Yersinia pseudotuberculosis のV抗原の役割:

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The Role of V Antigen in Mice Experimentally Infected with *Yersinia pseudotuberculosis*; Histopathological Study Using Enzyme Immunoassay

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ABSTRACT. A histopathological study using enzyme immunoassay (EIA) was carried out in mice experimentally infected with *Yersinia pseudotuberculosis* IB to elucidate the role of V antigen *in vivo*. The presence of V antigen and bacterial cells in the lesions was revealed in various organs by EIA using anti-V antigen serum and anti-O antigen serum. There was a distinct difference in the degree of V antigen production among the organs examined. V antigen was massively produced in the liver, spleen and mesenteric lymph nodes associated with lesions and bacteria. In the intestine, however, V antigen was weakly produced in the lesions under the intestinal epithelium despite the massive presence of a bacterial colony. The detailed staining pattern suggested that V antigen might be formed surrounding the bacterial cells in the lesions of the liver, spleen and mesenteric lymph nodes. *Yersinia pseudotuberculosis* IB mutant lacking in both VW antigen and plasmid did not cause lesions in any organs. In addition, fecal excretion of the organisms was closely associated with the formation of lesions in the intestine.—KEY WORDS: enzyme immunoassay (EIA), pathogenicity, V antigen, *Yersinia*.

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Pathogenicity of *Yersinia* was reported to be associated with different kinds of virulence factors [20]. These factors included enterotoxin production [18], VW antigen production [1], pili formation [11, 12], presence of plasmid [6], exogenous pigmentation [16, 17] and intracellular parasitism in macrophages [4, 19]. These factors exist independently or are associated closely together. Among these factors, VW antigen associated with about 42- to 48-M dalton plasmid recently has attracted much attention as an important one. Lawton *et al.* [13] first reported the purification of VW antigen and demonstrated that V antigen was the protein and W antigen was the lipoprotein. Recent studies have clarified the biological roles of VW antigen as a virulence factor. Avirulent *Yersinia* organism lacking VW antigen could survive and remain in mice when the mice were previously inoculated with V antigen [24]. Immunization with VW antigen provided protection in mice against homologous as well as heterologous challenge of virulent organisms [24, 25]. However, little is known about the actual role of VW antigen during the course of *Yersinia* infection *in vivo*. A histopathological approach may be essential to answer this question.

This study was undertaken to show histologically how V antigen acts as a virulence factor during the course of *Yersinia* infection in mice. EIA was employed to locate bacteria and V antigen in the lesions, which wre separately stained with anti-O or anti-V antigen serum.

MATERIALS AND METHODS

Bacteria: *Yersinia pseudotuberculosis* IB (the organism isolated from brown rat [8])

and IB mutant were used in this study. *Y. pseudotuberculosis* IB mutant was selected from the virulent strain of parent IB in magnesium oxalate agar as Ca^{2+}independent and plasmid-lacking mutant [7]. To prepare the working stock of each strain, the organisms were inoculated into Trypticase Soy Broth (BBL) and incubated aerobically at 25°C for 48 hr. After the incubation, cultured fluid was centrifugated at 4,000 rpm for 20 min. Then the bacterial pellet was suspended in a solution containing 5% lactose and 50% calf serum in double distilled water. The organisms were kept frozen at −80°C prior to their use.

Sera: Anti-O antigen serum was prepared in a rabbit by 10 successive inoculations of heat-treated *Y. pseudotuberculosis* IB. Anti-V antigen serum was also prepared in a rabbit by inoculations of partially purified V antigen. The V antigen for immunization was prepared as follows: The organisms carrying the plasmid were cultivated in the Ca^{2+} reduced medium [13] at 36°C for 36 hr, and removed from the cultured medium by centrifugation and filtration. This cultured medium was added to solid ammonium sulfate at a final concentration of 3.0 M. The precipitated proteins were collected by centrifugation, resuspended in phosphate-buffered saline (PBS) to a 100-fold concentration of the original, dialyzed overnight against PBS and used for immunization. The serum was absorbed with formalin-killed organisms lacking the plasmid (IB mutant) and used as the anti-V antigen serum.

