



A study on the efficacy of the recombinant *Yersinia* adhesin A vaccine against yersiniosis in the early phase

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ABSTRACT. *Yersinia pseudotuberculosis* (*Y. ptb*) is a zoonotic pathogenic bacterial species of the family Enterobacteriaceae and causes yersiniosis, an acute intestinal infection in humans and animals. *Y. ptb* is often implicated in lethal epidemics in zoo animals and reductions in the breeding population, but a valid prevention method has not been established. Therefore, this study aimed to develop a vaccine for yersiniosis control. The immunogenicity of one of the adhesion factors involved in pathogenic mechanisms of *Y. ptb*, *Yersinia* adhesin A (YadA), was investigated. BALB/c mice were divided into 3 groups: in group 1, mice received insoluble recombinant YadA (rYadA) produced in genetically engineered *Escherichia coli* (100 µg/dose); in group 2, mice received inactivated *Y. ptb* with strong expression of YadA (20 mg/dose); and in group 3, mice received phosphate-buffered saline (0.2 ml/dose). All interventions were administered subcutaneously twice at an interval of 1 week. One week after the second administration, *Y. ptb* (10⁷ cells/mouse) was inoculated orally. As a result, the survival rate was 100% in group 1, 60% in group 2, and 0% in group 3. The anti-YadA antibody titer increased in a stepwise fashion in groups 1 and 2. The present study results suggest that rYadA shows promise as a protective antigen against yersiniosis. This study concluded that vaccination against *Y. ptb* may become available as a new method to prevent lethal epidemics in animals.

KEY WORDS: recombinant, vaccine, *Yersinia* adhesin A, *Yersinia pseudotuberculosis*

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Yersiniosis is a zoonotic disease caused by *Yersinia pseudotuberculosis* (*Y. ptb*), which occurs sporadically or epidemically in many parts of the world [14, 15, 25]. *Y. ptb* is mainly transmitted via the oral route and causes severe acute intestinal infection, frequently accompanied by sepsis. In addition, *Y. ptb* forms lesions that resemble tuberculosis nodules in the liver and spleen [4].

Y. ptb affects various animal species and fatal cases have been reported in primates, birds, bats and rodents, as well as intensively farmed animals [4, 13, 28, 37, 39]. In particular, monkeys are highly susceptible to *Y. ptb* infection. In Japan, lethal epidemics have been reported in many species of monkeys in close proximity to apes and prosimians in exhibition facilities [40]. In these exhibition facilities, preventative measures against *Y. ptb* infection, including hygiene control and administration of antibiotics to carrier animals, have not been able to eliminate the epidemics. This situation represents a major hindrance to species preservation, including rare species. Under these circumstances, vaccination may be an effective preventative measure, but an effective vaccine has not yet been developed [14, 20, 25]. There are 3 *Yersinia* spp. that are pathogenic: *Y. pestis*, *Y. enterocolitica* and *Y. ptb*. Vaccines have been developed against *Y. pestis*, the cause of plague; although they have been used to reduce the risk of infection in endemic areas [1], the protective effects are inadequate [1, 12]. Therefore, *Y. ptb* has been studied as an immunogen for *Y. pestis* vaccine development [3, 10]. In addition, in New Zealand, an inactivated vaccine against *Y. ptb* is commercially available for intensively farmed animals, such as deer and cattle [26]. However, this vaccine is not available in Japan. Further, our preliminary trials using inactivated *Y. ptb* and inactivated *Y. ptb* with strong expression of *Yersinia* adhesin A (YadA) as immunogens in mice yielded no effect with the former vaccine and only a slight effect with the latter (private communication).

YadA is an extracellular membrane protein monomer with a molecular weight of 41–44 kilodaltons (kDa) and is encoded by the virulence plasmid (pYV). YadA is usually present as a trimer on the surface of the bacterium and has a so-called lollipop-shaped structure [16, 24, 29, 32, 36]. YadA has a variety of functions, such as inhibition of phagocytosis by macrophages, adhesion to intestinal epithelial cells and resistance to the bactericidal action of phagocytic cells [2, 11]. Therefore, we selected YadA as a possible immunogen for a vaccine against *Y. ptb*.

