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Interaction between *Vibrio cholerae* and Other Microorganisms: with Reference to Host Defence Mechanism of Mice against Cholera

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Abstract : The interaction between *Vibrio cholerae* and other microorganisms including *Escherichia coli*, *Enterococcus* and *Staphylococcus aureus* was examined in vitro with reference to the defence mechanism of a host against cholera. When *V. cholerae* and another microorganisms were inoculated into fresh media at the same time, *E. coli* suppressed the growth of vibrio to some extent. In the mixed culture with *E. coli*, maximum cell population of *V. cholerae* was reduced to below 10^7/ml. On the other hand, *Enterococcus* and *Staphylococcus* did not suppress the growth of vibrio, even when the inoculated proportion of *V. cholerae* to the cocci was 1 to 10^5. Moreover, these cocci seemed to protect the growth of vibrio in the tryptosoy broth.

The bactericidal effect of acid against *V. cholerae* was confirmed in detail. In the nutrient broth with pH 5, the organism maintained the initial viable cell count (2.5×10^6/ml) for 3 hours, and thereafter it went down. The same number of *V. cholerae* was completely killed within 2 hours in pH 4 and within 30 minutes in pH 3. *V. cholerae*, orally challenged to alkalinized stomach of mice, passed into the intestine, but it was soon and completely eliminated from the small intestine where pH was about 7.0.

Cholera toxin in the medium of 24 hour culture with *V. cholerae* (V86) was 2.6 mcg/ml, but it decreased when the other microorganisms were mixed in the culture.

INTRODUCTION

In the pathogenesis of infectious diseases, a host factor, as well as a pathogenic microorganism would play an important role to present clinical manifestations. In the experimental cholera in mice, it has been noticed that only infantile mice younger than 10 days of age were susceptible to oral challenge of *V. cholerae* (Ujiiye *et al.*, 1968).

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In the adolescent mice, however, neither disease nor multiplication of vibrio in the intestine at all. The mechanism of this resistance against invasion of *V. cholerae* in the adult and adolescent mice has not been clarified yet.

Therefore, we have investigated on the characteristics of the infantile mice in the relation to the pathogenesis of cholera, comparing with the adolescent mice in the viewpoints of bacterial flora, enzymes, morphology, histochemical composition and absorptive function of the intestine (Utsunomiya, 1969; Iwanaga, 1971). Many differences between infantile and adolescent mice were disclosed in these studies, but it was not clarified which factor was significant in the protective mechanism against the invasion of *V. cholerae*.

In the present study, the factors in eliminating the ingested vibrio from the gastro-intestinal tract were studied on the action of the intestinal flora and gastro-intestinal hydrogen ion concentration.

MATERIALS AND METHODS

Bacterial strains: *V. cholerae* El Tor (V86), *Staphylococcus aureus* (209−P), *Enterococcus* (ITM-01) and *E. coli* (HAM-773) were used. The latter two strains were isolated from human stool, and all strains were stocked in soft agar media in our laboratory. This *E. coli* was negative in ileal loop test.

Cultures: *V. cholerae* and another microorganisms were mixed in various proportions, and cultured in three types of media, i. e., the nutrient broth (Eiken), the tryptosoy broth (Difco) and pepton water with yeast extract consisted of 3% Bacto peptone (Difco), 0.5% yeast extract, and 0.5% NaCl. Cultures were kept in water bath at 37°C. Another type of mixed culture was made by adding small amount of *V. cholerae* to the nutrient broth in which each of the three other microorganisms had been cultured for 20 hours in advance. A part of this 20 hour cultured media was filtered to eliminate the precultured microorganisms, and *V. cholerae* was inoculated into this cell free filtrate for the single culture.

Test for the influence of pH on *V. cholerae*: *V. cholerae* in the concentration of 2×10^6/ml were incubated at 37°C in the nutrient broth with various pH of 3.0, 4.0, 5.0 and 6.0, and living cells were serially counted on agar plates by ten-folds dilution method.

Measurement of gastro-intestinal pH in mice: Following laparotomy under light anesthesia, small incisions were made in the bowels. Glass microelectrode (1.2 mm sencer and 2.0 mm coat in diameter, product by Microelectrode Inc. U.S.A., Type MI-410) was inserted through the incisions into the bowels. Hydrogen ion concentration was measured by pH meter (Hitachi Horiba) at several sites of the mucosa.

