Fimbriae of *Salmonella typhimurium* and Their Role in Mouse Intestinal Colonization of the Organism

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Abstract. In mice given orally 10^3 to 10^7 cells of a fimbriate descendant of *S. typhimurium*, the organism localized in between the middle to lowest parts of the small intestine and large intestine, especially in the walls 1 to 3 days postadministration. Thereafter, the organism multiplied in the tracts significantly, and with the increase of inoculating doses, remarkable multiplication of the organism in the tracts was shown. The recovery of the organism from the mesenteric lymph node, liver or spleen with or without gross lesions was found 3 to 5 days after administration, and the liver harbored 10^3 to 10^7 /g organisms between 5 and 14 days. A mouse given 10^6 cells died of sepsis on the 6th day, and 7 mice received 10^7 cells within 6 to 13 days. The O, H and fimbrial antibodies were found in the mice from the 7th day. On the other hand, in mice given a non-fimbriate descendant, no evidence was shown to prove the colonization of the organism in the digestive tracts. Exceptionally, a few mice given 10^5 to 10^7 cells produced O and H antibodies against the organism. On the contrary, it was reconfirmed that mice inoculated intraperitoneally with fimbriate and non-fimbriate descendants gave almost the same LD₅₀ value as reported previously. By scanning electron microscopy, fimbriate organisms were found to adhere to the epithelial surface of the villi (in vitro), while non-fimbriate organisms were not seen on the mucosal surface. These results suggest that the fimbriae may play a role in the colonization of *Salmonella* organisms in the intestinal mucosa.

In the previous study on the infectivity of *S. typhimurium* to mice [11], it was found that the fimbriate descendants of the organism showed higher infectivity than the non-fimbriate descendants when administered orally, and a descendant with a large number of fimbriate cells gave a smaller ID₅₀ value, while both fimbriate and non-fimbriate descendants showed almost the same infectivity when inoculated intraperitoneally. These findings suggested that the fimbriae might play some role in the establishment of intestinal infection.

Present paper deals with whether the fimbriae serve actually as the maintenance of intestinal colonization or not.

Materials and Methods

Animals: The animals used were SPF female mice of the NIH (gpc) strain 5 to 6 weeks old which had been raised in Funabashi Farm. For each inoculum, 5 to 6 groups of mice consisting of 3 or 5 animals were used. Care and management of the inoculated mice were carried out essentially the same way as reported previously [11]. After oral or intraperitoneal inoculation, mice were sacrificed on the 1st, 3rd, 5th, 7th, 10th and 14th days (oral), or on the 8th day (intraperitoneal). Autopsies were performed on mice died or sacrificed, and specimens were collected from all the mice for bacteriological examination. In the case of oral administration, the heart blood was also obtained from the sacrificed mice for serological examination.

Preparation of inocula: The fimbriate (F₁) and non-fimbriate (NF₁) descendants of *S. typhimurium* isolated from the feces of a carrier dog through

mouse passage [11] were submitted to the present experiment. It was reconfirmed that the fimbriate descendant has had richly fimbriate cells (91%) by the method reported previously [11]. From the non-fimbriate descendant, a colony having no fimbriate cells was reisolated using the same manner described previously [11]. These 2 descendants were cultured in Duguid and Gillies's broth [4] at 37°C for 48 hr. Tenfold serial dilutions were made with horse meat infusion broth for each culture. Of them, an appropriate dilution (1 ml/mouse) containing 10^3 , 10^5 or 10^7 cells was given orally to a group of mice by a stomach catheter. Certain dilutions (0.5 ml/mouse) containing 10^0 – 10^4 cells were inoculated intraperitoneally to another group of mice.

