Cellular Immunity After Oral Administration with *V. cholerae*

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Abstract

The mechanisms of oral vaccination have not been clarified yet. In this study, the mechanisms that prevent infection by oral vaccination with *Vibrio cholerae* (*V. cholerae*) were investigated. Mice were orally vaccinated with the whole cells, or IF 30 of *V. cholerae*. The splenic cells or Peyer’s patches (PP) cells of these mice were removed and their *in vitro* antibacterial activity against *V. cholerae* was determined. The splenic cells of mice orally vaccinated with the whole cells of *V. cholerae* showed suppression of the bacterium after 6 administrations, but no suppression after a single administration. The PP cells of mice orally vaccinated with the whole cells of *V. cholerae* showed suppression, irrespective of the number of times of administration. In the groups of mice orally vaccinated with IF30, suppression of *V. cholerae* was exhibited by the splenic cells of mice after 4 administrations and by the PP cells of mice after a single administration. The above mentioned suppression of *V. cholerae* was induced by B cells included in the splenic or PP cells of orally vaccinated mice. None of the humoral antibodies of orally vaccinated mice suppressed *V. cholerae*. Consequently, it is suggested that infection of *V. cholerae* is prevented locally in the intestine by the primary effect of oral vaccination and then by the splenic cells that recognize the antigen of *V. cholerae* through consecutive oral administrations.

Introduction

*Vibrio cholerae* (*V. cholerae*) invades the living body from the mouth to the intestines inducing diarrhea. Recently, Burrows et al.\(^1\) reported that diarrhea is induced by toxins produced by *V. cholerae*. Nelson et al.\(^2\) reported that diarrhea is induced by the colonization of *V. cholerae* in the intestines. However, the mechanisms of diarrhea have not been clarified. Since diarrhea is a local intestinal phenomenon, many investigators have considered local prevention. An oral vaccine of *V. cholerae* was developed recently. Clemens et al.\(^3\) reported a favorable efficacy of an oral vaccine, consisting of a mixture of cholera toxine and whole cells of *V. cholerae*, in a field-trial carried out in Bangladesh. However, they did not clarify the mechanisms of oral vaccination. The authors reported previously\(^4\) that the humoral IgM and vibriocidal antibodies were effectively produced in mice that were primed orally and then boosted intraperitoneally with whole cells of *V. cholerae*. An infection-preventing substance was also reported to be isolated from *V. cholerae*. This substance prevented against challenge of *V. cholerae* in the intestines of rabbits immunized with it. It also exhibited high potency in mice. The mechanisms of infectious prevention, however, have not yet been understood.

In this report, the authors investigated whether a potentant antibacterial activity can be induced *in vitro* by the lymphoid cell system of orally vaccinated mice.

Materials and Methods

*Mice:* Inbred female BALB/c mice were purchased from Shizuoka Breeding Laboratories and used at 6—8 weeks of age.

*Bacteria:* *V. cholerae* Inabe V86 was derived from the Japan Natl. Inst. Of Health. *V. cholerae* strains
were cultivated overnight on heart infusion agar (DIFCO).

**Preparation of IF30 and whole cells:** The preparation of IF30 followed the previously described method\(^5\). *V. cholerae* was incubated in heart infusion agar and suspended in phosphate buffer saline (PBS). Urea to final concentration of 2.5 M was added to a part of the suspension and treated with sonicater (BROWN). The supernatant was salted out with ammonium sulfate at a final concentration of 30% to obtain the IF30 of *V. cholerae*. The other part of the suspension of *V. cholerae* was heated at 121°C for 30 min. to obtain the whole cells of the bacterium.

**Immunization and vaccination:** 5 mice per group were intraperitoneally (I.P) administrated with 10 mg of IF30 or 10⁷ whole cells, simultaneously with alum. The same amount of IF30, or whole cells, were orally administered to the mice using feeding tubes. 5 mice per group were orally given once, 4 consecutive days, or 6 alternate days. In order to obtain the antibody, blood samples were taken from the mice 7 days after the single intraperitoneal administration or 3 days after the last oral administrations.

**Preparation and separation of cells:** As described in a previous report\(^6\), the spleen and Peyer's patches (PP) were removed from immunized and non-immunized mice. A single-cell suspension of these samples was made in RPMI 1640 medium (Gibco) containing 5% of normal murine serum. The single-cell suspension of the splenic or PP cells of 10 mice per group was passed through nylon-wool (Wako) columns\(^7\), to separate the adherent cells (B cell fraction viability of cell 95%) from non-adherent ones (T cell fraction, viability of cell 92%).

**Antibacterial assay\(^8\):** The previously reported antibacterial assay was that first reported by Steighigel et al.\(^9\) and modified by Lowell et al.\(^10\) in order to determine the leukocyte-mediated effect against bacterial growth. 10⁸ splenic or PP cells were suspended in 0.5 ml of RPMI 1640 medium, and 0.1 ml of the complement (Denka) was added. It was then incubated at 37°C for 1 hour and washed. The cells were resuspended in 0.5 ml of RPMI 1640 medium and 10⁵ or 10⁴ cells of *V. cholerae* were added. They were then incubated at 37°C for 1 hour. On the other hand, normal splenic or PP cells were suspended in 0.5 ml of RPMI 1640 medium and treated with 10-times-diluted antibody at 0°C for 1 hour. After washing, the cells were processed in the same manner as those from immunized mice. After incubation, the surviving *V. cholerae* (bacterium) were plated on HI agar and incubated overnight. The colony-forming units were then counted.