Oral challenge of *V. cholerae* after alkalinization of the stomach: Adult mice of

ICR strain dehydrated for 24 hours prior to the challenge. Shortly before the challenge, 7% sodium bicarbonate was given freely for 15 minutes as drinking water. Then, *V. cholerae* suspension, 2×10^8/ml in concentration, was given for 1 hour. The suspension was prepared in 2% sodium bicarbonate and 0.45% sodium chloride, from 12 hour culture on meat extract agar slant. The number of living vibrio in the intestine was counted 3, 6, 24 and 48 hours after the beginning of challenge.

Assay of cholera toxin: Pepton water with yeast extract (pH 7.3) was used for toxin production. In this media, *E. coli*, *Staphylococcus* and *Enterococcus* was respectively mixed with *V. cholerae*, and incubated at 30°C in the shaking water bath for 20 hours. Cholera toxin in the culture filtrates was assayed by rabbit skin test (Craig, 1965).

RESULTS

Growth curve of *V. cholerae* in various media: As shown in Figure 1, the viable cell count rapidly increased after a short period of resting phase. This logarithmic phase continued up to 6 hours from the beginning of incubation. When it was cultured in the nutrient broth or pepton water with yeast extract, the stationary phase continued for 24 hours at least. In the tryptosoy broth, however, viable cell count of vibrio decreased rapidly after reaching maximum number and no living vibrio was detected in the cultured medium in 24 hours. The pH of the former two media went down at the beginning of culture, and went up afterwards. After 24 hours, it became higher than that of the starting level. The pH of tryptosoy broth, however, steadily went down and was never restored. Growth curves of *E. coli* in the same conditions were shown in Figure 1 by dotted line, which indicated no decline even in the tryptosoy broth.

Interaction between *V. cholerae* and other microorganisms: When mixed cultures maintained in the tryptosoy broth (Fig. 2), a fall of final pH was not so marked as that in the single culture of vibrio. Although the pH after 24 hours was lower than the starting level, they were higher than that after 6 to 8 hours. In the mixed culture with *E. coli*,

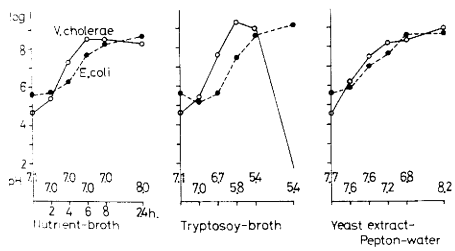

Fig. 1. Growth curve of *Vibrio cholerae* and *E. coli*, single culture of each in various media.
The values of pH indicate those of the media when *Vibrio cholerae* was cultured.

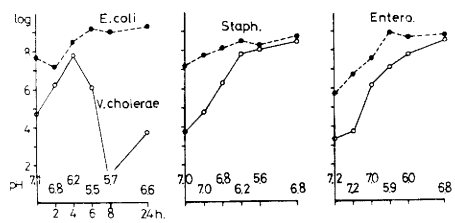

Fig. 2. Mixed culture in the tryptosoy broth.

multiplication of vibrio at the beginning was as rapid as in the single culture. But soon, the number of living vibrio started to fall. At 8 hours, it became less than 10^2/ml. When staphylococcus or enterococcus was mixed, the growth curve of vibrio was similar to that of the single culture in pepton water. The fall of viable cell count at 24 hours was not seen by mixing these microorganisms.

The growth curve of *V. cholerae* in the nutrient broth showed a different pattern from the culture in the tryptosoy broth, when it was cultured with *E. coli*. As shown in Figure 3-left, the growth of vibrio was about the same as that in the tryptosoy broth up to 4 hours. Although a fall of viable cell count was not seen, further multiplication seemed to be inhibited maintaining 10^7/ml of living cells until at 24 hours. Moreover, mixing a large number of *E. coli* (10^9/ml) against 3×10^4/ml of *V. cholerae* (Fig. 3-right), the number of vibrio did not increase within 8 hours. The growth pattern of *V. cholerae* in the mixed culture with staphylococcus or enterococcus in the nutrient broth were shown in Figures 4 and 5. No inhibition of the growth was observed.

