Detection of microcystins, a blue-green algal hepatotoxin, in drinking water sampled in Haimen and Fusui, endemic areas of primary liver cancer in China, by highly sensitive immunoassay

Yoshio Ueno1,6, Satoshi Nagata1, Tomoaki Tsutsumi1, Akihiro Hasegawa1, Mariyo F. Watanabe2, Ho-Dong Park3, Gong-Chao Chen4, Gang Chen5 and Shun-Zhang Yu5

1Faculty of Pharmaceutical Sciences, Science University of Tokyo, Ichigaya, Tokyo 162, 2Tokyo Metropolitan Research Laboratory of Public Health, Shinjuku-ku, Tokyo, 3Faculty of Science, Shinsyu University, Matsumoto, Japan, 4Haimen city Health and Anti-Epidemic Station, Jiang-Su Province, and 5School of Public Health, Shanghai Medical University, Shanghai, China

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Introduction

The incidence of primary liver cancer (PLC*) is very high in certain areas such as South-East Asia, Sub-Saharan Africa and China. Epidemiological surveys so far have suggested that environmental carcinogens such as aflatoxin B1 (AFB1), a mycotoxin often found contaminating foodstuffs, are closely connected with the development of PLC in such endemic areas (1, 2). Actually, by using a highly sensitive method combining high-performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA) and immunoaffinity column chromatography, a significant contamination of AFB1 was detected in the dietary foods in endemic areas (3). By a novel and sensitive ELISA, we also detected an AFB1 residue, a hepatoclostatic metabolite of AFB1, in the serum specimens of patients with various kinds of hepatic diseases in Nepal, where a high incidence of PLC and a high contamination of AFB1 in food were reported (4). As for other environmental risk factors connected with the high incidence of PLC, we have found a close correlation between the incidence of PLC and the intake of pond/ditch water or river water in daily life, after extensive epidemiological studies in the Haimen area, Jiang-Su province, one of the endemic areas of PLC in China (5, 6). The mortality rates from PLC in Haimen city and the Nanhui area in 1981–1984 were 100.13, 91.00 and 4.28 per 100 000 people employing ponds/ditches, rivers and wells as the source of drinking water, respectively. However, no close relationship was observed between exposure levels to dietary AFB1 and the incidence of PLC in this endemic area (5, 6). Furthermore, in a medium-term hepatocacinogenesis test, in which rats were hepatectomized, given phenobarbital, followed by a single i.p. injection of AFB1, the enhanced development of γ-glutamyl transferase-positive liver foci was demonstrated by an administration of the residues prepared from suspected pond/ditch water (7). This evidence suggests that unknown contaminants in the drinking water promoted the development of liver cancer.

Recently, a family of hepatotoxic cyclic peptides named MC has been isolated from certain species of blue-green algae such as Microcystis aeruginosa, as reviewed (8–10), and a two stage carcinogenesis test demonstrated its tumor promoting activity in rats (11).

Based on this accumulated evidence, we have aimed to demonstrate a possible correlation between the level of MC in drinking water and the incidence of PLC in Haimen city by introducing a simple and reliable monitoring method of MC in the drinking water. First, we established an monoclonal antibody (MAB) that selectively recognized MC (12), and by using this MAB we developed a highly sensitive ELISA for the quantification of MC in environmental water in levels of several 10 pg without complicated clean-up steps and concentration procedures (13).

*Abbreviations: PLC, primary liver cancer; MC, microcystin; ELISA, enzyme-linked immunosorbent assay; AFB1/M1, aflatoxin B1/M1; MAB, monoclonal antibody; HPLC, high-performance liquid chromatography.
This period is also linked to algal growth since high temperatures continue such as MC-LR, MC-RR and MC-YR (12), MC-LR, the most widely studied ELISA analysis during this season.

Water samples, 26 in total, were collected from different sources in October. County (Figure 1A), where the incidence of PLC was high and the drinking water samples, by humans from the different sources, the third sampling, 989 sites in total, was carried out in July 1994. This sampling composed of 324 pond/ditch, rivers, shallow well water samples and deep well water, respectively. Alphabetic sampling site codes are listed in Tables I and II.

