Yersinia enterocoliticaに対する新しい定量的経口免疫法並びに同経口免疫の持続期間

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A New Quantitative Method for Oral Vaccination of Killed cells and Persistence of the Vaccination against Fecal Excretion of *Yersinia enterocolitica*

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ABSTRACT. Mice were orally vaccinated and challenged with *Yersinia enterocolitica* 03 strain to determine the precise amount of killed bacterial cells, the number of doses and the interval for efficient oral vaccination. The rate of protection against fecal excretion reached 100% in the mice receiving three doses. Among the mice immunized with three doses, the rate of protection reached 100% in three groups of mice vaccinated with a total amount of 500 mg at 7-day intervals, or 250 mg at 7-day intervals, or at 4-day intervals. Mice were challenged after three doses of oral vaccine of a total amount of 250 mg of the killed cells at 4-day intervals to know persistence of the protection. The bacteria challenged 1 week to 6 months after the final vaccination were significantly blocked from colonizing in the intestines. Challenge 2 or 3 weeks after the final vaccination provided mice the maximum protection rate of 100%. There was a significant increase in the rate of mice excreting the bacteria 6 months after the final vaccination.—KEY WORKS: oral vaccination, *Yersinia enterocolitica*.

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There are a few reports on the oral vaccination with killed cells of *Yersinia enterocolitica*. Kaneko and Hashimoto [4] reported inhibition of fecal excretion of *Y. enterocolitica* in the mice vaccinated orally with killed bacteria and protection of the mice challenged 1 week after oral vaccination against fecal excretion of the bacteria. Persistence of the protection against fecal excretion by the oral vaccination, however, is still unknown. On the other hand, the usual method of oral vaccination allowing mice to drink the vaccine at will as drinking water has a disadvantage of unacculate amount of killed cells of the vaccine. It is necessary to develop an acculate vaccination method to provide mice with the protection against fecal excretion by oral vaccination with killed cells. The present study was planned to develop an acculate vaccination method to know persistant duration of the protection against fecal excretion by the oral vaccination.

MATERIALS AND METHODS

Bacteria, animal, challenge and culture methods: The bacterial strain used was *Y. enterocolitica* serovar 03 strain isolated from a brown rat. Biochemical characters of this strain (SD1416–11) were described in a previous report [3]. The strain was demonstrated to harbor the 42 Megadalton virulence-associated plasmid by the method of Kado and Liu [2] and to be Ca^{2+}-dependent according to the method of Higuchi and Smith [1]. It was cultured on Trypticase soy agar (BBL) at 25°C for 48 hr. The bacterial cells were suspended in a mixture of equal volumes of calf serum and a 10% lactose-water solution and stored at −80°C. Female 4-week-old SPF ICR mice (Shizuoka Agric. Coop. Assoc. Labo. Anim., Shizuoka) were used. The mice were shown not to harbor *Yersinia* species by culturing their feces before the experiment. A frozen stock strain was thawed and mixed in physiological saline to a concentration of 10^7 viable cells

Table 1. Dose effect of oral vaccine with killed cells on the protection against fecal excretion of *Y. enterocolitica* in mice

Exp. No.	Vaccination			Mice tested	Mice shedding bacteria, days after challenge[a)]	
	Total cells (mg)	Number of doses	Cells per dose (mg)		7	14
1	500	1	500	6	3 (4.34±2.31)	3 (4.53±1.78)
	0	0	0	7	7 (6.74±0.29)	7 (5.76±0.53)
2	500	2	250	6	2 (3.12±6.46)	2 (4.14±6.33)
	0	0	0	6	6 (6.14±1.17)	6 (5.53±0.49)
3	500	3	167	7	0	0
	0	0	0	7	7 (6.89±0.28)	7 (5.97±0.49)
4	500	4	125	6	1 (5.23)	1 (4.88)
	0	0	0	7	7 (6.27±0.61)	7 (5.86±0.29)
5	500	5	100	7	2 (6.20, 5.45)	2 (6.45, 6.03)
	0	0	0	7	7 (7.06±0.21)	7 (6.29±0.35)

a) Values in parentheses refer to $\log_{10}$ mean counts and standard deviations of mice shedding 10^2 or more viable cells per g of feces. When only one or two mice shed the bacteria, the values refer to the individual counts.

per 0.1 m*l*. The mice were intragastrically challenged with 0.1 m*l* of the mixture containing 10^7 viable cells through a gastric feeding tube. In experiments No. 1 to No. 8, mice were challenged 3 weeks after the final vaccination. Quantitative direct culture of feces was done as described previously [4].

