Acta Medica Okayama

Volume 64, *Issue* 3 2010 *Article* 2

JUNE 2010

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Abstract

In an attempt to prepare egg yolk immunoglobulin (IgY) to treat and prevent cholera, hens were immunized by a mixture of heat- or formalin-killed Vibrio cholerae O1 and O139 organisms, or by the recombinant cholera toxin B subunit (CTB). The IgYs were partially purified from egg yolk and orally administered to suckling mice before or after challenge with live O1 or O139 cells. The anti-O1 and O139 IgYs and the mixture of either IgY with anti-CTB IgY significantly protected the occurrence of cholera caused by both O1 and O139 infection. Since large amounts of IgY can be prepared very easily and at low cost, this seems to be a useful procedure for preventing and treating cholera.

KEYWORDS: Vibrio cholerae, O1, O139, IgY

Acta Med. Okayama, 2010
Vol. 64, No. 3, pp. 163-170

Acta Medica Okayama
http://escholarship.lib.okayama-u.ac.jp/amo/

Original Article

Passive Oral Immunization by Egg Yolk Immunoglobulin (IgY) to *Vibrio cholerae* Effectively Prevents Cholera

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In an attempt to prepare egg yolk immunoglobulin (IgY) to treat and prevent cholera, hens were immunized by a mixture of heat- or formalin-killed *Vibrio cholerae* O1 and O139 organisms, or by the recombinant cholera toxin B subunit (CTB). The IgYs were partially purified from egg yolk and orally administered to suckling mice before or after challenge with live O1 or O139 cells. The anti-O1 and O139 IgYs and the mixture of either IgY with anti-CTB IgY significantly protected the occurrence of cholera caused by both O1 and O139 infection. Since large amounts of IgY can be prepared very easily and at low cost, this seems to be a useful procedure for preventing and treating cholera.

Key words: *Vibrio cholerae*, O1, O139, IgY

Cholera is an intestinal infection caused by *Vibrio cholerae* that leads to a severe diarrheal disease. In 2007, a total of 177,963 cholera cases and 4,301 deaths were officially reported to the World Health Organization (WHO) [1]. Every year, more than 100,000 people are infected, of whom more than 90% are Asian or African [2]. The strains of *V. cholerae* that cause epidemics belong to serogroups O1 and O139. Many cases of *V. cholera* infection in travelers are also reported every year [1]. The principal symptom is the painless purging of voluminous stools that resemble rice water and that are caused by cholera toxin (CT). Since the patient becomes dehydrated, oral rehydration solution (ORS) is used as a general treatment [3]. Patients can recover by this treatment in many cases, but even so they suffer from serious symptoms for long periods, and sometimes the treatment is not successful. To prevent cholera, 2 kind of vaccines have been used: injectable vaccine and oral cholera vaccine (OCV). The injectable vaccine is made from phenol-inactivated *V. cholerae* O1 and is still available. However, this vaccine's efficacy ratio and its term reportedly are low and short, respectively, and it has some side effects. In OCV, 3 types of preparations have been developed. One is WC/rBS, which consists of inactivated *V. cholerae* O1 and recombinant cholera toxin B subunit (CTB). WC/rBS provides 90% protection in the first 6 months and 60% protection for at least 3 years. However, protection in children aged under 5 years declines more

Received October 21, 2009; accepted December 1, 2009.
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rapidly after 6 months, protection is not conferred against O139 infection, and the price is rather high (one dose is US$50). In Vietnam, the inactivated *V. cholerae* O1 and O139 without CTB were developed as the second type of OCV. The cost was very low (under US$1 per dose), but the protection rate was reported to be 66% at 8 months across all ages. The third OCV is the live O1 cells in which the gene of cholera toxin subunit A is deleted. This OCV showed high protection efficacy against *V. cholerae* O1 in challenge studies in the United States. On the other hand, a large field trial performed in Indonesia did not show convincing protection in a population exposed to cholera for a long time after immunization [4]. The vaccines may be effective if they are used precisely, but they may take a long time and require a lot of financial support. WHO has considered that *V. cholerae* O139 still has the potential to cause the next cholera pandemic, and it is possible that big outbreaks will occur after serious disasters such as earthquakes and floods.