**Vibriocidal antibody\(^11\):** The microtiter method was employed to determine the vibriocidal antibody. The titer was indicated as a dilution factor. Serum sample of mice in each group were diluted using microplates. A mixture of *V. cholerae* and complement was added to each well before incubation at 37°C for 1 hour. After adding 0.2 ml of HI broth into each well, the microplates were again incubated at 37°C for 4 hrs. The microplates were then left at 4°C overnight. The titer was indicated as the final dilution that inhibited the growth of *V. cholerae*.

**Statistical analysis:** Values of all figures were shown in the means ± standard deviation of bacterial numbers. Standard deviations were always from 0.636 to 0.771. The significance of differences among means of experiments were evaluated by Fisher's exact test; p<0.05 was considered significant.

**Results**

Whole cells or IF30 of *V. cholerae* were administered to mice orally or intraperitoneally at various intervals. The spleen was removed from the vaccinated mice and subjected to an assay for antibacterial activity (Fig. 1). Mice in the groups immunized intraperitoneally with whole cells, or IF30, showed a marked suppression of *V. cholerae* as compared with those in non-immunized control group (p<0.05). Marked suppression of the bacterium was also observed in mice orally vaccinated for 6 alternate days with the whole cells of *V. cholerae* (p<0.05). However, single or 4 consecutive daily vaccinations by the oral route
showed no significant effect in comparison to the control group. Oral vaccination with IF30 showed a marked suppression of the bacterium after 4 consecutive daily administrations \((p<0.05)\). Peyer's patches (PP) were also removed from the vaccinated mice and subjected to an assay for antibacterial activity (Fig. 2). Mice in the groups immunized intraperitoneally with whole cells or IF30 showed a weaker suppression of bacterium than the splenic cells of these mice. A stronger bacterial suppression than that induced by the splenic cells was observed in PP cells of mice vaccinated orally with whole cells \((p<0.05)\), irrespective of the administration schedule. Oral vaccination with IF30 showed a suppression of \textit{V. cholerae} after a single administration \((p<0.05)\). However, the other schedules of oral vaccination with IF30 showed no significant suppression of the bacterium compared to the control PP cells.

As mentioned above, \textit{V. cholerae} was suppressed by the splenic or PP cells of mice orally vaccinated for 6 alternate days with whole cells. Therefore, the following experiment was performed with this vaccination group. Oral vaccination with IF30 was also employed for reference. The splenic or PP cells were removed from the vaccinated mice and separated using nylon-wool columns. The antibacterial activity of the cells was then determined (Fig. 3). In both the whole cells and IF30 vaccination groups, B cells included in the splenic or PP cells suppressed bacterium \((p<0.05)\). On the contrary, increases of bacterium were observed in the presence of the T cell fraction of PP cells sampled from the above 2 groups, compared to the control group.

The next experiment was performed to find out whether the bacterium is suppressed by the humoral antibody. The vibriocidal antibody was determined in each serum of mice vaccinated orally or intraperitoneally with various antigens (Table 1). High titers of vibriocidal antibody, i.e., 1:256 and 1:128, were
Fig. 3 Cell-mediated in vitro activity against V. cholerae of B cells or T cells of spleen or PP cells mice after oral vaccination by 6 times on alternate days. 
△---△ non-vaccination ●○● vaccination with whole cells, ×---× vaccination with IF30.

Table 1 Vibriocidal antibody titer of serum in mice after oral vaccination or IP immunization with cholera whole cell or IF30

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<thead>
<tr>
<th>Vaccination or Immunization</th>
<th>Antigen</th>
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<tr>
<td></td>
<td>Whole cell</td>
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<tr>
<td>Intraperitoneal</td>
<td>1:256</td>
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<tr>
<td>One time oral</td>
<td>1:&lt;2</td>
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<tr>
<td>4 consecutive times oral</td>
<td>1:&lt;2</td>
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<tr>
<td>6 times on alternate days</td>
<td>1:&lt;2</td>
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indicated by the serum of mice immunized intraperitoneally with whole cells, or IF30. However, the antibody titers of mice vaccinated orally with whole cells and IF30 were 1:2 or less and 1:4 or less, respectively. These samples of humoral antibody were treated against splenic cells of normal mice and their antibacterial activity was determined (Fig. 4). The splenic cells treated with the serum of mice immunized intraperitoneally with whole cells, or IF30, strongly suppressed bacterium (p<0.05). However, the splenic cells treated with the serum of mice vaccinated orally showed no suppression of bacterium. PP cells of normal mice were also treated with samples of humoral antibody and subjected to the determination of antibacterial activity (Fig. 5). As in the case of splenic cells, the PP cells treated with the serum of mice immunized intraperitoneally strongly suppressed bacterium (p<0.05), while those treated with the serum of mice vaccinated orally showed no suppression of the bacterium.