Vaccine preparation: Killed vaccine was prepared as follows: The organisms grown on Trypticase soy agar (BBL) for 48 hr at 25°C were suspended in physiological saline solution. Formaldehyde was added to a final concentration of 1% to the suspension of the live organisms, and the suspension was maintained at room temperature for over one day. The formalin-killed cells were centrifuged to eliminate formaldehyde and the precipitate was suspended in physiological saline to a final concentration of 500 mg/m*l* (wet weight). This suspension was used as vaccine. Formalin-killed cells were given orally to mice through a gastric feeding tube.

Statistical analysis was made by Fisher's exact test.

RESULTS

The number of doses of oral vaccination with killed cells was appraised first. The total amount of the killed cells administered was 500 mg in all five experiments shown in Table 1. In all experiments but No. 1, mice were given oral vaccination at 7-day intervals and were significantly protected against fecal excretion ($P<0.03$).

It was demonstrated that the three doses of oral vaccination most effectively protected mice against fecal excretion. Then, the total amount of the killed cells and the intervals between doses were appraised. It was also demonstrated that the total amount of 500 mg of the killed cells protected mice in experiment No. 3 (Table 1). The total amounts of 125 and 250 mg of the killed cells were evaluated. Since the 7-day intervals of vaccination was demonstrated to be effective (Table 1), we attempted to shorten the intervals of vaccination. In experiment No. 6 (Table 2), mice were orally inoculated with three doses of 167 mg of vaccine at 2-,

Table 2. Effect of total amount of killed cells and vaccination intervals on the protection against fecal excretion of *Y. enterocolitica* in mice

Exp. No.	Vaccination		Mice tested	Mice shedding bacteria, days after challenge[b]	
	Total cells (mg)[a]	Intervals		7	14
6	500 (3×163)	7 days	7	0	0
		4 days	7	1 (6.83)	1 (6.98)
		2 days	7	2 (2.70, 4.30)	1 (5.04)
7	250 (3× 83)	7 days	7	0	0
		4 days	7	0	0
8	125 (3× 42)	7 days	7	1 (3.16)	1 (6.93)
	0		7	7 (7.26±0.18)	7 (6.15±0.49)

a) The formula in parentheses indicates the number of doses by each dose.
b) Values denote the same in Table 1.

Table 3. Persistence of protection against fecal excretion in mice orally vaccinated with three doses of 83 mg of killed cells of *Y. enterocolitica* at 4-day interval

Challenge after final oral vaccination	Mice	Mice tested	Mice shedding bacteria, days after challenge[a]	
			7	14
1 week	Vaccinated	8	1 (3.98)	1 (5.25)
	Unvaccinated	7	7 (6.42±0.31)	7 (6.22±0.34)
2 weeks	Vaccinated	9	0	0
	Unvaccinated	7	7 (6.73±0.30)	7 (6.42±0.30)
3 weeks	Vaccinated	9	0	0
	Unvaccinated	7	7 (6.41±0.32)	7 (6.49±0.32)
2 months	Vaccinated	9	2 (4.30, 5.81)	2 (4.48, 6.25)
	Unvaccinated	7	7 (6.92±0.48)	7 (6.37±0.34)
4 months	Vaccinated	9	3 (6.14±0.81)	3 (5.89±0.70)
	Unvaccinated	7	7 (7.04±0.33)	7 (6.28±0.62)
6 months	Vaccinated	9	4 (5.62±0.97)	4 (5.54±0.96)
	Unvaccinated	7	7 (7.12±0.19)	7 (6.40±0.54)

a) Values denote the same in Table 1.

