Method for Estimation of Toxic Endotoxin in Inactivated *Salmonella* Vaccine in D-Galactosamine-Sensitized Mice

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ABSTRACT. We developed a method to estimate the content of the toxic endotoxin in inactivated *Salmonella* vaccine in D-galactosamine-sensitized mice. Ten-fold serially diluted vaccines were injected intraperitoneally into D-galactosamine-sensitized mice. Lethality in the mice was judged 3 days after the injection. The best result was obtained when C3H/HeN mice were used for the test. Correlation was observed between the endotoxin content measured by *Limulus* amoebocyte lysate assay and the LD₅₀ in the mouse safety test ($r=0.81$). These results suggested that this test could be applied to the estimation of endotoxin content in inactivated vaccines of *Salmonella*.

KEY WORDS: endotoxin, *Salmonella*, vaccine.

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Endotoxin (lipopolysaccharide) is a surface component of gram-negative bacteria, and it exhibits various biological activities when it is released from bacterial cells [13]. Endotoxin causes various symptoms including a state of shock and pyrexia in the animal [1, 5, 9]. Rieder and Thomas [12] reported that intravenously injected *E. coli* endotoxin caused abortion in mice. Cattle manifest clinical signs of shock, tachypnea, dyspnea and a significant increase in the respiratory rate within a short time, and inflammatory cytokines of tumor necrosis factor, interleukin-1 rapidly raised in the blood within a few hours after injection of endotoxin [1, 10]. Kreeftenberg *et al.* [8] reported that free endotoxin in the supernatant of inactivated typhoid vaccine reacted stronger than the cell-associated endotoxin in the sediment in the rabbit pyrogenicity test. Hussaini and Ready [7] reported that a high level of free endotoxin contained in the vaccine caused abortion in sows, whereas a low level did not.

Limulus amoebocyte lysate (LAL) assay has been used to measure endotoxin content in the vaccine of gram-negative bacteria [6–8]. This assay is sensitive and rapid, recently used widely in many laboratories, and was introduced into the Japanese pharmacopoeia. However, this method is based on a clotting system of *Limulus* amoebocyte, and there is the possibility that LAL assay could not estimate the toxic endotoxin in cattle. Therefore, an *in vivo* assay system with laboratory animals is urgently required. D-galactosamine (GalN)-sensitized mice increased the sensitivity up to 100,000-fold to lethal effects of endotoxin [2–4]. Based on this knowledge, we applied GalN-sensitized mice for the estimation of the endotoxin content in inactivated *Salmonella* vaccine.

Fifteen batches of inactivated *Salmonella* vaccine containing various amounts of endotoxin ranging from 250 to 241,300 endotoxin units (EU)/ml measured by LAL assay (Endospecy, Seikagaku Kogyo Co., Tokyo, Japan) were prepared. These vaccines contained formalin-inactivated cells of *Salmonella enterica* serovar Typhimurium (ST) and

S. enterica serovar Dublin (SD) (approx. ST: 1.0×10^{10} cells/ml, SD: 2.5×10^{10} cells/ml).

Four strains of mice (ddY, ICR, BALB/c and C3H/HeN, 10-weeks old, Japan SLC, Hamamatsu, Japan) were used in this experiment. Each mouse was injected intraperitoneally with 10 mg of GalN (Wako Pure Chemical, Osaka, Japan) dissolved in distilled water. Immediately after the GalN injection, an aliquots (0.5 ml) of 10-fold serially diluted vaccines with Dalbecco modified phosphate buffer saline (Nissui Pharmaceutical, Tokyo, Japan) were injected intraperitoneally. Lethality in the mice was judged 3 days after the injection. All of the experiments in this study were performed according to the Guidelines for Animal Experiments of The Kitasato Institute, Saitama, Japan.

In the first experiment, the lethality of the vaccine (batch No. 4) in 4 mice strains was examined. As shown in Table 1, ddY and ICR mice were less sensitive to the vaccine than BALB/c and C3H/HeN mice. The LD₅₀ value for each strain of mice was reproducible in the repeated experiments. In the next experiment, the lethality of 4 batches of vaccine with different endotoxin contents was tested on BALB/c and C3H/HeN mice. In both tests with different mice strains, the LD₅₀ values correlated well with the amounts of endotoxin (Table 2), but in the tests for batch No.2 of BALB/c mice,

Table 1. Lethality of the inactivated *Salmonella* vaccine against 4 strains of D-galactosamine-sensitized mice

Vaccine ^{a)} dilution	Strain of mice			
	ddY	ICR	BALB/c	C3H/HeN
10 ¹	2/5 ^{b)}	1/5	5/5	5/5
10 ²	2/5	1/5	2/5	5/5
10 ³	0/5	0/5	1/5	3/5
10 ⁴	0/5	0/5	0/5	0/5
LD ₅₀ (log)	0.9	0.9	2.1	3.1

a) Vaccine batch No.4 (241,300 endotoxin unit/ml measured by LAL assay) was used in this experiment.

b) Dead mice/tested mice.