Experimental infection: The bacteria were suspended with PBS and were adjusted to the concentration of 10^8 colony forming units/m*l*, respectively. Eight-week-old mice (C57BL/6) were used for the experimental infection. They were injected with 0.1 m*l* of bacterial suspension per the oral (p.o.) route by the aid of gastric catheterization [9, 22]. Then immune mice were prepared by teh oral and intraperitoneal (i.p.) administrations of formalin-killed organisms [10, 21]. The immune mice prepared via the oral route were challenged intraperitoneally with 0.1 m*l* of bacterial suspension, and the immune mice prepared via the i.p. route were challenged via the p.o. route. And then the feces of the challenged mice were collected and examined for the presence of organisms. Feces were suspended in physiological saline at concentrations of 10%. Trypticase Soy Agar (BBL) and McConkey Agar (Eiken) plates were used as the tive medium. Identification of the colonies grown on the plates was done by the slide agglutination test using antiserum against O antigen of *Yersinia pseudotuberculosis* IB.

Preparation of tissue section: At optimal intervals after the inoculation, three mice were sacrificed and fixed with paraformaldehyde-lysine-phosphate (PLP) buffered solution by the cardiac perfusion method. Then the intestine, spleen, liver and mesenteric lymph nodes from the infected mouse were removed and immersed in PLP solution. After two days, the organs were dehydrated with alcohol and embedded in paraffin. The paraffin blocks were sliced with a microtome to 4 μm in thickness. The sections were placed on a slide glass and kept at 4°C until their use.

Staining by enzyme immunoassay (EIA): One of the sections on the slide glass was stained with hematoxyline eosin method and the other was stained by EIA. EIA was performed as follows. i) The section was treated with methanol and 0.1% hydrogen peroxide to reduce the nonspecific staining and then rinsed three times with PBS. ii) The specimen was mounted with antiserum against O antigen or V antigen with optimal dilution and was incubated at 37°C for 1 hr. After incubation, the specimen was rinsed three times with PBS. iii) The specimen was mounted with horseradish peroxidase labeled anti-rabbit IgG goat serum (Cap-

Table 1. Occurence and degree of lesions in mice produced by *Yersinia pseudotuberculosis* IB parent and IB mutant without virulence plasmid

Inoculated strain	organ	Feature of lesion[b]	Degree of lesions after challenge[a]								
			Hours						Days		
			6	12	24	48	72	96	5	7	14
Y. pseudo-tuberculosis IB (plasmid$^+$)	Small intestine	1	−	−	−	+++	+++	+++	+++	+++	+++
		2	−	−	−	−	−	++	+++	+++	+++
		3	−	−	+	+++	+++	+++	+++	+++	+++
	Cecum	1	−	−	−	++	+++	+++	+++	+++	+++
		2	−	−	−	−	−	−	++	+++	+++
		3	−	−	−	++	+++	+++	+++	+++	+++
	Large intestine	1	−	−	−	−	+++	+++	+++	+++	+++
		2	−	−	−	−	−	−	++	+++	+++
		3	−	−	−	−	+++	+++	+++	+++	+++
	Liver	1	−	+	++	+++	+++	+++	+++	+++	+++
		2	−	−	−	−	−	++	+++	+++	+++
		3	−	−	−	−	−	−	+++	+++	+++
	Spleen	1	−	−	+	+++	+++	+++	+++	+++	+++
		2	−	−	−	−	−	−	+++	+++	+++
		3	−	−	−	−	−	−	++	+++	+++
	Mesenteric lymph nodes	1	−	−	−	−	++	+++	+++	+++	+++
		2	−	−	−	−	−	−	++	+++	+++
		3	−	−	−	−	−	−	+	+++	+++
Fecal excretion of organisms			+++	+++	+++	+++	+++	+++	+++	+++	+++
IB mutant (plasmid$^-$)	Small-large intestines	1	−	−	−	−	−	−	−	−	−
		2	−	−	−	−	−	−	−	−	−
		3	−	−	−	−	−	−	−	−	−
	Liver, Spleen Mesenteric lymph nodes	1	−	−	−	−	−	−	−	−	−
		2	−	−	−	−	−	−	−	−	−
		3	−	−	−	−	−	−	−	−	−
Fecal excretion of organisms			+++	+++	+++	+	−	−	−	−	−

a) The number of positive mice having lesions or fecal excretion per three mice; +++: positive in all 3 mice, ++: positive in 2 mice, +: positive in 1 mouse, −: negative in all 3 mice.
b) Feature of lesion was indicated as 1; inflammation, 2; necrosis and 3; bacterial colonization.

pel). It was incubated at 37°C for 1 hr and then rinsed 3 times with PBS. iv) This specimen was immersed in a substrate solution composed of 10 mg of 3,3 diaminobenzidine and 10 μl of 30% H_2O_2 in 100 m*l* of 10 mM tris-HCl buffer (pH 7.4). Following incubation for 5 min at room temperature, the specimen was rinsed with distilled water and then stained with hematoxylin for 2 min to give contrast. v) After the specimen was rinsed in water for more than 30 min, it was dehydrated with alcohol and embedded with Entellan (Merck). The specimen was then observed under a microscope.