In this manuscript, we have investigated whether or not the egg yolk immunoglobulin (IgY) against cholera organisms (a mixture of O1 and O139 inactivated with heat or formalin) and the B subunit of cholera toxin can be used to prevent and treat cholera because 1) large quantities of IgY can be isolated from the yolk by simple, low-cost methods and without distress to the birds [5], and 2) their value has already been reported in infection with rotavirus, parvovirus, *E. coli*, *S. typhimirium*, *S. mutans*, *H. pylori*, and *P. gingivalis* [6–9]. These antibodies were designated as anti-V.C IgY and anti-CTB IgY, and each antibody or a mixture of them was orally inoculated into suckling mice before or after the challenge with *V. cholerae* O1 or O139.

Materials and Methods

Bacterial strains. *V. cholerae* O1 El Tor (Inaba) and (Ogawa), isolated from a river in Bangladesh, were kindly provided by Dr. S. Miyoshi, Department of Environmental Health and Microbiology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences. Also, *V. cholerae* O139, isolated from patients in India, was provided by Dr. K. Okamoto, Department of Gene Function, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences.

Anti-V. cholerae IgY and anti-CTB IgY preparation. IgY against *V. cholerae* organisms (anti-V.C IgY) and CTB (anti-CTB IgY) were prepared. *V. cholerae* O1 El Tor (Inaba) and O139 were grown in 400 ml of brain heart infusion (BHI) broth for 15 h at 37℃ with continuous shaking at 100 rpm. Bacteria were harvested by centrifugation for 15 min at 11,380 × g at 4℃. These were washed twice with sterile phosphate-buffered saline (PBS; pH 7.2) and suspended. O1 and O139 cells (each 1.5×10^{10} CFU/ml) were inactivated by treatment with 0.5% formalin-PBS overnight, or by heat (80℃ for 20 min). These formalin- or heat-inactivated *V. cholerae* O1 and O139 organisms (a total of 4) were mixed together in equal proportions and used as an immunogen to hens.

Also, recombinant CTB prepared as reported previously was used as an immunogen [10]. These antigens were mixed with an equal volume of Freund's complete adjuvant (FIA) and immunized into 5-month-old White Leghorn hens (strain Hyline W36; GHEN Corporation, Gifu, Japan) according to the method described by Yokoyama *et al.* [11]. To prepare anti-V.C IgY, the mixture (0.5 ml) was injected into both breast muscles, and a booster was given in the same manner at 6 weeks after the initial immunization. Also, anti-CTB IgY was produced by immunization with 0.1 mg/ml CTB, followed by a booster (0.5 mg CTB/ml) at 6 weeks after the initial immunization. The eggs were harvested daily throughout the third and fourth weeks after the booster and stocked at 4℃. The yokes were separated carefully from the albumin and yolk membrane. The yokes was pooled, homogenized, filtrated through Teflon filter cloth, and then partially purified by ammonium sulfate. The precipitated IgY was suspended in PBS, dialyzed, and freeze-dried in a freeze-drying machine (Labconco LL-12, Labconco Corp., Kansas City, MO, USA). Control IgY powder was prepared from eggs from non-immunized hens in the same manner.

Reactivity of IgY against V. cholerae and CTB. The reactivity of the IgY preparations (0.1 mg/ml) to the cells and CTB was analyzed by enzyme-linked immunosorbent assay (ELISA). A 96-well microtiter plate was coated with CTB (1 μg/100 μl/well) or inactivated O1 or O139 cells (OD600 = 0.1 7.5×10^7 CFU/ml, 100 μl/well) in 0.1 M carbonate-bicarbonate buffer (pH 9.6) at 4℃

overnight. After blocking with 200 μl of PBS containing 10% skim milk, the wells were incubated with 100 μl of 1 μg/well and 100 μg/well of IgY for 2 h at room temperature. The wells were then incubated with horseradish peroxidase (HRP)-labeled anti-chicken IgY (Medical & Biological Laboratories, Nagoya, Japan). Between each step, the cells were washed extensively with PBS containing 0.05% Tween 20. Color was developed with o-phenylenediamine and H_2O_2. The reaction was terminated by 6 N H_2SO_4 and optical density (OD) was measured at 490 nm. All treatments were replicated three times and reported as averages.

