable in the target tissues such as kidney and brain of an autopsy case of Minamata disease examined in this report, by use of Prussian-blue and Turnbull-blue reactions. The hemosiderin iron in tissues of hemosiderosis selected from other autopsy cases was not reactive for this procedure, in spite of being intensely reactive for Prussian-blue reaction. The islet zinc of pancreas in rats and human autopsy cases was also not reactive for this procedure. Zinc was also not detectable histochemically in the liver, kidney and occipital lobe and cerebellum in brain of Minamata disease, using two methods proposed by Okamoto *et al.* Cadmium which was experimentally administered repeatedly in considerable amounts to rats (2.7 mg/100 mg) for another purpose was not demonstrated in the kidney and liver by this method, although it was easily detected histochemically by fluorescence method (Sato, 1974) and Timm's impregnation technique.

DISCUSSION

Techniques for the demonstration of mercury in tissues have been proposed by several investigators as mentioned in the introduction. However, no suitable histochemical method has been reported for selectively forming nonreactive products of any of the other metals, so as to specifically demonstrate individual metal deposits.

Okamoto *et al.*, 1944, have recommended the previous use of chloride vapor dried in advance of preparing diphenylthiocarbazide or dithizone methods to obtain a specific reaction, but other metals could remain in a reactive state and form complexes with diphenylthiocarbazide or dithizone. Therefore, even though Okamoto *et al.* stated that metals other than mercury can be identified by differences in the color of the various metal complexes, their method still lacks chemical specificity. It was also discovered through the use of the human materials affected by Minamata disease that the results of the methods of Okamoto *et al.* were not so sensitive in the case of mercury determination.

There are several methods for the demonstration of mercury, but these methods can not specifically demonstrate it, and so the sensitive reaction for metal precipitates should be selected and then the metal should be identified chemically. Practically applied, the technique for demonstration should be used for the stored materials of human autopsy cases, too. The previously proposed techniques were not always suitable for the demonstration of mercury in tissues stored in formalin or in paraffin block. The method proposed in this paper was best fitted to undertake those purposes. The sensitivity of the reaction was higher than that of Voight's and Timm's methods which was previously examined by Shiraishi, 1963, and Matsumoto, Kameda and Takeuchi, 1969. However, the specificity of the reaction should be examined by the selective technique for demonstration of other metals.

It was found by the control experiments that metals such as iron, zinc and cadmium were not reactive to the procedure of this proposed method. It was, therefore, considered that mercury is presumably converted easily by silver in this

technique because of the similarity of ionization tendency. The mechanism of this silver impregnation is still unknown, but the study is continued by Umeda *et al.*

Although the chemical identification for metals should be undertaken by assay procedures, the histochemical preparation in advance may help the diagnosis of metal poisoning.

The prior preparation by Kardasewitsch's procedure to present an erratic deposition of formalin pigments in this method was not always needed. However, the prior staining by hematoxilin-eosin was needed more than the post-staining.

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