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Table 1. The primer of *yadA* for transformation in *E. coli* strain BL21

Strain		Primer sequence (5'–3')	
PS	Insert	<i>yadA</i>	F GGAATTCATGACTAAAGATTTTAAG
			R CCGCTCGAGTTACCACTCGATATTAATAA
	Vector	pGEX-6P-1	F GGGCTGGCAAGCCACGTTTGGTG
			R CCGGGAGCTGCATGTGTGACAGAGG
TF	Insert	<i>yadA</i>	F CCGCTCGAGATGACTAAAGATTTTAAG
			R CGAATTCTTACCACTCGATATTAATAA
	Vector	pCold-TF	F CCACTTTCAACGAGCTGATG
			R CAGAATCTAAGATCCCTGCC

MATERIALS AND METHODS

Preparation of recombinant *YadA*

In this study, *YadA* protein was produced using genetic recombination in *Escherichia coli*. The array of *yadA* (ACCESSION NC_010635 VERSION NC_010636.1) was obtained by analyzing the full length of 1,299 base pairs (bp) in the *Y. ptb* serotype 4b RIMD 2503048 strain (Table 1). Primers created with reference to the array of *yadA* were inserted into multiple cloning sites of 2 vectors: pGEX-6P-1 (TAKARA, Otsu, Japan) and pCold-TF (TAKARA). *E. coli* strain BL21 (DE3) (TAKARA) was implanted with the vectors, thereby producing 2 types of recombinant *E. coli* strains: PS with the pGEX-6P-1 vector and TF with the pCold-TF vector. The recombinant *YadA* (r*YadA*) proteins expressed by the 2 strains were analyzed by polyacrylamide gel electrophoresis. The r*YadA* of the PS strain (GST-*YadA*) was expressed as 67–70-kDa insoluble inclusion bodies, and the r*YadA* of the TF strain (His-TF-*YadA*) was expressed as an 89–92-kDa soluble component. The TF strain was purified by ultrasonic fragmentation and affinity chromatography using a His60 Ni Gravity Column (Clontech, Mountain View, CA, U.S.A.), and the PS strain was purified by ultrasonic fragmentation and centrifugation. The r*YadA* proteins were purified; the PS strain-derived insoluble r*YadA* was used as an immunogen, and the TF strain-derived soluble r*YadA* was used as a measurement antigen for *YadA* antibody in serum. The reason is that soluble r*YadA* derived from the TF strain could be purified with high accuracy but yields a small amount, whereas insoluble r*YadA* from the PS strain could be purified with low precision but the purification is easy to perform and yields a large amount.

Furthermore, to investigate the antibody reacts what domains of *YadA*, *YadA* head domain (*YadA*-HD) which is the domain of *YadA* was made and used for ELISA. The gene of *YadA*-HD exists from 76 to 681 bp from the 5' of *yadA* and a total length is 605 bp. Also, *YadA*-HD exists from 26 to 227 from the N-terminal of the full-length *YadA* and is about 22.2 kDa [21]. The method of making up the *YadA*-HD expression recombinant *E. coli* strain (YH) was same for PS, using pGEX-6P-1 and *E. coli* strain BL21, the primer of *YadA*-HD gene was wrote in Table 1. YH was cultured performed ultrasonic disruption. Since recombinant *YadA*-HD was expressed as a soluble protein, the supernatant including *YadA*-HD purified using GSTrap FF (GE Healthcare Japan, Tokyo, Japan).

Mice immunization and challenge with *Y. ptb*

The immunogenicity of the 3 treatments was evaluated by the survival rate and *YadA* antibody titer in mice. The mice were BALB/c, male and 3 weeks of age (Japan SLC, Hamamatsu, Japan). Mice were divided into 3 groups: in group 1 (n=5), mice received PS strain-derived insoluble r*YadA* (100 µg/dose); in group 2 (n=5), mice received 1% formalin-inactivated *Y. ptb* serotype O4b strain RIMD2503048 with strong expression of *YadA* (20 mg/dose); and in group 3 (n=3), mice received phosphate-buffered saline (PBS) (0.2 ml/dose). All dosages were administered subcutaneously twice at an interval of 1 week. *Y. ptb* serotype O4b strain RIMD2503048 (10⁷ cells/mouse) was administered by oral inoculation 1 week after the second administration. Mice were observed for 2 weeks, and any surviving mice were humanely killed. The survival rate and pathological findings in each group were investigated. Blood was collected from each mouse before vaccine administration and each week thereafter (a total of 4 times). The serum antibody titers in transition were measured by enzyme-linked immunosorbent assay (ELISA). The present study was carried out with the approval of the Azabu University Animal Care and Use Committee (approval number: 140303).