CHO assay. Chinese Hamster Ovary (CHO) cells were maintained in DEME with 10% fetal calf serum (FCS) [12]. The cells were trypsinized and re-suspended in DEME with 1% FCS. Cells were pretreated with 2–60 μg/ml of anti-CTB IgY, 10 ng/ml CT (List Biological Laboratories, Campbell, CA, USA) was added to the cells. After 12 h, morphological change was observed under a microscope.

Effect of IgY on cholera in infant mice. Since *V. cholerae* causes diarrhea and death in suckling mice younger than about 10 days [13], 4-day-old suckling mice were infected [14, 15]. O1 and O139 cells were separately cultured for 12 h at 30℃ in 400 ml of BHI broth with shaking, washed twice with PBS, and re-suspended into PBS at 7.5×10^8 CFU/ml. Four h after the mice were separated from their mother, each cell suspension (50 μl) thus prepared was intragastrically inoculated into the mice using a syringe with a flexible needle by oral administration [14]. Three h after this inoculation, the mice were then administered 50 μl of each (1 mg/ml) of 3 IgYs (anti-V.C, anti-CTB, or the mixture of them), and returned to their mothers. Thereafter, the surviving mice were repeatedly administered with the same IgY for up to 72 h with different intervals (every 2, 4, 6, or 12 h) in order to study the therapeutic effects. To assess the prevention effect, each IgY preparation described above was mixed with O1 or O139 cells (the final IgY and cell concentrations were 1 mg and 7.5×10^8 CFU/ml, respectively), and 50 μl of the mixture was orally inoculated.

Results

Reactivity of anti-V.C IgY and anti-CTB IgY to bacterial cell or cholera toxin. The reactivity of anti-V.C-IgY and anti CTB-IgY to the different antigens was measured by ELISA (Table 1). Anti-CTB IgY well reacted with not only recombinant CTB but also whole cholera toxin. The anti-V.C IgY that was obtained by immunization with the mixture of O1 El Tor (Inaba) and O139 reacted with all 6 kinds of bacterial antigens including heat- or formalin-killed *V. cholerae* O1 Ogawa. As expected, anti-CTB IgY and anti-V.C IgY did not react with bacteria and CTB (or cholera toxin), respectively.

Prevention of anti-CTB IgY against morphological change in CHO cells. CHO cells changed morphologically from a round or flat shape to an elongated spindle shape after the addition of 10 ng/ml CT (Fig. 1A–1 and 2) [12]. The effect of anti CTB-IgY on this change was observed. CHO cells were treated with 2–60 μg/ml of anti CTB-IgY and then challenged with CT (10 ng/ml). The IgY prevented the morphology change dose-dependently (0–20 μg/ml) (Fig. 1A–(3) and 1B).

Therapeutic effect of oral administration of IgY. Suckling mice were infected with *V. cholerae* O1 El Tor (Inaba) or O139, and then, 3 h later, were treated with 50 μl of anti-V.C IgY, anti-CTB IgY, or a mixture of them (each 1 mg/ml) was performed at a 2 h interval, as described in Materials and Methods. Anti-V.C IgY prevented the death of almost all the mice infected with *V. cholerae* O139 (Fig. 2B), but did not prevent the death of mice infected with *V. cholerae* O1 El Tor (Inaba). In the latter case, death was highly prevented by the mixture of anti-V.C IgY and anti-CTB IgY (Fig. 2A). We then, investigated the effect of IgY administration at different intervals. The mixture of anti-V.C IgY and anti-CTB IgY was administered at 2, 4, 6, 8, or 12 h intervals after 3 h of *V. cholerae* O1 El Tor (Inaba) infection (Fig. 2C). The mice survived at high rates after the 4, 6, and 8 h intervals as well as after the 2 h interval; the rates were 80% after 4 h and 70% after 6 or 8 h (Fig. 2C).