When 5×10^4/ml of vibrio was added to nutrient broth in which each of the other three microorganisms had already been cultured for 20 hours prior to inoculation of vibrio (Fig. 6), multiplication of vibrio was partially suppressed, especially in the *E. coli* cultured

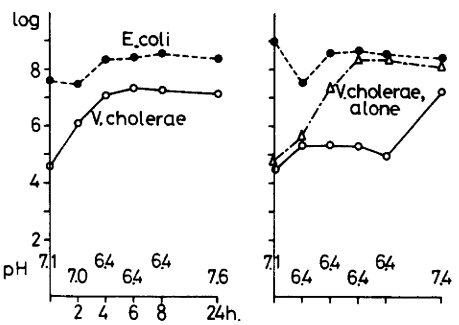

Fig. 3. Mixed culture in the nutrient broth, *Vibrio cholerae* and *E. coli*.

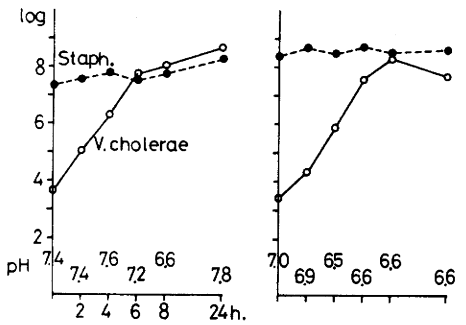

Fig. 4. Mixed culture in the nutrient broth, *Vibrio cholerae* and *Staphylococcus aureus*.

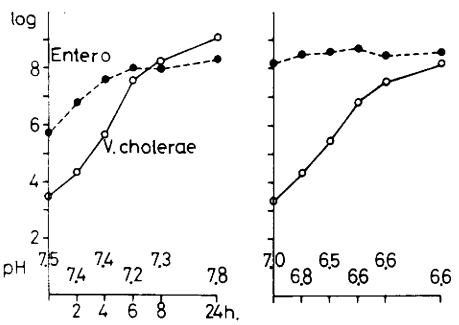

Fig. 5. Mixed culture in the nutrient broth, *Vibrio cholerae* and *Enterococcus*.

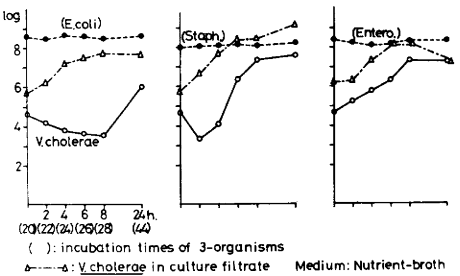

Fig. 6. Survival and growth of *Vibrio cholerae* inoculated to cultured media with the other organisms, and to the culture filtrates.

medium. Viable count of vibrio added to the *E. coli* cultured medium slowly went down for 8 hours. In the case of enterococcus, no decrease of vibrio was seen, but multiplication of vibrio was rather slow. The maximum number of vibrio in these three media during 24 hours were 1×10^6/ml, 3×10^7/ml, and 2×10^7/ml in *E. coli*, staphylococcus and enterococcus cultured media respectively. In this figure triangle spots connecting with dott-dash line indicate multiplication of vibrio in the cell-free filtrates of 20 hour culture with each of the other three microorganisms. The growth of vibrio in the filtrate of the staphyloccus culture was so fast and full as in the fresh medium. The number of vibrio at 24 hours of the culture was 1.2×10^9/ml. On the contrary, the growth of vibrio in the other two filtrates were rather slow, and the number of vibrio after 24 hours of culture was 4×10^7/ml and 2×10^7/ml respectively.

Gastrointestinal pH of mice: As shown in Table 1, gastric pH of five adult mice ranged from 1.5 to 5.0. Regarding the small intestine, it ranged from 6.7 to 8.1. While pH of the large intestine ranged from 6.4 to 7.2. Gastric pH of infantile mice was higher than that of adult ones, which showed a range from 4.2 to 5.5 (Table 2). Giving 0.5–1.0ml of 7% sodium bicarbonate to adult mice dehydrated for 24 hours in advance, gastric pH elevated to 7.0 and maintained the level for an hour at least (Table 3).

