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A food-poisoning incident caused by *Clostridium botulinum* toxin A in Japan

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SUMMARY

Food poisoning caused by *Clostridium botulinum* toxin A occurred in Japan. Eleven (31%) of 36 patients from 14 different areas died of botulism. Most of the patients had eaten commercial fried lotus-rhizome solid mustard without heating. The food, which implicated one of the special local products used for gifts in Kumamoto, was found to have been produced by a manufacturer in Kumamoto prefecture.

In Fukuoka prefecture, two of three patients died on days 4 and 8 after eating the food; they had typical symptoms of botulism. A total of 42 packages of the food bought as gifts was collected from different districts in Fukuoka prefecture for examination for both organism and toxin. Thirteen of these (31%) were contaminated with the organism, and in 11 (26%) a small amount of toxin A had been produced.

INTRODUCTION

Clostridium botulinum toxin A has the highest lethal activity in the genus *Clostridium*. Only one case of food poisoning caused by the organism has been reported in Japan (Komai *et al.* 1977; Sakai *et al.* 1977). It is probable, however, that the organism is distributed widely in the environment, since *Cl. botulinum* producing type A toxin has been isolated from soil in different areas in Japan (Wakamatsu, 1953; Nohdomi, 1957; Kodama *et al.* 1963).

In June 1984, food poisoning caused by *Cl. botulinum* occurred in 14 different districts in Japan. Food poisoning appeared only in persons who ingested the fried lotus-rhizome solid mustard, Karashi-Renkon, which was produced in Kumamoto prefecture. Normally, this food is vacuum packed for storage, and some is sold to tourists as gifts.

The present study was conducted to determine the quantity and the lethal doses of toxin produced in the food.

MATERIALS AND METHODS

Preparation of materials and cultivation

The fried lotus-rhizome solid mustard consisted of two original materials, lotus-rhizomes and mustard. After the mustard was completely mixed into the lotus-

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rhizomes, the product was fried and packed in vinyl under a vacuum. The products used in this study were parts of the remnants of the samples ingested by two patients (No. 5 from case A and No. 13 from case B) and 40 other samples that had been bought in Fukuoka prefecture. For examination of these materials, 50–80 g of each sample was homogenized with a Stomacher (Lab Blender 4000), and divided into two parts. One part was inoculated into cooked meat broth medium (Eiken-Kagaku Co Ltd), and cultured anaerobically at 37 °C for a week. The other part was used for detecting toxin.

For isolating *Cl. botulinum*, the cooked meat culture was inoculated on to GAM medium (Nissui Seiyaku Co Ltd) to which 5% egg yolk was added as a supplement, and the plate was incubated in an anaerobic jar for 48 h by using a GasPak (BBL Microbiology Systems, Becton Dickinson and Co.).

Detection of toxin and determination of LD_{50}

Male 4-week-old DDY mice (Seiwa Experimental Animals Co Ltd) were used for detection of toxin; 0·5 ml quantities of homogenized food samples or serum of venous blood of case A were inoculated intraperitoneally into two mice per sample and the animals were observed for 4 days. Lethal doses (LD_{50}) of toxin were determined by intraperitoneal inoculation into 50 mice (10 mice per group) with 0·5 ml of the sample diluted with phosphate buffer (pH 6·0). The quantity of toxin in the food was determined by intravenous injection reported by Sakaguchi, Sakaguchi & Kondo (1968); 0·1 ml portions of the sample being inoculated into mice *via* the tail vein.

Antisera against toxins A, B, E and F purchased from Chiba Serum Institute, Japan, were used for determining the toxin type.

Purification of toxin produced by a wild strain

The toxin produced by *Cl. botulinum* isolated from one sample (No. 5 of case A) was partially purified as follows; the cells were inoculated into Duff medium (Duff *et al.* 1957) and cultured at 37 °C for 4 days. The cells, which had grown to 10^8 per ml, were removed by centrifugation at 6000 rev./min for 30 min, and the supernatants were dialysed against ammonium sulphate to 60% saturation. The precipitates were collected by centrifugation at 10000 rev./min for 15 min. For purifying toxin A, the sample was applied on a DEAE cellulose column, and adsorbed toxin was eluted with a sodium chloride gradient from 0·1 to 0·5 M in 0·05 M Tris-HCl buffer (Gerwing, Dolman & Baines, 1965).

RESULTS AND DISCUSSION

Clinical characteristics of the patients

Eleven of the 36 persons reported as patients in this food poisoning incident died of botulism (Fig. 1). All 11 had ingested a certain amount of food which was probably contaminated by the botulinum toxin, and it was confirmed that they had typical symptoms of botulism. The majority of the patients and the deceased were thought to have eaten the food without heating it because no symptoms were observed among persons who ingested the food after heating.