Protective effect of passive immunization with IgY. Different amounts of anti-V.C IgY (2 mg, 1 mg, 0.5 mg/ml) were mixed with *V. cholerae* O1 El Tor (Inaba) and then orally inoculated into suckling mice. The anti-V.C IgY showed a strong protective effect even though 0.5 mg/ml was employed (Fig. 3A–1), and a similar effect was observed when

Table 1 Reactivity of IgY to *V. cholerae* and cholera toxin

Antigen		IgY			
Source	Concentration	Concentration	Control IgY OD	Anti VC-IgY OD	Anti CTB-IgY OD
Cholera Toxin	10 μg/ml	100 μg/ml	0.139 ± 0.004	0.069 ± 0.005	3.000 ± 0.105
		1 μg/ml	0.044 ± 0.008	0.036 ± 0.001	0.660 ± 0.054
Cholera toxin B subunit	10 μg/ml	100 μg/ml	0.065 ± 0.002	0.069 ± 0.003	3.000 ± 0.123
		1 μg/ml	0.038 ± 0.002	0.035 ± 0.002	0.839 ± 0.082
V. cholerae O1 E1 Tor (inaba) 7.5×10^6 CFU/ml					
heat shock		100 μg/ml	1.641 ± 0.174	3.000 ± 0.100	0.592 ± 0.014
		1 μg/ml	0.103 ± 0.010	0.734 ± 0.035	0.106 ± 0.054
formalin		100 μg/ml	1.702 ± 0.071	3.000 ± 0.125	0.812 ± 0.027
		1 μg/ml	0.220 ± 0.018	0.944 ± 0.020	0.167 ± 0.017
V. cholerae O1 E1 Tor (ogawa) 7.5×10^6 CFU/ml					
heat shock		100 μg/ml	1.165 ± 0.132	2.460 ± 0.538	0.421 ± 0.105
		1 μg/ml	0.086 ± 0.013	0.656 ± 0.060	0.065 ± 0.010
formalin		100 μg/ml	1.438 ± 0.088	3.000 ± 0.040	0.709 ± 0.186
		1 μg/ml	0.128 ± 0.087	0.874 ± 0.259	0.108 ± 0.049
V. cholerae O139	7.5×10^6 CFU/ml				
heat shock		100 μg/ml	1.430 ± 0.161	2.908 ± 0.392	0.582 ± 0.094
		1 μg/ml	0.103 ± 0.010	0.743 ± 0.040	0.075 ± 0.003
formalin		100 μg/ml	1.588 ± 0.100	3.000 ± 0.183	0.884 ± 0.032
		1 μg/ml	0.220 ± 0.007	1.229 ± 0.040	0.173 ± 0.004

the *V. cholerae* O139 was mixed with 1 mg/ml of anti-V.C IgY (Fig. 3A–2). We then investigated the protective effect of IgY administered before cell infection. Suckling mice were administered a mixture of anti-V.C and CTB IgY (each 1 mg/ml). After 1 h, *V. cholerae* O1 El Tor (Inaba) was challenged. The survival time became longer in the IgY-treated group than in the control, and 30% of those in the former group survived (Fig. 3B).

Discussion

We have shown in a mouse model that the mixture of anti-V.C and anti-CTB IgYs can be used to prevent or treat cholera caused by either O1 or O139. Administration of anti-V.C IgY alone also showed some therapeutic effect, whereas anti-CTB IgY alone was not so effective. These results indicate that the elimination of the bacteria in the gut is more important than neutralization of the toxin. This seems reasonable. In the case of oral vaccination that has been tried in humans, the vaccine inducing antibodies against both bacteria and toxin is most effective [16].