Discussion

N. lycke et al. investigated the mechanisms of oral vaccination and reported that memory cells of antigen were present in the lamina propria and Peyer’s patches of mice for 2 years after oral administration with cholera toxin\[12\]. The authors previously reported that the production of IgE antibody was suppressed and vibriocidal antibody was increased by the oral priming and intraperitoneal boosting with whole cells of V. cholerae\[13\]. These results suggested an enhancement of antibacterial antibodies by oral administration. Therefore, in this report, the authors studied the mechanisms of antibacterial activity in relation to oral vaccination with whole cells, or IF30 of V. cholerae.

V. cholerae was suppressed in vitro by the splenic or PP cells of mice immunized intraperitoneally. Serum obtained from these mice showed high titers of vibriocidal antibody. The normal splenic or PP cells
treated with these serum also suppressed *V. cholerae*. These results suggest that *V. cholerae* is suppressed by the humoral antibody.

On the other hand, in the orally vaccinated groups, *V. cholerae* was strongly suppressed by the splenic cells of mice vaccinated 6 alternate days with whole cells. The PP cells of mice vaccinated orally with whole cells also suppressed *V. cholerae*, irrespective of the administration schedule. However, no humoral antibody was detected in mice vaccinated orally. Consequently, the suppression of *V. cholerae* observed in these groups was showed to be a cell-mediated reaction of the splenic, or PP cells.

The splenic and PP cells were separated using nylon-wool columns. It was showed that B cells contained in the splenic and PP cells suppressed *V. cholerae*. This result suggests that B cells contained in the PP cells recognize the antigen in the early phase of oral vaccination and induce the antigen specific Fc receptor on B cells and suppress *V. cholerae*. Also it suggested that B cells contained in the splenic cells recognize the antigen though multiple oral administration and suppress *V. cholerae* such as PP cells.

In the groups of oral vaccination with IF30, the splenic cells of mice immunized for 4 consecutive days suppressed *V. cholerae*. The PP cells of mice vaccinated by a single oral administration with IF30 also suppressed *V. cholerae*. These results suggest that the PP cells also recognize soluble antigen, such as IF30 in the early phase of oral vaccination.

Clemens reported that a mixed vaccine of cholera toxin and whole cells of *V. cholerae* was effective in a
field-trial carried out in Bangladesh and that whole cells of the bacteria were essential for the oral vaccination\(^3\). Kagnoff et al. reported that PP cells recognized the antigen in the oral vaccination and that the memory cells were transmitted to the splenic cells\(^{14}\). Consequently, it has been argued that early infection might be protected in PP cells of mice vaccinated orally.

The humoral vibriocidal antibody was not enhanced by oral vaccination alone. However, other components in the serum considered to suppressed the bacterium. In order to clarify this point, the splenic and PP cells of normal mice were treated with the serum of mice vaccinated with *V. cholerae* orally or intraperitoneally. The antibacterial activity of these treated cells was then determined. It was showed that a strong suppression of *V. cholerae* is induced in the cells treated by intraperitoneally immunized serum, but not induced by orally vaccinated ones. These results suggest that *V. cholerae* also was suppressed in the splenic or PP cells treated with antigen specific antibody.

Tagliable et al. reported that cellular immunity was induced in volunteers orally vaccinated with live attenuated strains of *Salmonella typhi*\(^{15}\). They also reported that cellular immunity was detected in the intestines of mice orally vaccinated with *Salmonella typhimurium*\(^{16}\). Consequently, the lymphoid cell system in the intestines is considered to play an important role in the intestinal infection and exhibit a preventive activity in the early phase of infection.

This study suggests that infectious defense of *V. cholerae* was exhibited by the lymphoid cell system in the intestine at the early phase of infection and that the humoral antibody became active at the later phases.

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**References**


コレラに対する細胞性免疫

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（昭和62年12月23日受理）

経口 vaccination 機構はまだ明らかにされていない。そこで我々はこれを明らかにするためにコレラ菌体及び IF 30を経口投与し、in vitro でのコレラ菌に対する Antibacterial activity を検討した。コレラ菌体を経口投与した場合に脾臓細胞では 1 回のみの経口投与によって、コレラ菌は抑制されないが、6 回経口投与では抑制された。そして peyer's 板細胞では 1 回だけの経口投与から全ての群でコレラ菌が抑制された。また IF 30を経口投与した場合に脾臓細胞では 4 回投与群で、peyer's 板細胞では 1 回投与だけでもコレラ菌が抑制された。このようなコレラ菌の抑制は経口投与したマウスの脾臓、peyer's 板細胞の B 細胞が示した。

また、経口投与マウスの血中抗体では、いずれの群でもコレラ菌が抑制されなかった。

従って、コレラ菌の感染は経口投与の始めに腸管所で防御し、次に、くり返し経口投与することにより、コレラ菌の抗原を認識した脾臓細胞で防御することが示唆された。