RESULTS

Correlation between the degree of lesions and the presence of virulence plasmid: To observe the correlation between the degree of the lesions and the presence of virulence plasmid in *Yersinia pseudotuberculosis*, both strains of IB parent and IB mutant, lacking virulence plasmid, were inoculated into mice through the oral route. The mice were

Table 2. Effect of immunization route on the formation of the lesions and the prevention of the fecal excretion of organisms

Organ	Feature of lesion[c]	Degree of lesions in mice[a]															
		Days after IP challenge[b]								Days after PO challenge[b]							
		PO immune[b]				Non-immune				IP immune[b]				Non-immune			
		1	3	5	7	1	3	5	7	1	3	5	7	1	3	5	7
Small intestine	1	−	−	−	−	−	+++	+++	+++	−	−	−	+++	−	+++	+++	+++
	2	−	−	−	−	−	−	−	+++	−	−	++	+++	−	−	+++	+++
	3	−	−	−	−	+	+++	+++	+++	−	+	−	+++	+	+++	+++	+++
Cecum	1	−	−	−	−	−	+++	+++	+++	−	−	++	+++	−	+++	+++	+++
	2	−	−	−	−	−	−	−	+++	−	−	−	+++	−	−	++	+++
	3	−	−	−	−	−	+++	+++	+++	−	−	++	+++	−	+++	+++	+++
Large intestine	1	−	−	−	−	−	+++	+++	+++	−	−	−	+++	−	+++	+++	+++
	2	−	−	−	−	−	−	+	+++	−	−	−	+++	−	−	++	+++
	3	−	−	−	−	−	+++	+++	+++	−	−	−	+++	−	+++	+++	+++
Liver	1	−	++	++	++	+	+++	+++	+++	−	−	++	+++	++	+++	+++	+++
	2	−	−	−	++	−	−	+++	+++	−	−	−	+++	−	−	+++	+++
	3	−	−	−	++	−	−	+++	+++	−	−	−	+++	−	−	++	+++
Spleen	1	−	+	++	++	−	+++	+++	+++	−	−	−	+++	+	+++	+++	+++
	2	−	−	−	++	−	−	+++	+++	−	−	−	+++	−	−	+++	+++
	3	−	−	−	++	−	−	+++	+++	−	−	−	+++	−	−	++	+++
Mesenteric lymph nodes	1	−	+	+	−	−	+++	+++	+++	−	−	++	+++	−	+++	+++	+++
	2	−	−	−	−	−	−	++	+++	−	−	−	+++	−	−	++	+++
	3	−	−	−	−	−	−	−	+++	−	−	−	+++	−	−	−	+++
Fecal excretion of organisms		−	−	−	−	+	+++	+++	+++	++	+	++	+++	+++	+++	+++	+++

a) Indications were the same as in Table 1; +++: positive in all 3 mice, ++: positive in 2 mice, +: positive in 1 mouse, −: negative in all 3 mice.

b) PO indicated per oral and IP indicated intraperitoneal.

c) Feature of lesion was indicated as 1; inflammation, 2; necorsis and 3; bacterial colonization.

sacrificed at intervals and lesions were examined in tissue sections from various organs under a microscope. Fecal excretion of the organisms was also monitored at intervals. One of the typical features of lesions at later period of infection was shown in Fig. 1 (A, B, E, F) which represents the spleen and the large intestine of mice 14 days after oral infection of IB parent strain. The lesions in these two organs at 14 days were essentially the same and showed the massive necrosis with the bacterial colonization and the inflammatory change with infiltration of neutrophil. The infiltration of neutrophil seemed to be stronger in spleen than in small intestine. The features and degrees of lesions in various organs were different during early course of infection and were expressed as the rate of appearnce of inflammation, necrosis and bacterial colonization in three mice (Table 1). After administration of the virulent strain, the inflammation characterized by infiltration of neutrophils was first detected in the liver at 12 hr post infection in one out of three mice and at 24 hr post infection in two out of three mice. At 24 hr, the inflammation was also detected in the spleen in one out of three mice and at 48 hr in the small intestine, cecum and spleen in most of the mice. At 72 hr, the large intestine and mesenteric lymph nodes also showed the inflammatory changes. The necrosis in the intestines appeared later (4 to 5