Anti-V.C IgY was very effective for O139 infection but was not very effective for O1 infection. The reason for this difference is not clear. The behaviors of toxin production and ELISA reaction were not different between O1 and O139 cells. Furthermore, the mice challenged with O139 died earlier than those challenged with O1 (Fig. 2A and B), indicating that the pathogenicity of O139 is stronger than that of O1 in this mouse model. On the banding profile of Western blotting, however, some differences were observed (data not shown). This time, we immunized hens with mixed cells of O1 and O139. In order clarify the above-mentioned phenomena, we plan to

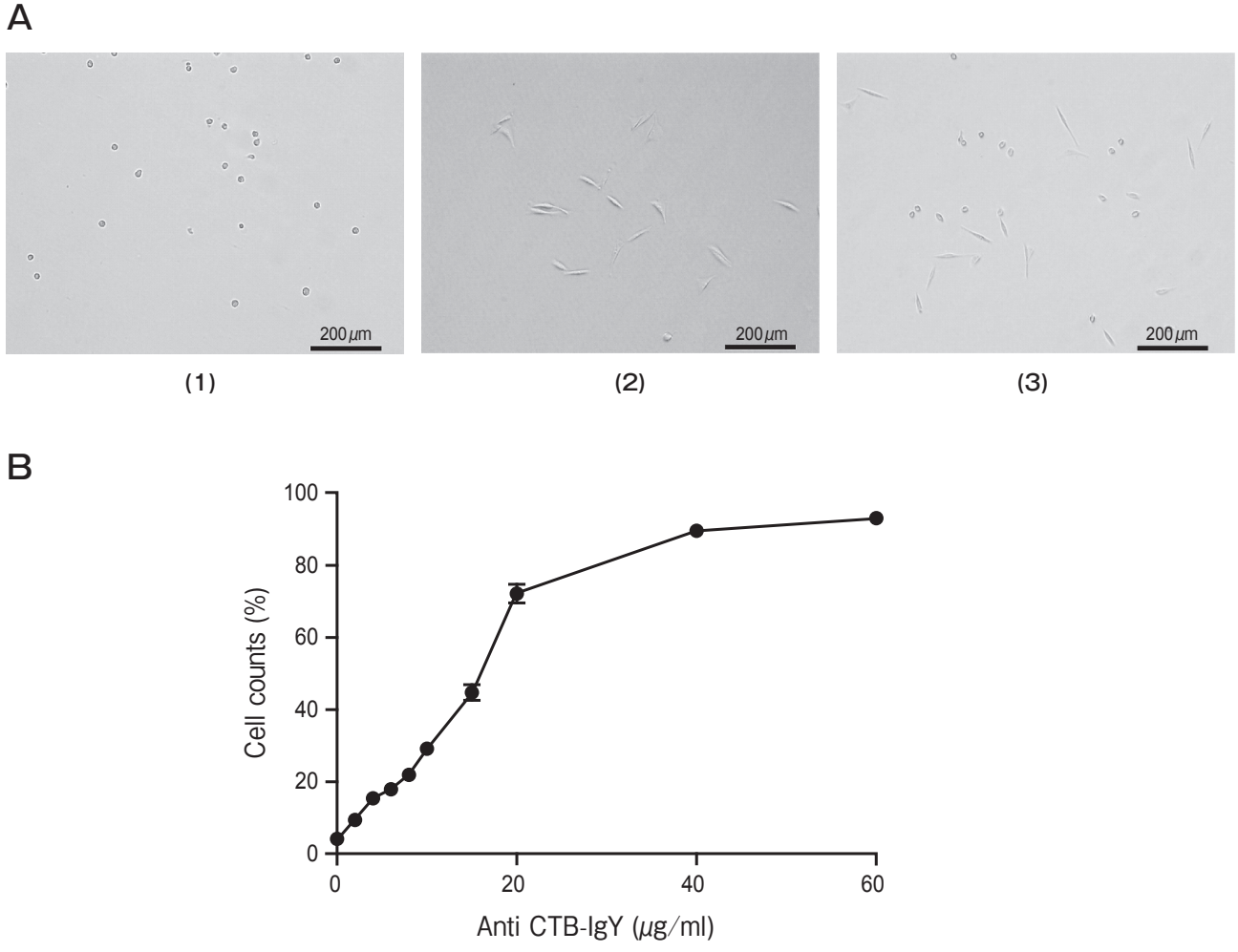

Fig. 1 Effect of anti-CTB IgY on elongation of CHO cells. **A**, Morphology of the CHO cells. CHO cells were cultured in 24-well dishes **(1)**. CT treatment changed the cell morphology **(2)**. This morphological change was inhibited by preincubation of CT with 15 μg/ml anti-CTB IgY **(3)**; **B**, Effect of different amounts of IgY on elongation. CT was treated with different amounts of anti-CTB IgY and then reacted to the CHO cells. The percentage of round cells to total cells was obtained by averaging the counts obtained by 10 microscope view fileds.

immunize the hens with each of these cells and characterize the antibodies obtained.

The therapeutic effects in the mice were similar across the range of administration intervals, from 2 h to 8 h. The mice were challenged with huge numbers of cells (3.75×10^{10} CFU); this amount is speculated to cause cholera more than 60% of the time in humans [17, 18]. This indicated that administration of IgY 3 or 4 times per day may be effective in humans.

In prevention studies, the occurrence of cholera was inhibited almost completely by mixing the anti-V.C IgY with live O1 or O139 cells. Also, the IgY (mixture of anti-V.C and anti-CTB) demonstrated some good effects even though it was administered 1 h before the challenge with O1 Inaba cells. It has been reported that oral administration of IgY in humans has good effects on *S. mutance* and *H. pylori* infection without severe side effects including egg allergy [19, 20]. Therefore, we think the IgYs prepared this time can be used for cholera in humans, too. We plan to carry out these studies while carefully considering the dosage and dose interval of IgY administration, as well as egg allergy. We think the best administrative method is to mix them into dairy products, powdered milk,