In Fukuoka prefecture, two of the three patients who developed botulism after

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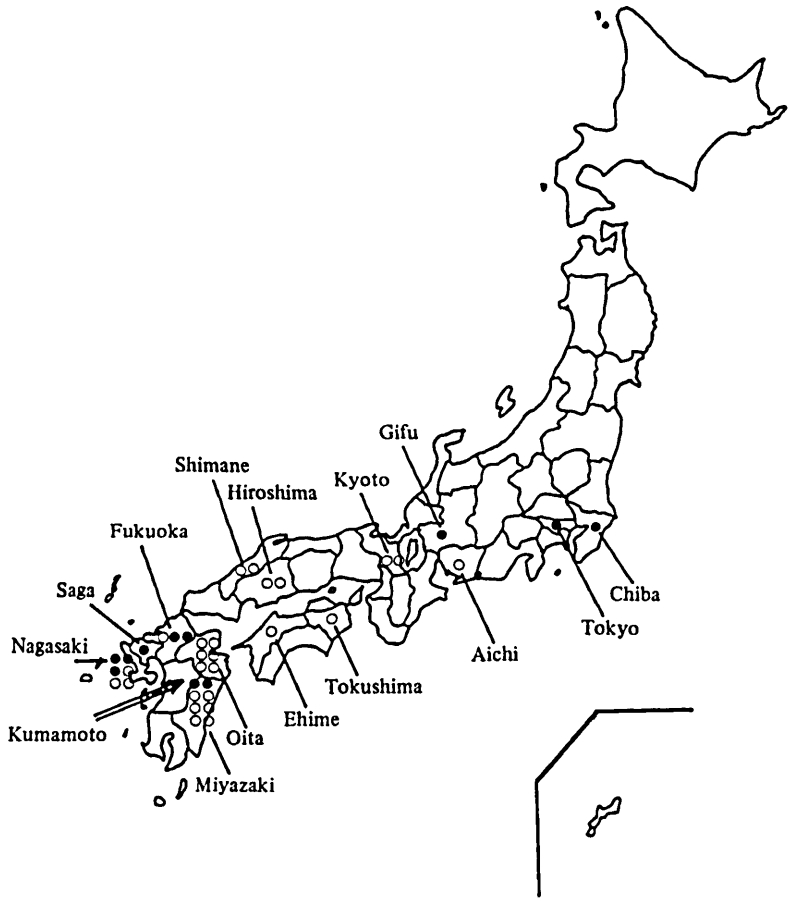

Fig. 1. Distribution of patients and the deceased in a food-poisoning caused by *Clostridium botulinum* in Japan. ○, patients; ●, deceased.

eating the food died. In both fatal cases (case A, a 64-year-old male and case B, 61-year-old female), the typical symptoms began as vomiting more than 10 times a day commencing 10 h after ingestion of the food, which was bought and presented as a gift, and subsequently, thirst and characteristic oculomotor disturbance with dizziness appeared, but there was no fever or diarrheoa. At that time, blood pressure of both patients showed a marked increase from 140 to 210 mmHg for case A and 210 mmHg for case B. Both died of dyspnoea accompanied by respiratory and pharyngeal paralysis after 8 and 4 days, respectively. The patient in case B died before the food-poisoning incident was discovered.

Isolation of Cl. botulinum *and determination of toxin*

The isolation of *Cl. botulinum* and the determination of toxin production are summarized in Table 1. Food samples were cultured in cooked meat medium and plated on GAM agar for isolation of *Cl. botulinum* as described in Materials and Methods. Thirteen (31 %) of 42 samples tested were found to be contaminated by typical *Cl. botulinum*. The isolated strains withstood boiling for 60 min, but were killed completely at 110 °C for 5 min.

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Table 1. *Isolation and determination of* Clostridium botulinum *and its toxin in fried lotus-rhizome solid mustard*

			Route of inoculation				
			Intraperitoneal		Intravenous		
Sample no.	Isolation of *Cl. botulinum*	No. of mice tested	No. of mice died	Death time (h)	Death time (min) after inoculation	LD_{50}'s/g of food	Calculated quantity of toxin (μg of N/g)
5	+	2	2	3 and 5	130	$2{\cdot}0 \times 10^4$ (A)*	0·123
13	+	2	2	3 and 4	197	$6{\cdot}0 \times 10^3$ (A)	0·037
1	+	2	2	4 and 5	—	< 3000 (S)	—
6	+	2	2	96 and 96	—	< 3000 (A)	—
10	+	2	2	24 and 28	—	< 3000 (A)	—
14	+	2	2	24 and 28	—	< 3000 (A)	—
15	+	2	2	48 and 72	—	< 3000 (A)	—
16	+	2	2	24 and 24	—	< 3000 (A)	—
20	+	2	2	2 and 3	78	$9{\cdot}0 \times 10^4$ (A)	0·549
21	+	2	2	6 and 8	230	$3{\cdot}0 \times 10^3$ (A)	0·023
22	+	2	2	22 and 26	—	< 3000 (A)	—
25	+	2	0	—	—	—	—
26	+	2	0	—	—	—	—

* (A), Toxin type.