Survival and growth of vibrio in various pH: As shown in Figure 7, *V. cholerae* could grow without inhibition in the medium with pH 6.0. But no growth was seen in pH 5. The number of vibrio in the medium with pH 5.0 maintained the initial level until three hours after incubation, and thereafter it went down. When it was incubated in pH 3, all cells were killed within 30 minutes.

Table 1. Gastrointestinal pH of adult mice

No. of Mice	Stomach	Small intestine	Large intestine
1	1.5–4.0	7.2–7.4	7.0–7.2
2	1.5–2.1	7.7–8.1	×
3	3.0–5.0	7.2–7.6	6.8–7.2
4	2.8–3.3	6.8–7.2	×
5	3.0–5.0	6.7–7.3	6.8–7.1

Table 2. Gastric pH of infantile mice

No. of Mice	pH of stomach
1	4.2–4.6
2	4.3–4.8
3	4.9–5.1
4	4.5–5.0
5	4.7–5.5

Table 3. Change of gastric pH after giving 7% $NaHCO_3$ ad libitum for 15 minutes

Time after dose (Minutes)	Gastric pH
1	6.8–7.2
15	7.0–8.0
25	7.0–7.2
40	7.6–8.0
60	6.8–7.4

Fate of vibrio in the intestine of adult mice: By alkalinizing the stomach of the animals, orally challenged vibrio appeared in the intestine (Fig. 8). Three hours after challenge, the concentration of vibrio was highest in the large intestine. The vibrio in the small intestine decreased rapidly and was completely eliminated within a day.

Cholera toxin in mixed culture: Showing in Figure 9, *V. cholerae* strain V86 produced 2.6 mcg/ml of cholera toxin in the medium of 20 hour single culture. In the mixed culture with *E. coli*, however, 1.1 mcg/ml of toxin was detected. Less amount of toxin was detected in the mixed culture with staphylococcus or enterococcus.

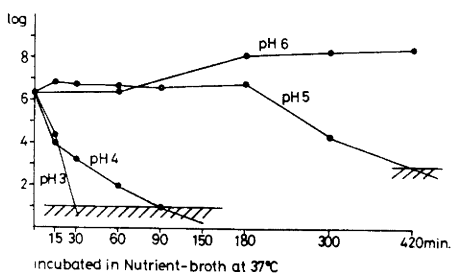

Fig. 7. Bactericidal effect of pH against *Vibrio cholerae*.

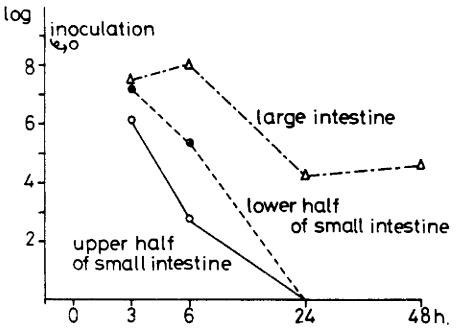

Fig. 8. Fate of *Vibrio cholerae* in the intestine.
Challenged after alkalinization of the stomach (adult mice).

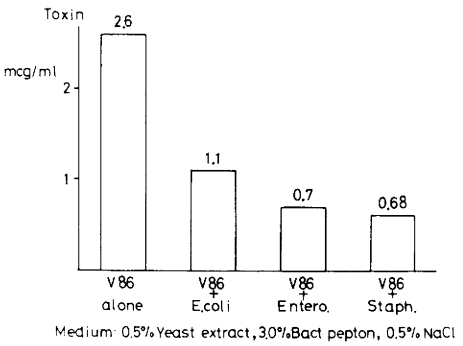

Fig. 9. Toxin production of *Vibrio cholerae* (V86) in mixed culture with *E. coli*, *Enterococcus*, and *Staphylococcus aureus*.