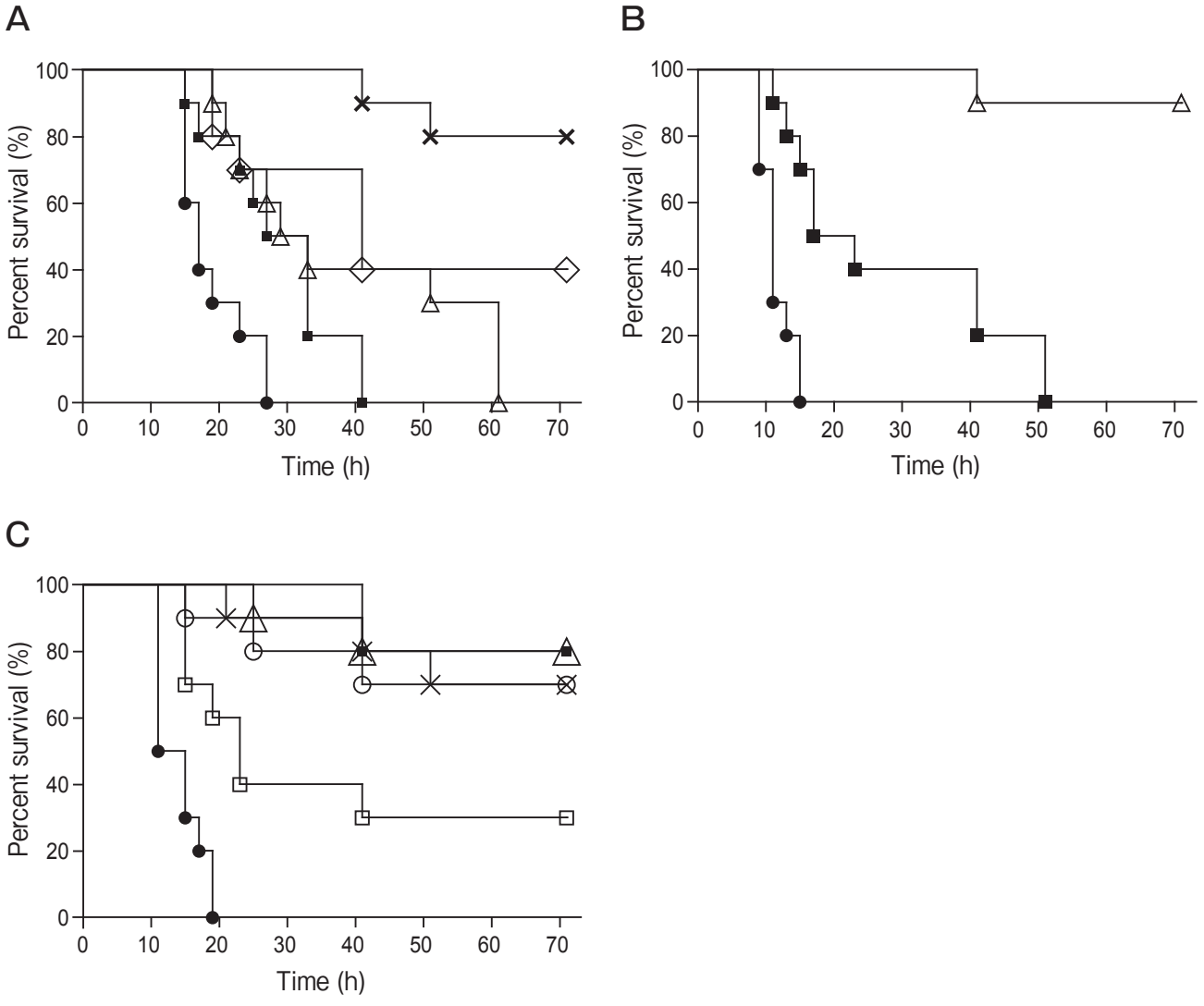

Fig. 2 Therapeutic effect of IgY on cholera in infant mice. Four-day-old suckling mice were gastrointestinally inoculated with 50 μl of O1 or O139 cells (7.5×10^8 CFU/ml). After 3 hr, the mice were then administered 50 μl of anti-V.C IgY, anti-CTB IgY, or a mixture of 1 mg/ml of each at different intervals. **A**, Inoculation of *V. cholerae* O1 El Tor (Inaba). The mice were then administered the following at 2 h intervals: PBS, (●); control IgY, (■) or anti-CTB IgY, (△); anti-V.C IgY, (◇); or both anti-CTB IgY and anti-V.C IgY, (×); **B**, Inoculation with *V. cholerae* O139. The mice were then administered the following at 2 h intervals: PBS, (●); anti-CTB IgY, (■); and anti-V.C IgY, (△); **C**, Effects of different intervals. After the challenge with *V. cholerae* O1 El Tor (Inaba), the mice were administered with PBS, (●); or a mixture of anti-V.C and -CTB IgYs at intervals of 2 h, (■); 4 h, (△); 6 h, (×); 8 h, (○); or 12 h, (□).

foods, water, or ORS to prevent or treat cholera in humans, because they can supply water and nutrition in addition to antibody activity. A large amount of IgY can be easily prepared in dry powder form and can be easily transferred, without reducing its activity, to a place where a big outbreak of cholera might occur. IgY is very simple to administer. It can be easily given to people of all ages, from babies to the elderly, even under serious or miserable conditions such as those occurring after a natural disaster.