Recovery of *Salmonella*: From sacrificed mice after oral administration, specimens of the liver, spleen, mesenteric lymph node, stomach, small intestine, cecum and colon were obtained. The small intestine was divided into 3 equal segments (S-I, -II and -III). After removing the contents with a pair of forceps, the gastrointestinal walls were washed thoroughly with phosphate-buffered saline (PBS, pH 7.0). One part of the liver or gastrointestinal walls was emulsified in 9 parts of PBS by a blender. Tenfold serial dilution was made on each emulsion up to 1:100,000 dilution. To count viable *Salmonella* organisms, 0.1 ml of each dilution was seeded directly on a mannit lysine crystalviolet (MLCB) agar (Nissui) plate. Four plates were used for each dilution and incubated at 37°C for 24 hr. The remaining portion of a 1:10 dilution of each specimen which took for viable count was examined qualitatively. The content of the S-I segment, cecum or colon was cultured quantitatively (MPN) and qualitatively, and the gastric content only qualitatively. The both culture methods used were exactly the same as those reported previously [12]. A small piece of the spleen or mesenteric lymph node was cultured with Hajna's tetrathionate broth (Eiken) at 37°C for 24 hr. After the enrichment culture, a loopful of the broth was streaked on MLCB agar plates and incubated at 37°C for 24 hr.

In cases of died mice after oral or intraperitoneal inoculation, the heart blood, liver, spleen, mesenteric lymph node, and contents of the small intestine (S-I segment), cecum and colon were examined qualitatively as mentioned above.

Serological tests: The sera obtained from the heart blood of sacrificed mice after oral administration were examined for fimbrial agglutinin as well as O and H agglutinins against *S. typhimurium* following the method reported previously [11].

Adherence of the fimbriate and non-fimbriate *S.*

typhimurium organisms to mouse intestinal mucosa: The terminal ileum (3 cm in length) was isolated from the sacrificed SPF mouse, and washed by shaking it thoroughly in 100 ml of PBS to remove the content adhered to mucosal surface. The washing was repeated 3 more times in new PBS. Then square of the intestinal tissue (about 10 mm×5 mm) was cut by a scalpel and pinned to a block of wood. The fimbriate and non-fimbriate organisms grown on Duguid and Gillies's broth at 37°C for 48 hr were washed 2 times with PBS by centrifugation at 3,000 rpm for 15 min, and finally, each bacterial suspension containing 10^9 cells was made with PBS. One tenth ml of the suspension was placed on the tissue pinned which held in a petri dish, and left at room temperature for 15 min. Then, the tissue was washed with PBS thoroughly. The washed tissue was fixed in 2.5% glutaraldehyde at 4°C for 2 hr and washed in PBS, and then fixed in 2% osmium tetroxide at 4°C for 1 hr. The fixative was dehydrated in 50, 70, 95, 100 and 100% acetone, and amyl acetate added to acetone (1:1) and 100% amyl acetate. It was dried in critical-point drying apparatus using CO₂ gas phase, coated with gold and examined under a scanning electron microscope.

Results

1. Multiplication of *S. typhimurium* in the digestive tracts of mice inoculated orally with the fimbriate descendant

The mice given 10^3 , or 10^5 cells of the fimbriate descendant (F_1) of *S. typhimurium* were sacrificed 1 to 14 days postadministration respectively (Table 1). None of mice given 10^3 cells died, whereas one of mice administered with 10^5 cells died of sepsis on the 6th day. In the sacrificed mice, the organism was isolated from some digestive tracts of all mice except two sacrificed on the 1st or 14th day in case of 10^3 cells, or one on the 14th day in case of 10^5 cells. As for the organs, the recovery of the organism from the mesenteric lymph node, liver or spleen was shown from the 7th day in mice inoculated with 10^3 cells, or from the 5th day in mice with 10^5 cells. Some of these organs harboring the organism had gross lesions such as enlargement, or small, white,

Table 1. Recovery of *S. typhimurium* from the gastrointestinal tract and organs of mice after oral administration with a fimbriate descendant