days post infection) than the inflammatory chanages. The bacterial colonization first appeared in the small intestine almost at the same time (24 to 48 hr) of the onset of the inflammatory change. In the cecum and large intestine, the bacterial colonization on the inflammatory change appeared also at the same time. The bacterial colonization was seen under intestinal epithelium with edema and led to the necrosis and ulceration. The necrosis was also observed at Payer's patch. In the liver, spleen and mesenteric lymph nodes, however, the bacterial colonization were detected later than in the intestines, almost at the same time of the onset of the necrosis. Fecal excrtetion of the organisms was observed in all three mice during the observation period. However, when the IB mutant strain lacking the plasmid was administrated, no lesions were detected in any tissue and the organisms were recovered in the feces only until 48 hr of post administration. These results support the previous observation that the lesions formed by *Y. pseudotuberculosis* are closely associated with the presence of the virulence plasmid. They may also indicate that the liver is the organ where the lesions characterized by the inflammatory change are first formed after oral infection of *Yersinia.*

Effects of the immunization route on the formation of the lesions and prevention of fecal excretion of organisms: Our previous study showed that oral immunization of mice with formalin-killed *Yersinia* prevented the continuous fecal excretion of the organisms after oral challenge [10, 21]. To see which organ or organs play a vital role in this phenomenon, mice were immunized with the p.o. or i.p. administration of formalin-killed organisms and challenged by the i.p. or p.o. routes, respectively (Table 2). When mice were immunized via the p.o. route and challenged afterward via the i.p. route, any of the lesions such as inflammation, necrosis and bacterial colonization were not observed in the small intestine, cecum or large intestine. At the same time, the fecal excretion of organisms was also prevented. However, the lesions such as inflammation, necrosis and bacterial colonization were detected in the liver, spleen and mesenteric lymph nodes. The control experiment using non-immune mice challenged via the i.p. route showed that the lesions existed in all the organs tested and that prevention of the fecal excretion of the organisms did not occur. The i.p. route of immunization did not prevent the fecal excretion of the organisms, even though the formation of the lesions in the liver, spleen and mesenteric lymph nodes was delayed as compared with that in the control non-immune mice. These results indicated that the absence of the lesions in the intestines from the mice immunized via p.o. route correlated well with the inhibition of the fecal excretion of organisms even in the presence of lesions in other organs, and that only the p.o. route of immunization could provide protection against lesion formation in the intestine.

The distribution of O and V antigens in organs infected with virulent IB parent strain: To elucidate the distribution pattern of the organisms in the organs and the role of V antigen during the pathologic process, sereal sections of various tissues were examined by both methods, hematoxylin-eosin staining and EIA, using antisera against O and V antigen, respectively. Typical results obtained in the intestine and spleen were shown in Fig. 1. The lesions in the intestine observed by EIA were strongly stained with anti-O antigen serum (Fig. 1, C) but weakly stained with anti-V antigen serum (Fig. 1, D), and the same was true in the cecum and large intestine. In contrast, the lesions in the spleen were weakly stained with anti-O antigen serum (Fig. 1, G) but strongly stained with anti-V antigen serum