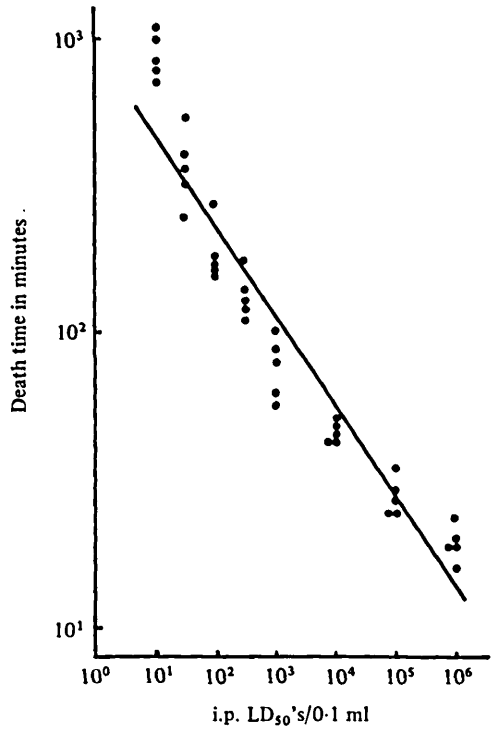

Fig. 2. Relationship between the dose in i.p. LD_{50}'s and the time from intravenous injection to death.

Part of each homogenized food sample was centrifuged at 5000 rev./min for 30 min, and 0·5 ml amounts of the supernatant were inoculated intraperitoneally into mice for detecting toxin. As indicated in Table 1, 11 (26·2%) of the 42 samples tested were found to contain toxin. Almost all of the mice died of characteristic neurosis with respiratory paralysis; the toxigenic activity was inactivated by antitoxin A.

The partially purified toxin of strain No. 5 contained $1{\cdot}63 \times 10^8$ lethal doses (LD_{50}'s) per mg of total nitrogen. The toxin was inactivated by heating at 100 °C for 3 min. Then the short-term test described by Sakaguchi, Sakaguchi & Kondo (1968) was adopted for determining the quantity of toxin in the food. This method was convenient for determining the amount of botulinum toxin because a close correlation was observed between the time in minutes from injection to death of each mouse and each lethal dose determined by the intraperitoneal injection method (Fig. 2). On the basis of the results of intravenous injection, the quantity of toxin in the fried lotus-rhizome solid mustard was then estimated (Table 1). The quantities of toxin in samples 5, 13, 20 and 21 were estimated to be in the range of 0·023–0·549 μg per gram of the food. It was impossible to determine the quantities of toxin in the other materials (samples 1, 6, 10, 14, 15, 16 and 22) because the amounts of toxin were too small. Samples 5 and 13 were part of the remnants of food that had been ingested by two deceased patients (cases A and B). If the patients had ingested 200 g of the fried lotus-rhizome solid mustard, the possible doses of botulinum toxin ingested were 24·6 and 7·4 μg, respectively. This fact

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suggests that the lethality of the toxin for humans was very high although the toxin was produced in extremely small amounts.

On the other hand, about 10 ml of venous blood in case A was taken on day 4 after the onset of illness, and the number of LD_{50}'s of the toxin in the blood was determined by inoculation into mice. The serum was found to contain $1{\cdot}1 \times 10^2$ i.p. LD_{50}'s per ml. This amount was a little less than that in the food of sample 5 in case A, and was calculated to be 0·67 ng of total nitrogen per ml of serum. Therefore, the amount of toxin A in the serum was found to be much higher than that in two patients observed by Sakai *et al.* (1977).

This fried lotus-rhizome solid mustard is one of the special products of Kumamoto prefecture, and is sold by the manufacturer packed with vinyl in a vacuum for storage. For investigating the sources of contamination by the organism, 11 kinds of mustard used for the food were cultured in cooked meat medium, and isolation of the organism was attempted. No *Cl. botulinum* could be isolated from the mustard samples. There is a possibility that, in lotus-rhizomes contaminated by the organism, germination of *Cl. botulinum* might be initiated by some agent such as heating in the process of manufacturing the food, and that packaging in a vacuum resulted in marked promotion of growth and toxin production by *Cl. botulinum*. In Kumamoto prefecture, the fact that no cases of botulism have occurred can be explained by the custom that most people in this area eat the non-vacuum packed product after heating.

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