Dose (cells)	Days after administ- ration	No. of mice	Gastrointestinal tract												
			Wall						Content						
			Small intestine												
			St	S-III	S-II	S-I	Ce	Co	St	S-I	Ce	Co	Li	Sp	ML
10^3	1	3	0	1*	1	2	2	2	0	1	2	2	0	0	0
	3	3	0	0	1	1	3	3	0	1	2	3	0	0	0
	5	3	0	1	1	3	3	2	0	2	3	2	0	0	0
	7	3	0	0	2	3	3	2	0	2	3	3	1 (1)	1 (1)	1 (1)
	10	3	1	2	1	3	3	3	1	2	3	3	2 (1)	2 (1)	2 (1)
	14	3	0	0	0	1	1	1	0	1	0	2	2 (2)	1 (1)	0
10^5	1	5	1	3	3	4	5	4	***	0/3	1/3	3/3	3/3	0	0
	3	5	1	2	2	4	5	5	0/3	2/3	2/3	2/3	0	0	1
	5	5	1	2	3	5	5	5	0/3	1/3	2/3	2/3	2 (1)	1 (1)	2 (1)
	7	4	2	2	2	4	4	4	0/2	2/2	2/2	1/2	3 (3)	2 (2)	4 (2)
	10	3	0	1	1	2	3	2	0	2	2	2	2 (1)	1 (1)	2
	14	3	1	0	2	2	2	2	0	1	2	1	2 (2)	1 (1)	1 (1)

St, Stomach; Ce, Cecum; Co, Colon; Li, Liver; Sp, Spleen; ML, Mesenteric lymph node.

* Figure indicates the number of *Salmonella* positive case. ** Figure in parentheses indicates the number of cases having gross lesions. *** The number of *Salmonella* positive cases/the number of cases examined.

Fig. 1. Number of *S. typhimurium* (/g) in the segments of the small intestine, cecum, colon and liver of mice given 10^3 cells of a fimbriate descendant.

Number of *S. typhimurium*/g transformed into logarithm. □ The wall; ▨ The content.

+ wall; ⊕ content; △ liver: *S. typhimurium* was isolated by qualitative culture only, and the wall and liver are shown less than 10^2 /g and content 10^1 /g.

The vertical bar indicates a range of the number of organisms/g.

St, Stomach; Ce, Cecum; Co, Colon; Li, Liver.

irregular foci. The organism was recovered from all the specimens examined of the died mouse. From the 7th day, all the sacrificed mice other than two received 10^3 cells produced O, H and fimbrial agglutinins. The antibody titer ranged 1:10 to 1:40 in the O or H agglutination test, and 1:10 to 1:20 in the fimbrial test.

In mice given 10^3 cells, a small number (10^2 /g or less) of organisms was recovered from several parts of the intestinal tracts on the 1st and 3rd days (Fig. 1). The numbers in the walls of lower parts of the small intestine and upper parts of the large intestine were slightly higher than that in the contents of these tracts. On the 5th and 7th days, the small and large intestinal walls harbored the organisms in numbers of 10^2 to 10^4 /g, but the number of the organisms in the contents was 10^1 to 10^2 /g or less. On the 10th day, the multiplication of the organism in the digestive tracts became prominent (10^2 to 10^5 /g). The numbers in

the contents increased following the multiplication in the walls. On the 14th day, however, the decrease of number of the organisms was shown in the gastrointestinal tracts. The liver harbored 10^4 to 10^5 /g organisms on the 7th to 14th day.

In mice administered with 10^5 cells, the distribution pattern with number of the organisms in the digestive tracts on the 1st and 3rd days was similar to that seen on mice with 10^3 cells, but high number of the organisms was found always in the terminal segments (S-I) of the small intestine and upper parts of the large intestine, especially in the walls (Fig. 2). On the 5th and 7th days, significant evidence was observed for the multiplication of the organism in the digestive tracts (10^1 to 10^6 /g), but the numbers in the walls and contents of the tracts rather decreased to 10^1 to 10^4 /g on the 10th and 14th days. As for the liver, 10^4 to 10^6 /g organisms were recovered 5 to 14 days.