Immunoglobulin G enzyme-linked immunosorbent assay (IgG-ELISA) for *YadA* antibody levels

TF strain-derived soluble r*YadA* dissolved in carbonate buffer (pH 9.4) was placed in a 96-well microtiter plate at 50 ng/well and allowed to stand for 24 hr at 4°C; then, 300 µl of blocking solution was placed in each well and allowed to stand for 15 min. Mouse serum diluted 400-fold with 0.05% PBS-Tween 20 (PBS-T) was added to each well at 50 µl/well. The plate was allowed to stand at room temperature for 1 hr, and then washed 5 times with PBS-T. Then, 50 µl of horseradish peroxidase (HRP)-labeled anti-mouse IgG diluted 1,000-fold was placed into each well and allowed to stand for 1 hr at room temperature. The plate was washed 5 times with PBS-T, and a coloring solution was added. Measurements were then performed using an iMark microplate reader (Bio-Rad, Hercules, CA, U.S.A.) at 405 nm. In addition, ELISA of inactivated *Y. ptb* and *YadA*-HD was also performed in the same method. All the solutions and methods used were the same, inactivated *Y. ptb* coated 8 µg / well of antigen and serum of mice diluted 50-fold, *YadA*-HD antigen coated 400 ng / well and serum diluted 100-fold.