In the present experiment, this new technique was adopted for the survey of MC in the mass samples of drinking water collected in Haimen city and Fusui county, high risk areas of PLC, in 1993 and 1994, along with the survey of blue-green algal species. A part of the present data was reported in a preliminary form (14-16).

Materials and methods

Water samples

The sampling of drinking water from the different sources such as ponds/ditches, rivers, shallow and deep wells was carried out during 1993 and 1994 in Haimen city and Fusui county (Figure 1A). A pond/ditch was defined as a water storage place, about 5-6 m in width, 100 m in length, and 1-2 m deep, usually located nearby residential areas. A river was defined as being >10 m wide and >2 m deep, flowing through the city at a slight distance from residential areas. A shallow well was defined as being <3-5 m in depth, and a deep well as being >100-200 m deep, both usually found relatively near residential areas.

In the first sampling 14 water specimens were collected from different pond/ditches in September 1993. The sampling sites are shown in Figure 1B. Approximately 30-40 ml aliquots of the water samples were directly collected from the surface of the water, packed in plastic tubes and capped (Corning 1730), added to this was a 1/100 volume of 10% (w/v) sodium azide. The tubes were subsequently kept frozen at -20°C until an open circles, triangles and squares indicate the sampling sites of pond/ditch, river, shallow-well and deep-well water, respectively. Alphabetical sampling site codes are listed in Tables I and II.

In order to estimate the MC exposure level in the drinking water consumed by humans from the different sources, the third sampling, 989 sites in total, was carried out in July 1994. This sampling composed of 324 pond/ditch water samples, 188 river water samples, 323 shallow well water samples and 154 deep well water samples.

An additional sampling of drinking water was carried out in 1994 in Fusui county (Figure 1A), where the incidence of PLC was high and the drinking water was suspected as a risk factor, similar to Haimen (6). The drinking water samples, 26 in total, were collected from different sources in October. This period is also linked to algal growth since high temperatures continue during this season.

ELISA analysis

The procedure for a competitive ELISA of MC was reported in a preliminary form (13) and is summarized briefly as follows. Since the anti-MC MAb used in the ELISA recognized almost equally the major MC derivatives such as MC-LR, MC-RR and MC-YR (12), MC-LR, the most widely studied MC, was used as the standard of the ELISA. MC-LR was isolated in our laboratory from blue-green algae sampled from Suwa Lake, Japan (12). An HPLC analysis revealed over 95% purity. The water specimens to be analyzed were treated twice by freeze-thawing followed by filtration over glass filters (Whatman GF/C, 25 mm in diameter). The samples or the standards were mixed with an appropriate dilution of MAb and added to a 96 well microtiter plate (Coaster) that had been coated with an MC-LR bovine serum albumin conjugate. After washing, the bound MAb was detected with horseradish peroxidase-labeled goat anti-mouse IgG (TAGO 4550) and its substrate (0.1 mg/ml of 3,3',5,5'-tetramethylbenzidine, 0.005% H₂O₂ in 0.1 M acetate buffer (pH 5)). The MC concentration was determined from the standard competitive curve of MC-LR. In this ELISA, the recovery of MC-LR spiked to pond and tap water at 25–500 pg/ml was 101% on average, and the minimum detection limit, based on 20% inhibition, was 20 pg/ml (13). To minimize minor false positive results, 50 pg/ml was set as a minimum detection limit in the present experiments.

Statistics

For a statistical evaluation on the MC positivity among the four different types of water sources, the proportions of MC positive samples were compared for all possible combinations of water types using a Chi square analysis of the resulting 2×2 contingency table.