Table 2. Lethality of 4 batches of the vaccine against BALB/c and C3H/HeN mice

Vaccine dilution	BALB/c mice				C3H/HeN mice			
	Vaccine batch No.				Vaccine batch No.			
	1	2	3	4	1	2	3	4
10 ⁰	8/10 ^{a)}	5/10	9/10	10/10	9/10	10/10	10/10	10/10
10 ¹	8/10	6/10	8/10	10/10	5/10	8/10	10/10	10/10
10 ²	2/10	8/10	7/10	10/10	0/10	4/10	9/10	10/10
10 ³	0/10	5/10	5/10	6/10	0/10	0/10	0/10	4/10
10 ⁴	NT	0/10	0/10	0/10	NT	NT	NT	0/10
LD ₅₀ (log)	1.3	1.7	2.4	3.1	0.9	1.7	2.4	2.9
Endotoxin (EU/ml)	4,200	10,500	104,600	241,300	4,200	10,500	104,600	241,300

a) Dead mice/tested mice.

the dilution rate of the vaccine and the number of dead mice did not correlate (Table 2). These phenomena, did not often, occur except when BALB/c mice were used for the tests.

In order to confirm the toxicity factor contained in the vaccine, the lethality to C3H/HeN mice and the endotoxin low-responders among C3H/HeJ mice, harboring the mutation in the endotoxin receptor (TLR-4) gene [2, 11], were compared. As shown in Table 3, all C3H/HeN mice died at the endotoxin dose of 0.5 µg/mouse, whereas C3H/HeJ mice survived even at the dose of 5 µg/mouse. Next, the effect of polymyxin B (Wako Pure Chemical, Osaka, Japan), which neutralizes endotoxin [14], was examined. Polymyxin B (500-fold the weight of the endotoxin) was mixed with vaccine, the mixture was incubated at room temperature for 30 min, and tested for lethality. All of the mice injected with the non-treated vaccine were dead, but all of the mice injected with the polymyxin B-treated vaccine survived (Table 4). These results clearly indicated that the toxic factor in the vaccine is the endotoxin.

As reported previously by other groups [7, 8], the toxicity of endotoxin is supposed to be associated with free endotoxin. This hypothesis was confirmed also in this study as follows. The vaccine (batch No. 4) was centrifuged (1,400 × g) for 15 min, and the mouse lethality of the supernatant was compared with that of the original vaccine. About 90% of the lethal activity was found to be present in the supernatant, indicating that the free endotoxin released from the bacterial cell membrane is responsible for the lethal toxicity. In this experiment, the lethal toxicity of the supernatant (without the aluminum adjuvant) and the original vaccine (with the adjuvant) had almost the same score. This suggests that the influence of the adjuvant on the lethality is not large in this test (Table 5).

BALB/c and C3H/HeN mice were shown to be more sensitive to endotoxin than ddY and ICR mice. In addition, the lethality in C3H/HeN mice was more stable and reproducible than that in BALB/c mice. Therefore, we propose to use C3H/HeN mice for this test developed in this study, designated as the mouse safety test.

Since a correlation was observed between the lethality and LAL assay value in the experiments shown in Table 2, the data for both tests were further compared with all

Table 3. Lethality of the inactivated *Salmonella* vaccine and the authentic LPS against D-galactosamine-sensitized C3H/HeN and C3H/HeJ mice

Mouse	Vaccine		LPS ^{b)}	
	Endotoxin by LAL (µg/ml) ^{a)}		(µg/ml)	
	10	1	10	1
C3H/HeN	10/10 ^{c)}	10/10	10/10	10/10
C3H/HeJ	0/10	0/10	0/10	0/10

a) Measured by LAL assay with LPS from *E. coli* O111:B4 as reference (1 µg=2,400 EU/ml).b) LPS from *S. enterica* serovar Typhimurium (Sigma).

c) Dead mice/tested mice.

Table 4. Neutralization of the lethality against D-galactosamine-sensitized C3H/HeN mice by polymyxin B

Polymyxin B	Vaccine		LPS ^{b)}	
	Endotoxin by LAL (µg/ml) ^{a)}		(µg/ml)	
	10	1	10	1
+	0/10 ^{c)}	0/10	0/10	0/10
-	10/10	10/10	10/10	10/10

a) Measured by LAL assay with LPS from *E. coli* O111:B4 as reference. (1 µg=2,400 EU/ml).b) LPS from *S. enterica* serovar Typhimurium (Sigma).

c) Dead mice/tested mice.

Table 5. Lethality of free and cell associated endotoxin in inactivated *Salmonella* vaccine

Test	Original vaccine ^{a)}	Supernatant	Sediment
LAL (EU/ml)	241,000	148,000	20,500
Lethality in mouse test (LD ₅₀ log)	3.1	3.2	1.9

a) Vaccine batch No. 4 (241,300 EU/ml) was used in this experiment.

batches of vaccine prepared. As shown in Fig. 1, the data for both tests correlated well with the value, $r=0.81$ (Spearman's correlation coefficient), and the value of significance, $P<0.01$, but the deviation of the data was rather high. This is probably due to the difference between the bacteria and methods used in the LAL assay and the mouse safety test.

Fig. 1. Correlation between endotoxin content and LD₅₀ in the mouse safety test. Each batch of the vaccine was tested by LAL assay and the mouse safety test. C3H/HeN mice were used for the test.

The *in vivo* assay with GalN-sensitized mouse is more suitable to estimate the toxicity of biologically active endotoxin, or free endotoxin, in cattle than *in vitro* LAL assay. Moreover, there is the possibility that endotoxin content can not be measured by LAL assay, in case the vaccine contains oil adjuvant. Therefore, this mouse safety test can be used for the estimation of the content of the toxic endotoxin in the inactivated *Salmonella* vaccine, and might also be applied to inactivated vaccines of the other gram-negative bacteria.

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