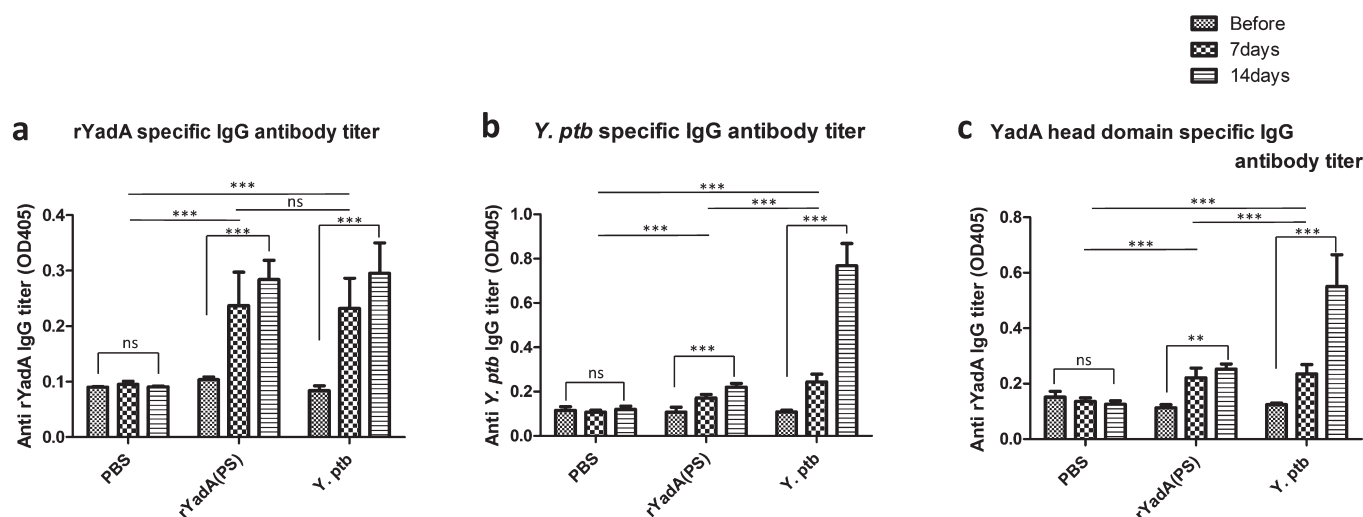


Fig. 1. Serum IgG antibody titers by ELISA in each group. The group1 was rYadA administrated mice, group2 was inactivated *Y. ptb* administrated and group3 was PBS administrated. a) The rYadA-specific IgG antibody titers are significantly elevated in the rYadA-administered group and inactivated *Y. ptb*-administered group ($***P<0.0001$). There was no increase in antibody titer in the PBS-administered group (ns, $P=0.6124$). Comparing antibody titers between the rYadA-administered group, *Y. ptb*-administered group and PBS-administered group, there were significant differences in the antibody titer increases in the rYadA-administered group and *Y. ptb*-administered group compared with the PBS-administered group ($***P=0.0002$). On the other hand, when comparing the rYadA-administered group and *Y. ptb*-administered group, there was no significant difference in the antibody titer increases (ns, $P=0.8966$). b) *Y. ptb* specific IgG antibody titers were significantly increased over time compared to baseline values in those groups ($***P<0.0001$). Comparing antibody titers between groups 1, 2 and 3, there was a significant difference in antibody titer increases in groups 1 and 2 compared with that in group 3 ($***P=0.0001$). Furthermore, there was significant difference in antibody titer increases between groups 1 and 2 ($***P=0.0001$). c) YadA-HD specific IgG antibody titers were significantly increased over time compared to baseline values in those groups (group1: $**P=0.0037$, group2: $***P<0.0001$). Comparing antibody titers between groups 1, 2 and 3, there was a significant difference in antibody titer increases in groups 1 and 2 compared with that in group 3 ($***P=0.0001$). Furthermore, there was significant difference in antibody titer increases between groups 1 and 2 ($***P=0.0001$).

Pathological examination

Dead and sacrificed mice underwent an autopsy and macroscopic examination. Sampled heart, lung, liver, spleen, jejunum, ileum, cecum and colon specimens were fixed in 10% phosphate-buffered formalin solution, paraffin-embedded and sectioned in 3–4- μ m slices using conventional methods. Then, hematoxylin-eosin (HE) staining was performed for histopathological observation. Furthermore, samples were immunohistochemically searched using rabbit anti-*Y. ptb* O4 antibody ($\times 400$, Trypsin) (Denka Seiken, Tokyo, Japan) and horseradish peroxidase (HRP)-labeled goat anti-rabbit antibody (Nichirei Biosciences, Tokyo, Japan) by the conventional method.

Statistical analysis

Serum IgG antibody titers of mice were compared with a two-way repeated-measures analysis of variance (ANOVA) test and a one-way ANOVA test, and the survival of mice was compared with the Kaplan-Meier test. For both tests, GraphPad Prism 5 (GraphPad Software, La Jolla, CA, U.S.A.) was used.

RESULTS

Serum *YadA*-specific IgG antibody titers

In groups 1 and 2, anti-*YadA* specific IgG antibody titers in the serum were significantly increased over time compared to baseline values in those groups (Fig. 1a) ($P<0.0001$). Also *Y. ptb* specific IgG antibody titers were increased (Fig. 1b) ($P<0.0001$), and rYadA-HD specific IgG antibody titers were significantly increased (Fig. 1c) (group1: $P=0.0037$, and group2: $P<0.0001$). The average of rYadA in group 1 increased from 0.103 to 0.301, *Y. ptb* increased from 0.107 to 0.220 and rYadA-HD increased from 0.113 to 0.252 and the values of rYadA in group 2 were 0.083 to 0.295, those of *Y. ptb* were 0.108 and 0.773 and those of rYadA-HD were 0.124 to 0.550 at 0 days to 14 days, respectively (Fig. 1a–c). There were no increase in the antibody titers of rYadA, *Y. ptb* and rYadA-HD in group 3 (rYadA; $P=0.6124$, *Y. ptb*; $P=0.6124$, rYadA-HD; $P=0.5152$). In addition, when comparing antibody titers of rYadA, *Y. ptb* and rYadA-HD between groups 1, 2 and 3, there were significant differences in antibody titer increases in groups 1 and 2 compared with that in group 3 (rYadA; $P=0.0002$, *Y. ptb*, rYadA-HD; $P=0.0001$). On the other hand, there was no significant difference in antibody titer of rYadA increases between groups 1 and 2 ($P=0.8966$). But there was significant difference in antibody titers of *Y. ptb* and rYadA-HD increases between groups 1 and 2 ($P=0.0001$).

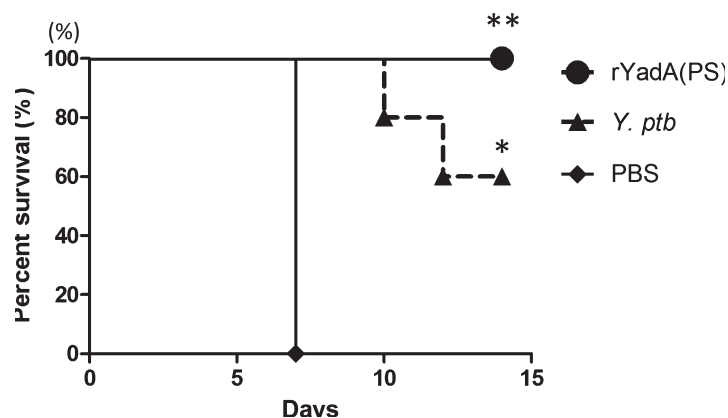


Fig. 2. Survival rate in each group. The survival rate is significantly increased in the rYadA-administered group (100%; $**P=0.0044$) and inactivated *Y. ptb*-administered group (60%; $*P=0.0121$). On the other hand, the survival rate of the PBS-administered group is 0%.