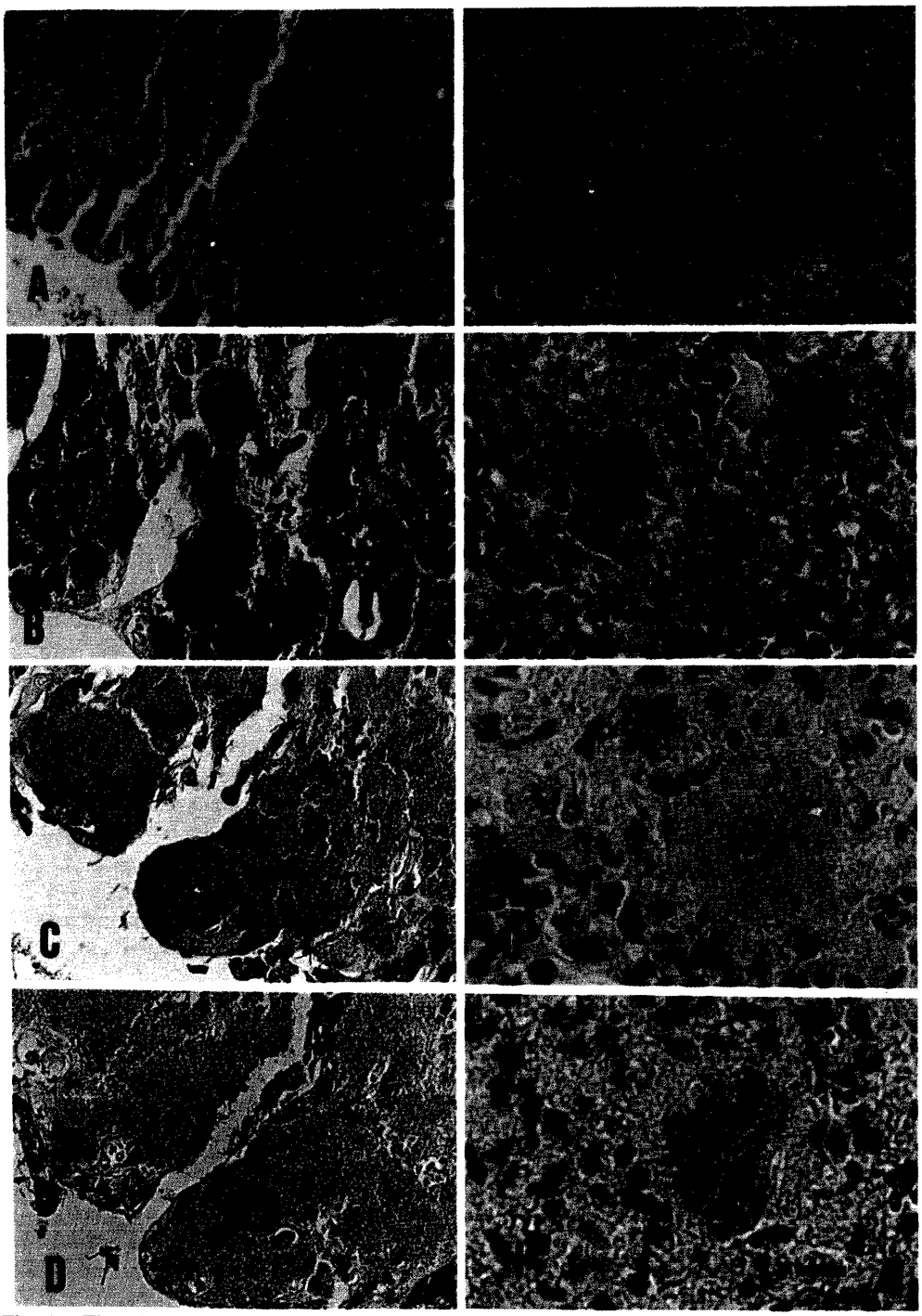

Fig. 1. The micrographs show the large intestine (A, B, C, D) and spleen (E, F, G, H) of a mouse at 14 days after challenge of *Y. pseudotuberculosis* IB per the oral route. The tissue sections were stained by the hematoxylin-eosin technique (A, B, E, F) and by the EIA technique using anti-O antigen serum (C, G) or anti-V antigen serum (D, H). The tissue sections applied to EIA were counter-stained with hematoxylin. Their magnifications were as follows: (A, E); ×85, (B, F); ×340, (C, D); ×340 and (G, H); ×680. Scale: 10 μm.

(Fig. 1, H) by EIA, and the same was true in the liver and the mesenteric lymph nodes. The staining pattern of the intestine with anti-O antigen serum showed fine granules; however, the staining pattern of the spleen, liver and mesenteric lymph nodes with anti-V antigen serum showed coarse granules and sometimes a ring formation with an empty spot inside (Fig. 1, H). The granules stained with anti-V antigen serum had a larger diameter than those with anti-O antigen serum in the spleen, liver and mesenteric lymph nodes. These results showed that V antigen was weakly produced in the intestines despite the intensive growth of the organisms, but produced massively in the spleen, liver and mesenteric lymph nodes during the course of *Yersinia pseudotuberculosis* infection in the mice.