Of 18 mice given 10^7 cells (not illustrated),

Fig. 2. Number of *S. typhimurium* (/g) in the segments of the small intestine, cecum, colon and liver of mice given 10^5 cells of a fimbriate descendant.

See the remarks on Fig. 1.

Table 2. Recovery of *S. typhimurium* from the gastrointestinal tract and organs of mice after oral administration with a non-fimbriate descendant

Dose (cells)	Days after administ- ration	No. of mice	Gastrointestinal tract												
			Wall						Content						
			Small intestine				Ce	Co	St	S-I	Ce	Co	Li	Sp	ML
			St	S-III	S-II	S-I									
10 ⁵	1	5	0	0	1	1	2	2	0	1	1	3	0	0	0
	3	5	0	0	0	0	1	1	0	1	1	1	0	0	0
	5	5	0	0	0	0	0	0	0	1	1	1	0	0	0
	7	5	0	0	0	0	0	0	0	1	1	1	0	0	1
	10	3	0	0	0	0	0	0	0	0	0	0	0	0	0
	14	3	0	0	0	0	0	0	0	0	0	0	0	0	0
10 ⁷	1	3	0	0	1	2	3	2	0	2	3	3	0	0	0
	3	3	0	0	0	1	1	0	0	1	2	2	0	0	0
	5	3	0	0	0	0	1	0	0	0	3	3	0	0	0
	7	3	0	0	1	1	1	1	0	1	2	1	1 (1)	0	0
	10	3	0	0	0	0	0	0	0	0	1	1	0	0	0
	14	3	0	0	0	0	0	0	0	0	0	0	0	0	0

See the remarks on Table 1.

7 animals died of sepsis on the 6th to 13th day after administration. In the mice sacrificed, the multiplication of the organism in the walls between the terminal segments (S-I) of the small intestine and upper parts of the large intestine was found already on the 3rd day. On the 7th or 10th day, very high number (10^4 to 10^7 /g) of the organisms was recovered from the gastrointestinal tracts. The liver yielded 10^5 to 10^7 /g organisms on the 5th to 10th day. Bacterial findings, and pathological and serological results on these sacrificed mice were almost the same to those on mice with 10^5 cells or more significant.

2. Multiplication of *S. typhimurium* in the digestive tracts of mice inoculated orally with the non-fimbriate descendant

Mice were given orally 10^3 , 10^5 or 10^7 cells of the non-fimbriate descendant (NF₁) and examined bacteriologically, pathologically and serologically between 1 and 14 days postadministration. None of the mice

died during the period in each case of inoculating dose.

In case of 18 mice given 10^3 cells (not illustrated), all the mice except one gave negative results in all the examinations. Only one mouse harbored a small amount of the organisms (less than 10^2 /g) in the cecum and colon on the 3rd day after administration.

In mice given 10^5 or 10^7 cells, the organism was isolated from the intestinal walls and contents of the sacrificed mice on the 1st to 10th day (Table 2). However, no organism was recovered from the digestive tracts after 7 days (10^5 cells) or 14 days (10^7 cells). A total of 44 mice inoculated with 10^5 or 10^7 cells, only each one mouse on the 7th day respectively was found to harbor the organism in the mesenteric lymph node, or liver showing gross lesions. Three animals including these two produced antibody. The O and H antibody titers ranged 1:10 to 1:20, while none of them showed fimbrial agglutinin titer.

On the 1st to 3rd days, a small number

Fig. 3. Number of *S. typhimurium* (/g) in the segments of the small intestine, cecum, colon and liver of mice given 10^5 cells of a non-fimbriate descendant.

See the remarks on Fig. 1.