Fig. 3. Comparison of the gross lesions. a) The spleen of a non-surviving mouse in the group administered PBS. As was the case in all the mice that died during the observation period, the spleen shows marked enlargement and a large number of small white nodules. b) The spleen of a surviving mouse in the group administered rYadA. As was the case in mice that survived the *Y. ptb* infection, the spleen shows mild-to-moderate enlargement. Although white nodules are observed, this was not the case in all the surviving mice.

Clinical symptoms and survival

The survival rate after *Y. ptb* infection was 100% in group 1, 60% in group 2 and 0% in group 3 (Fig. 2). The fur of mice inoculated with *Y. ptb* appeared dull and ruffled from day 4. All mice in group 3 and 2 mice in group 2 showed reduced vitality, a hunched posture and depression, and died between day 7 and day 13 after *Y. ptb* inoculation. No clinical abnormalities were observed during the immunization period and no side effects resulted from vaccine administration.

Pathological findings

In all 5 mice that did not survive, marked enlargement of the spleen, Peyer's patches and mesenteric lymph nodes were grossly visible; in addition, the spleen and liver showed numerous multifocal white nodules (Fig. 3a). On the other hand, in surviving mice, the spleen and liver lesions were mild, but the enlargement of the Peyer's patches and mesenteric lymph nodes were comparable to those in the non-surviving mice (Fig. 3b).

On histological examination, the non-surviving mice displayed severe necrotic lesions with *Y. ptb* growth. In the spleen and liver, consistent with the white nodules, areas of severe necrosis were observed with colonies of *Y. ptb* in the center and with surrounding inflammatory cell infiltration consisting mainly of neutrophils (Fig. 4a and 4c). Additionally, in the liver, small foci of inflammation consisting of a small number of inflammatory cells, mainly neutrophils, were observed. Peyer's patches were almost