DISCUSSION

In this study the roles of plasmid and plasmid-associated V antigen were clarified by analysing the prevalence of lesions, bacteria and V antigen in the organs examined during the pathologic process of *Yersinia pseudotuberculosis* infection in mice. The most important finding was that V antigen was well formed in the lesions in the liver, spleen and mesenteric lymph nodes. By using EIA with anti-O antigen serum and anti-V antigen serum, the bacteria and V antigen could be observed in tissue sections from various organs under a microscope. The lesions in the liver, spleen and mesenteric lymph nodes were strongly stained with anti-V antigen serum and weakly stained with anti-O antigen serum. Histopathologically, V antigen of *Yersinia pseudotuberculosis* was actually produced *in vivo* and associated with the lesion and the organisms in *Yersinia pseudotuberculosis* infection in mice.

Detailed histological observation revealed that, in the liver, spleen and mesenteric lymph nodes, the particles stained with anti-V antigen serum had a larger diameter than those stained with anti-O antigen serum. Furthermore, the particles in these organs stained with anit-V antigen serum sometimes showed a ring shape with an empty space inside, as if the bacteria were surrounded by V antigen. When tissue sections of the liver, spleen and meseteric lymph nodes were treated with trypsin and stained with sera, anti-O antigen serum gave a stronger staining reaction in the lesions and anti-V antigen serum gave a weaker reaction as compared with trypsin non-treated sections (Sugiyama, unpublished data 1985). Considering the protein nature of V antigen and the lipopolysaccharide nature of O antigen, these results further suggest the above mentioned assumption that the bacteria were surrounded by V antigen. Concerning the biological role of V antigen, Une and Brubaker [24] demonstrated that avirulent strain lacking V antigen could survive and remain in mice previously treated with V antigen. Wake *et al.* [25] and Une and Brubaker [24] reported that VW antigen used as vaccine in mice induced protection against the challenge of *Yersinia* spp.. These works in combination with our histopathological findings may lead to the common assumption that the bacteria were covered with V antigen in order to resist host-defense mechanisms, to survive and to cause the lesions in the organs such as the liver, spleen and mesenteric lymph nodes.

Factors to facilitate the production of V antigen *in vivo* were not clearly identified but they might be related to various host-defense mechanisms which inhibited the growth of bacteria. In the other hand, it was speculated that V antigen might not be produced in the condition which allowed the abundant bacterial growth *in vivo*. V antigen was strongly produced in the liver, spleen and mesenteric lymph nodes in which

the bacterial growth was delayed and strong inflammatory reaction characterized by neutrophil infiltration was observed. In contrast, V antigen was not much produced in intestines in which strong bacterial colonization and weak inflammatory reaction were seen. Production of V antigen *in vitro* was known to be associated with the poor conditions for bacterial growth such as low concentration of Ca^{2+} and to occur against a bactericidal action of macrophages in tissue culture.

Plasmid carrying organisms could produce the lesions and led to the fecal excretion of the organisms. Plasmid lacking mutant did not produce any lesions in the organs and also did not show continuous fecal excretion of organisms. These observations agreed with those of other studies [2, 3, 5, 15, 23]. In addition to these, Nakajima *et al.* [14] also showed that the subcutaneous route of immunization was not effective for the protection of fecal excretion of the organisms. When the animal was immunized via the p.o. route and challenged with the organisms via the i.p. route, no lesions were observed in the intestine and no fecal excretion of the organisms was detected even if lesions were formed in the liver, spleen and mesenteric lymph nodes. The p.o. route of immunization was essential to prevent the fecal excretion of the organisms, whereas the i.p. route of immunization was not effective to do so. By the EIA technique using anti-O antigen serum, there is an evidence that the massive presence of bacteria under the intestinal epithelium of the mouse caused the shedding of organisms into feces.

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Yersinia pseudotuberculosis のV抗原の役割：実験感染マウスモデルにおける酵素抗体法を用いた病理組織学的研究：杉山芳宏・金子賢一[1)]・高島郁夫・橋本信夫（北海道大学獣医学部獣医公衆衛生学講座，[1)]東京農工大学農学部獣医学科家畜衛生学講座）——*Yersinia* 属菌の病原性解析の一環としてV抗原の生体内における役割を，酵素抗体法を用いて病理組織学的解析した。*Yersinia pseudotuberculosis* 感染マウスの脾臓，肝臓および腸間膜リンパ節において，V抗原は菌体を包む様に存在した。一方，本菌は腸管でよく増殖してコロニーを形成したが，V抗原はほとんど産生されていなかった。本抗原は宿主の感染防御に抵抗して菌体を保護すると推測され，本菌が容易に増殖できる環境ではV抗原は産生されないと考えられた。また，*Yersinia* 属菌の腸管定着現象には，plasmid$^+$ 菌による腸管病変の形成が関連すると判断された。