Fig. 4. Number of *S. typhimurium* (/g) in the segments of the small intestine, cecum, colon and liver of mice given 10^7 cells of a non-fimbriate descendant.

See the remarks on Fig. 1.

of organisms (10^2 /g or less) was recovered from the intestinal walls and contents of mice given 10^5 or 10^7 cells (Figs. 3, 4), but thereafter, the numbers decreased gradually up to less than 10^2 /g by the 7th day (10^5

cells) or 10th day (10^7 cells). Only one case inoculated with 10^7 cells showed relatively high numbers in the tracts (10^1 to 10^3 /g) or liver (10^4 /g) on the 7th day.

3. Infectivity of the fimbriate and non-fimbriate descendants of *S. typhimurium* to mice in the intraperitoneal inoculation

Mice were inoculated intraperitoneally with 10^0 to 10^4 cells of the fimbriate and non-fimbriate descendants. Fifty percent lethal dose (LD_{50}) calculated by the method described in the previous report [11] was 1.1 in case of the fimbriate descendant and 1.3 in case of the non-fimbriate descendant. There was no significant difference in LD_{50} value between the two descendants. The infectivity between the fimbriate (F_1) and non-fimbriate (NF_1) descendants in the intraperitoneal inoculation was almost the same as obtained in the previous paper [11].

4. Scanning electron microscopic observation on adherence of the fimbriate and non-fimbriate organisms to the intestinal mucosa

In the intestinal specimen washed thoroughly with PBS, no bacteria were observed on the epithelial surface of the mucosa before the bacterial suspension was placed on it (Fig. 5).

When the fimbriate bacterial suspension was put on the intestinal specimen, numbers of bacteria were found to adhere to the apical or lateral region of villi (Figs. 6, 7). The adherent bacteria were rod-shaped and dimension of them was about $0.5\ \mu\text{m}$ in width and 1.0 to $2.0\ \mu\text{m}$ in length. While the specimen was also treated with the non-fimbriate bacterial suspension, no bacteria were observed on the epithelial surface.

Discussion

In the current studies, it has been reported by several workers [1, 5–10] that fimbrial antigens of enterotoxigenic *E. coli* play a role in intestinal colonization of the organism in the swine (K88), calf

(K99) or human (CFA/I and CFA/II), which are morphologically similar to each other, but antigenically and functionally different. As for *Salmonella* organisms, it was suggested by the experiment on mice that the fimbriae of *S. typhimurium* might somewhat promote the intestinal infection by facilitating adhesion of the organism to the mucosal surface [2, 4], but Tannock et al. [13] reported that such surface structures of *S. typhimurium* as fimbriae, O antigen and flagella seemed not to be essential for association with mucosal surface of mouse ileum. In the previous experiment on mice, the authors [11] obtained a result suggesting that the fimbriae of *S. typhimurium* might be essential for the establishment of intestinal infection. Nevertheless, some factor such as O antigen was suspected to be participated in the overall infectivity of the organism to mice. In this paper, an attempt was made to study whether the fimbriae served actually as a role such as the maintenance of intestinal colonization or not.

In case of oral administration of a fimbriate descendant of *S. typhimurium*, *Salmonella* organisms localized in between middle to lowest parts of the small intestine and large intestine, especially in the walls at early stages (1 to 3 days). Thereafter, the multiplication of the organism in the tracts became more significant. The organism reached already the mesenteric lymph node, or liver and spleen, and these organs produced gross lesions. On the 6th day or more, one to 7 mice died of sepsis. Almost all the mice showed antibodies against *S. typhimurium* from the 7th day after administration. With the increase of inoculating doses, remarkable multiplication of the organism in the digestive tracts was proved, and the number of infected or died mice increased. On the other hand, in case of oral administration of a non-fimbriate