4- and 7-day intervals. These mice were significantly protected against fecal excretion of the challenged organism ($P=0.01$, $P=0.002$ and $P=0.003$, respectively). In experiment No. 7 with a total amount of 250 mg of the killed cells, fecal excretion was inhibited in all mice receiving at 7-day intervals and also in all at 4-day intervals. In experiment No. 8, fecal excretion was also significantly inhibited in the mice having received three doses of 42 mg of the killed cells ($P=0.002$). All seven unimmunized mice excreted the bacteria for 14 days after challenge with 10^6 to 10^7 cells.

To see duration of protection against fecal excretion mice were challenged 1, 2 and 3

weeks and 2, 4 and 6 months after the third dose of 83 mg of the killed cells by the 4-day interval method. The mice challenged a week after vaccination were significantly inhibited from excreting the bacteria in their feces (P=0.001). All mice challenged 2 or 3 weeks after vaccination were inhibited from excreting the bacteria. The mice challenged 6 months after vaccination were still significantly inhibited from excreting the bacteria in feces (P=0.029). Two of nine mice challenged 2 months after vaccination and three of nine mice challenged 4 months after vaccination excreted the bacteria in their feces for 14 days. Four of nine mice challenged 6 months after vaccination shed the bacteria for 14 days. The rate of mice shedding the bacteria began to increase 2 months after the vaccination. There was a significant difference in the rate of mice shedding between those challenged 2 or 3 weeks and those challenged 6 months after the vaccination (P=0.041).

DISCUSSION

Uchida *et al.* [5] reported that oral administration of formalin-killed *Y. enterocolitica* cells for 4 weeks protected the mice against fecal excretion. The final concentration of vaccine of the previous report was 1 mg/m*l*. A total of 500 mg of the killed bacteria was estimated to be needed to protect mice against fecal excretion. In the present study, we found that three doses of vaccination were the best to provide the protection, a total amount of 250 mg of the killed cells provided mice with the maximum protection, and the 4-day interval method also provided mice with the maximum protection rate as did the 7-day interval method. The mice can be more quantitatively vaccinated against fecal excretion by the method devised in the present study.

In the present study, *Y. enterocolitica* was significantly inhibited from colonizing in the intestines of mice challenged a week or 6 months after the final oral vaccination. It was also demonstrated that the protection rate of the mice challenged 6 months after the final oral vaccination was significantly lower than that of those challenged 2 or 3 weeks after that. These facts suggest that the protection starts as early as a week after the final vaccination, persist for 6 months and begins to decrease 6 months after the final vaccination. Kaneko and Hashimoto [4] reported that formalin-killed cells of serovar 03 of *Y. enterocolitica* provided cross-protection against serovar 09 and vice versa and that serovar 06 of *Y. enterocolitica* did not furnish cross-protection against serovar 03. These facts may suggest that the protection against fecal excretion with oral killed vaccine is due to acquired immunity and that this immunity would persist for at least 6 months.

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要　　約

Yersinia enterocolitica に対する新しい定量的経口免疫法並びに同経口免疫の持続期間：柳沢順子・金子賢一・林谷秀樹・小川益男（東京農工大学農学部家畜衛生学教室）—— *Yersinia enterocolitica* 03菌に対する定量的経口免疫法を検討するために，同菌のホルマリン死菌を用いて投与回数，投与菌量および投与間隔を変えて最適条件を検討した。(1) 総菌量 500mg または，(2) 同 250mg を 1 週間隔で 3 回に分けて経口投与，あるいは，(3) 同 250mg を 4 日間隔で 3 回に分けて経口投与するといずれのマウス群でも100％の腸管定着阻止率を示した。(3) の方法によって経口免疫されたマウスについて，経時的に生菌攻撃を行って免疫の持続期間を検討したところ，最終死菌投与後 6 ヵ月において腸管定着阻止率の有意な低下が認められた。