Results and discussion

As for the MC problem in China, the presence of toxic cyanobacteria, such as Microcystis aeruginosa, was reported in drinking sources in central China (17). Chemical analysis also revealed the contamination of MC-LR and 3-desmethyl-MC-LR in drinking water (K. Harada, personal communication). However, a systematic survey of MC has not been carried out in China. This study was first conducted in Haimen city, located northwest of Shanghai, China. Two tributaries of the Chang Jiang, the Haimen River and the Haigi River, run across the city from west to east (Figure 1). There are a lot of artificial small ponds and ditches. Prior to the 1980s in Haimen, a limited number of families used water from shallow or deep wells. The rest of the families used pond/ditch water or river water, or both, as drinking water sources. In later years, well water became available to greater numbers of people. However, due to the difficulty in repairing damaged wells, some families are still using pond/ditch water or river water, or both as water sources for domestic usage. At present, ~50% of the total number of families in the city seem to use well water.

By using the ELISA method, we analyzed the content of MC in samples of pond/ditch water collected in September 1993. As shown in Table I, three out of 14 samples in Haimen...
city were positive (>50 pg/ml) for MC, with a range of 90–460 pg/ml. Microscopic observation revealed the presence of various species of cyanobacteria, shown in Table I. Among the species identified, *Oscillatoria agardhii* was reported to produce microcystins (18–20). *Anabaena* sp. observed in an *Oscillatoria* and *Lyngbya* sp. identified cannot be ruled out. A further survey of the toxigenic cyanobacteria is underway.

In order to demonstrate the seasonal variation of MC contents in the pond/ditch water, water specimens were sampled every month from limited numbers of different water sources. Two out of five pond/ditch water areas were selected from the MC positive sampling sites in the first trial. A second ELISA analysis on 132 samples revealed that four out of five ponds/ditches and one out of two rivers were positive for MC during the period between May and September, and no detectable amount of MC was found in the other seasons, as shown in Table II. From these data, it was suggested that the water in ponds/ditches and rivers is hazardous to the people who use it for drinking in the summer season when algal growth as well as toxin production are favorable.

Based on the present two trials, we systematically investigated the levels of MC in water specimens collected from 989 different sampling sites in Haimen. The results, as summarized in Table III, showed that MC was positive for 17% and 32% of the total samples of pond/ditch and river water respectively. Their respective averages of MC were 101 and 160 pg/ml. The MC positive rates were significantly higher than those of the shallow and deep well water. Among the 989 samples examined, two samples from the river showed MC levels over 1000 pg/ml, with the highest being 1558 pg/ml (Table III).

Since higher levels of MC were found in the samples of pond/ditch and river water collected in Haimen, we examined...
of pond/ditch water or river water, or both, was significantly expected to clarify exposure to MC as a whole.

### Acknowledgements
This work was supported in part by grants from Asahi Glass Foundation (Tokyo), Nippon Life Insurance Foundation (Osaka) and the Ministry of Education, Science and Culture (Japan).

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**Table III. Microcystin levels in different drinking water types collected in July 1994 in Haimen city**

<table>
<thead>
<tr>
<th>Water type</th>
<th>No. of samples assayed&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of samples with indicated levels of microcystin (pg/ml)</th>
<th>No. of positives&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Mean conc. in positives (pg/ml)</th>
<th>Maximum conc. (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pond/ditch</td>
<td>324</td>
<td>268 36 15 5</td>
<td>56 (17)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>101</td>
<td>300</td>
</tr>
<tr>
<td>River</td>
<td>188</td>
<td>128 30 22 8</td>
<td>60 (32)</td>
<td>160</td>
<td>1,558</td>
</tr>
<tr>
<td>Shallow-well</td>
<td>323</td>
<td>309 13 1 0</td>
<td>14 (4)</td>
<td>68</td>
<td>106</td>
</tr>
<tr>
<td>Deep-well</td>
<td>134</td>
<td>154 0 0 0</td>
<td>0 (0)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>All samples were collected at different sites.  
<sup>b</sup>Number in parentheses indicate percentage. There are significant differences in the ratio of positive samples between water types: pond/ditch > shallow-well (<0.001); pond-ditch > deep-well (<0.001); river > shallow-well (<0.001); river > deep-well (<0.001); shallow-well > deep-well (<0.01).

the MC contents in 26 samples of drinking water collected from a different district, Fusui in Guangxi province, where a close relationship was observed between the high incidence of PLC and the intake of ditch/pond water as a drinking source (6). Four out of nine specimens of pond/ditch water and three out of 87 river water specimens were positive for MC with averages of 103 and 221 pg/ml respectively. No MC was detected in shallow and deep well water. This additional trial in Fusui supported the finding in Haimen city that the MC levels in pond/ditch and river water were higher than those observed in shallow and deep well water.