descendant, it was demonstrated that the intestinal tracts harbored a small number of organisms in the earlier stage, but the organism disappeared gradually with the lapse of time even though given the highest number (10^7 cells). None of mice died regardless of inoculating doses. Accordingly, mice given orally the fimbriate descendant were shown to become infected easily with colonization of the organism in the intestinal tracts and the organism invaded into the organs following the multiplication in the tracts, while in mice received the non-fimbriate descendant, no evidence was shown to prove the colonization of the organism in the digestive tracts and almost all the animals were found not to be infected. However, there were still a few exceptional cases that showed O and H antibody levels with or without recovery of organism or gross lesions in case of oral administration with a large number (10^5 to 10^7 cells) of the non-fimbriate descendant, but possessed no fimbrial antibody.

By scanning electron microscopic observation, numbers of *S. typhimurium* organisms adhered to the surface of villi of the isolated ileum were seen in case of the fimbriate descendant, while no organisms were found on the epithelial surface of the tract in case of the non-fimbriate descendant.

On the contrary to these findings, no significant difference in the infectivity was recognized between the fimbriate and non-fimbriate descendants when they were inoculated intraperitoneally into mice. From the results obtained, it seemed, therefore, that the difference observed in the infectivity between the two descendants in the oral administration depended upon whether the organism colonized in the mucosa of the intestinal tracts at initial stage of infection or not, and the fimbriae partici-

pated in the intestinal colonization by promoting adhesion of the organism to the mucosa.

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要 約

Salmonella typhimurium の線毛とそのマウス腸管定着における役割: 田中 饒・勝部泰次・武藤 健・今泉 清 (国立予防衛生研究所獣疫部)——*S. typhimurium* 線毛株の $10^3 \sim 10^7$ 個をマウスに経口投与した場合, 本菌は投与後 1~3 日目に小腸の中部および下部, ならびに大腸, 特にその壁に定着した。その後, 腸管においては投与菌の明確な増殖が認められた。そして, 投与菌量の増加に伴ない, 菌の増殖は顕著であった。菌投与後 3~5 日目から, 腸管膜リンパ節, 肝および脾から菌が回収され, これらの臓器に肉眼病変が形成された例がみられた。肝では, 菌投与 5~14 日の間に $10^2 \sim 10^7/\text{g}$ の菌が回収された。 10^5 個菌投与マウスでは, 菌投与後 6 日目に 1 頭が, 10^7 個菌投与マウスでは, 菌投与後 6~13 日の間に 7 頭が敗血症死した。菌投与 7 日目から, マウスでは投与菌に対する O, H および線毛抗体が検出された。一方, 非線毛株をマウスに投与した場合, その腸管における菌の定着は認められなかった。しかし, 例外的に $10^5 \sim 10^7$ 個菌を投与された少数のマウスでは, 接種菌に対する O および H 抗体の産生がみられた。これに対し, 線毛および非線毛株をマウス腹腔内に接種したところ, 両者共に前報におけるものとほぼ同様な LD_{50} 値を示した。In vitro の実験で, 供試線毛菌はマウスの腸管粘膜の絨毛の表層に多数付着している所見が走査電顕によって観察された。一方, 非線毛菌による実験では粘膜表層部に菌を検出することが出来なかった。以上の成績は, 投与菌が腸管粘膜に定着する上に, その線毛が役割を演じていることを示唆しているものと思われる。

Explanation of Figures

Fig. 5. Villi from the terminal ileum (segment-1) of a normal mouse. Epithelial surface is apparently free of bacteria (Scanning electron micrograph. $\times 410$).

Fig. 6. Bacterial adherence of a fimbriate descendant (F_1) of *S. typhimurium* to the apical region of villus of a mouse ileum (segment-1).

(Scanning electron micrograph. $\times 2,300$).

Fig. 7. Bacterial adherence of a fimbriate descendant (F_1) of *S. typhimurium* to the lateral region of villus of a mouse ileum (segment-1). Rod-shaped morphology of adherent bacteria can be seen (Scanning electron micrograph. $\times 4,200$).