Acknowledgments. This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

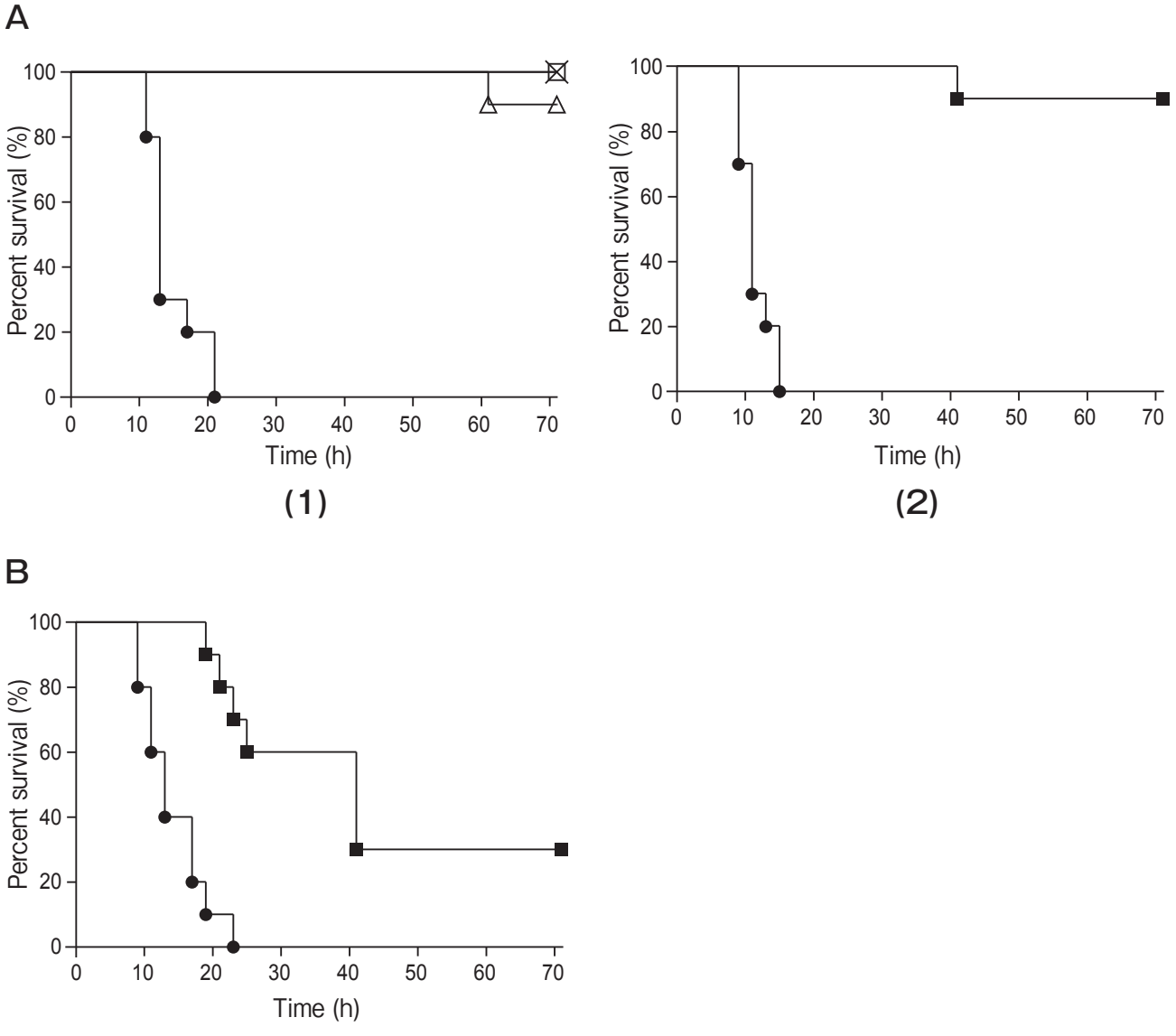

Fig. 3 Protective effect of IgY. **A**, The simultaneous administration of cells and IgY. O1 (Inaba) or O139 cells were mixed with anti-V.C IgY and then injected into the stomach of the suckling mice. **(1)** O1 cells alone, (●); O1 cells with anti-V.C IgY [0.5 mg/ml, (□); 1 mg/ml, (△); or 2 mg/ml, (×)]. **(2)** O139 cells alone, (●); O139 cells with anti-V.C IgY 1 mg/ml, (■); **B**, Effect of pretreatment with IgYs. The suckling mice were first administered PBS, (●); or 1 mg/ml of anti-V.C and -CTB mixed IgYs, (■). After 1 h, 50 μl (7.5×10^8 CFU/ml) of O1 organisms was challenged. After 2 h, the mice were returned to their mothers and observed for up to 71 h at 30℃.

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