As shown above, it is highly possible that the water in the ponds/ditches and rivers is widely contaminated by MC, an algal hepatotoxin, and that the water in deep wells is mostly free from MC, in the endemic areas of China.

In the present experiment, we used a newly developed ELISA method for the detection of MC in the drinking water sampled from the different sources. According to this ELISA method, MC at the level of several 10 pg/ml was able to be detected without clean-up processes. This detection limit is about 1000 times higher than that of the ordinary HPLC method with a UV detector. This simple and sensitive method allowed us to monitor the MC level in a large number of water specimens (1161 in total) during these two years.

From Table III, we have calculated an exposure level to MC in Haimen city as follows: the average MC content in the ponds/ditches (101 pg/ml) and river water (160 pg/ml) were averaged to 130 pg/ml. If an adult person took at least 1.5 l of pond/ditch water or river water during June to September, they would be exposed to 0.19 pg of MC per day intermittently for the 4 months of the summer season, with the average adult being exposed to these levels of MC over a period of 40–50 years.

At present, no information on chronic toxicity, particularly on the carcinogenicity of the purified MC given by an oral route is available. In mice given toxic cyanobacterial cell-free extracts in drinking water for short periods and periods of up to 1 year, acute and chronic liver injuries developed (22,23). From further experiments with growing pigs given drinking water added with toxic algal extracts for 3 months, conservative estimates for water safety were proposed to be 1.0 μg MC/l (1000 pg/ml) for a short term exposure and 0.1 μg/l (100 pg/ml) for long term exposure (24,25).

As originally reported by Yu et al. (5,6), and reviewed by Carmichael (9), the incidence of PLC in people who commonly use pond/ditch water or river water, or both, was significantly higher than those who use shallow and deep well water. The present data summarized in Table III clearly shows that the average content of MC in the ponds/ditches and rivers (130 pg/ml) was close to the above conservative estimates for long term exposure even if the source of MC is limited to the drinking water. Therefore, the guideline of water safety for long term exposure should be at least ten times lower (13 pg/ml) than that of the average content of MC detected in pond/ditch and river water in the high endemic areas of PLC. Such a low concentration of MC is below the detection limit employed in the present ELISA, however, by a combination of ELISA and affinity column chromatography, even such a low content of MC in the water samples could be detected, as will be reported elsewhere.

MC is reported as a potent promoter of hepatic liver tumors in rats (11). We have also demonstrated that the development of glutathione-S-transferase P-positive foci in the hepatic tissue of rats given AFB1 was enhanced by a following administration of MC (26). However these experiments (11,26) were performed with an i.p. injection of MC to rats initiated by carcinogens. Therefore, it is hard to apply these data directly to human cases who are suspected of MC intake by an oral route. In this respect, further experiments are required to demonstrate whether MC in drinking water is associated with the development of PLC, by estimating MC residues in human serum and/or hepatic tissues.

With an ELISA analysis of AFB1, this potent hepato-carcinogen was often found contaminating corn kernels and flour, which were the major food stuffs in the past in Haimen city (27). Thus, the combined effect of AFB1 derived from foodstuffs and MC from the drinking water may result in the high incidence of PLC in Haimen city and other endemic areas in China.

Microbial toxicants as well as environmental pollutants enter into the food chain system. We have presumed the drinking water to be a major source of MC at the present time, based on the water intake of people every day for a long time, however we cannot exclude the possibility of exposure to MC through the food chain. On this line, a survey of MC residue in edible tissues of fish, shellfish and ducks is being studied by an application of the present ELISA technique combined with affinity column chromatography. This experiment is expected to clarify exposure to MC as a whole.
References


Microcystins in drinking water


Received on August 1, 1995; revised on February 13, 1996; accepted on February 13, 1996.