DISCUSSION

A number of studies on interaction between enteropathogenic microorganisms and intestinal flora has been reported by Sarkar and Tribedi (1953), Ramson *et al.* (1961), Barua *et al.* (1963), Hattori *et al.* (1965), Bhattacharya and Mukerjee (1968), Mohan *et al.* (1969), Hentges (1970), Ozawa (1971) and Miller and Feeley (1975). In these

studies, some of the resident flora such as *E. coli* were found to play an important role in eliminating an invaded pathogen, especially shigella (Hentges, 1970; Ozawa, 1971). But as far as the interaction between *V. cholerae* and enteric flora is concerned, any marked inhibition against *V. cholerae* was not reported. Only Ramson *et al.* (1961) reported that the growth of *V. cholerae* was partially suppressed in the presence of growing enterococci and lactobacilli; but the mechanism of this phenomenon is not understood. In our study, however, enterococci did not suppress vibrio at all. Although it has been reported by many workers that *E. coli* did not suppress the growth of *V. cholerae* in vitro or in vivo, present study did not completely coincide with the results of the previous workers.

Ramson *et al.* (1961) applied a continuously fed culture probably to maintain nutrition, pH and others constant. It must be important to study the bacterial interaction in an environment similar to the intestine as far as intestinal infections are concerned. There must be many workers who prefer continuous flow culture to study the interaction between intestinal flora and enteric pathogen, on the basis of this idea of similar environment. But the environment of the intestinal lumen must be far from constant. Ingested materials (foods) are variable in composition, amount, ingesting intervals, etc. Even pH must be ready to change. In short, these environments in vitro are quite different from that in vivo. And it may be said that the continuous flow culture method is not necessarily reflected the intestinal environment any better than other culture methods.

It has been well known that *V. cholerae* is readily killed by acid, but the detail knowledge of the survival rate in a certain pH has been poor (Reimann, 1973; Sarkar and Tribedi, 1954). Gastric pH of the adult mice was enough to kill *V. cholerae* in a short time, and alkalinization of the stomach permit the organism passing through the stomach and getting into the intestine. Although pH of the small intestine is about 7.0 or higher, *V. cholerae* in the small intestine was rapidly elminated from the site.

The present study and previous reports by many workers give us a speculation that the factor which eliminates *V. cholerae* from the small intestine is not likely related to the floral organism. Although *V. cholerae* can stay as an intestinal resident flora in germ free mice, this animal is not only "conventional mice without microorganisms" but also physiologically quite different from so called conventional ones (Sasaki, 1971). In these points of view, the host factor to eliminate *V. cholerae* should be studied not only on bacteriological action but also on intestinal physiology as a non-specific defence mechanism against infection.

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大腸菌，ブドウ球菌，腸球菌がコレラ菌におよぼす作用－コレラに対するマウスの防御機構に関連して－

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コレラ菌の侵入を初めて受けた個体が，コレラの発症を免れる要因を解明するため，今回は腸内先住菌とコレラ菌の相互作用，及び酸度がコレラ菌におよぼす影響などを主に検討した．

腸内先住菌として選定した大腸菌，ブドウ球菌，腸球菌をそれぞれ種々の培養条件で，コレラ菌と混合培養を行った．その結果大腸菌はある程度までコレラ菌の増殖を抑制したが，バクテリオシン様物質の存在は否定的であった．ブドウ球菌，腸球菌はコレラ菌の増殖を全く抑制せず，逆に，トリプトソイブイヨン使用の場合，コレラ菌単独培養で8時間目以後にみられる死滅を抑制し，発育増殖を保護する所見が得られた．

酸度がコレラ菌におよぼす影響は普通ブイヨンのpHを調整して検討した．その結果，pH 6.0では充分増殖したが，pH 5.0では3時間まで接種菌量を維持し，その後減少して7時間目で消失した．さらにpH4.0では2時間で，pH3.0では30分間で生菌は検出されなくなった．成熟マウスの胃内容または胃粘膜のpHは平均して3.0前後という測定結果であった．そこで重炭酸ソーダの前投与によって胃をアルカリ化した直後にコレラ菌を経口投与すると，菌は小腸に到達したが24時間以内に検出されなくなり，その後大腸において少なくとも48時間までは菌が検出された．

コレラ菌と他の細菌を混合培養することにより，培地中から検出されるコレラ毒素は減少した．コレラ菌（V86）単独培養20時間の培地からは2.6mcg/mlの毒素が検出されたが，ブドウ球菌を混合培養すると毒素量は0.68mcg/mlに減少した．

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