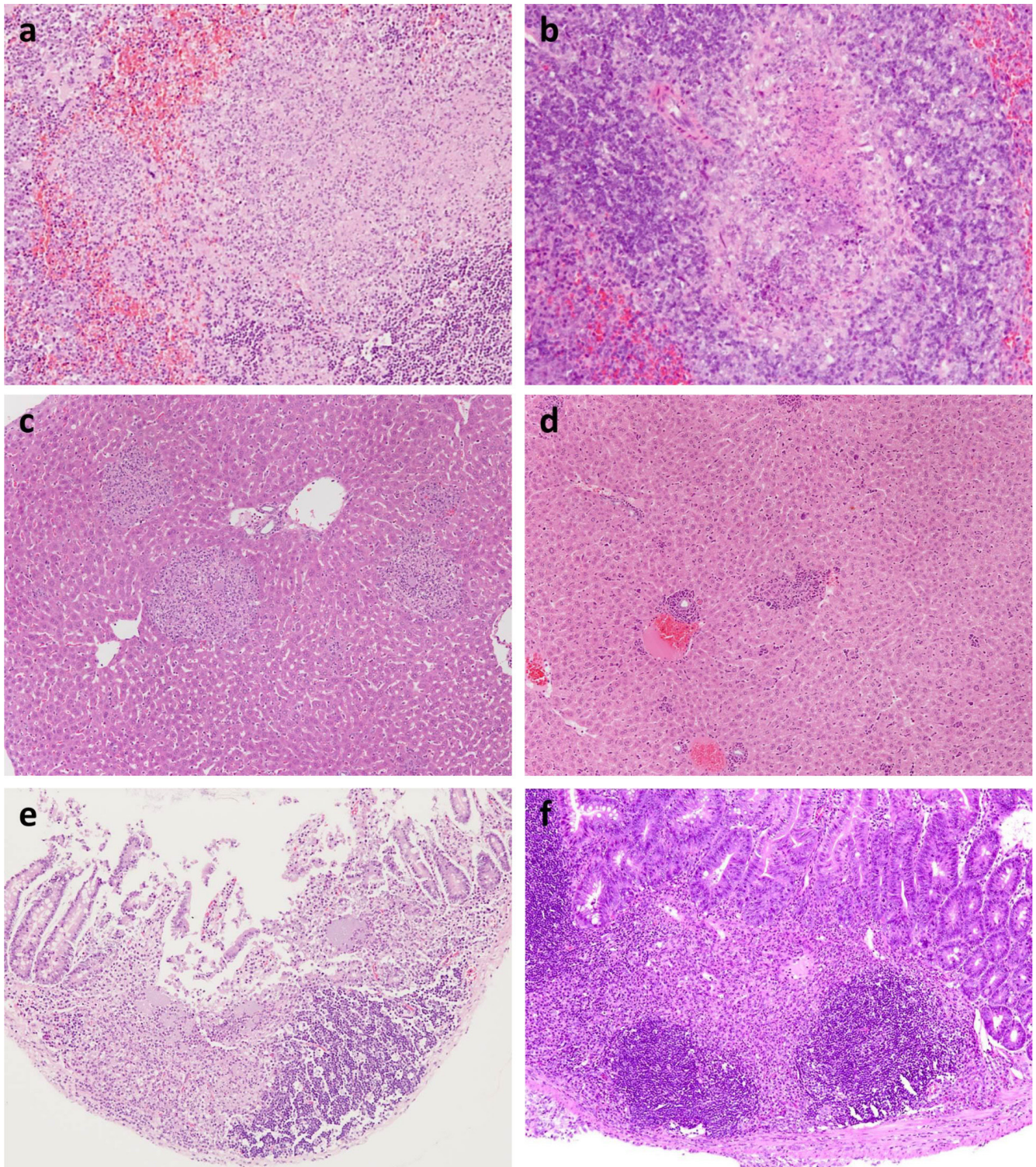


Fig. 4. Comparisons of histological lesions. a) The spleen of a non-surviving mouse in the group administered PBS. Lymphoid follicles are substantially reduced in size and the number of lymphocytes is decreased. In addition, a large number of severe necrotic foci containing *Y. ptb* colonies are observed (Bar=100 μ m). b) The spleen of a surviving mouse in the group administered rYadA. Scattered moderate necrotic foci containing *Y. ptb* colonies are observed, and the surrounding tissue is organized. In addition, infiltration by mainly macrophages, lymphocytes and fibroblasts is seen at the periphery of the necrotic foci (Bar=100 μ m). c) The liver of a non-surviving mouse in the group administered PBS. Numerous large and small severe multifocal necrotic foci are observed (Bar=100 μ m). d) The liver of a surviving mouse in the group administered rYadA. A limited number of small areas of granulomatous inflammation without necrosis and large areas of granulomatous inflammation with necrosis are observed (Bar=100 μ m). e) Peyer's patches in a non-surviving mouse in the group administered PBS. Severe necrotic foci containing *Y. ptb* colonies are observed. Mucosal epithelial cells display necrosis and exfoliation, and infiltration of neutrophils is seen in the gut lamina propria mucosae (Bar=100 μ m). f) Peyer's patches in a surviving mouse in the group administered rYadA. Scattered moderate necrotic foci and infiltration by mainly macrophages, lymphocytes and fibroblasts are seen (Bar=100 μ m).

replaced by necrotic tissue containing *Y. ptb* colonies. The mucosal epithelial cells of the small intestine, cecum, and colon were necrotic and exfoliated (Fig. 4e).

In the surviving mice, the necrotic lesions were mild, and granuloma formation and cellular tissue repair reactions were advanced. The spleen had a small number of mild necrotic foci with *Y. ptb* colonies, and organization and infiltration of mainly fibroblasts, macrophages and lymphocytes were present around these necrotic foci (Fig. 4b). In the liver, a small granuloma without necrosis and a small number of granulomas with necrosis were observed (Fig. 4d). Peyer's patches were interspersed with moderate necrotic foci, but the tissue surrounding these foci was organized (Fig. 4f). Immunohistochemically, *Y. ptb* was found in large amounts in the spleens, livers and Peyer's patches of all dead mice (Fig. 5a, 5c and 5e). On the other hand, in the surviving mice, no bacteria were found in the spleens or livers (Fig. 5b and 5d). However, in some surviving mice, *Y. ptb* was present in a mild-to-moderate amount in the Peyer's patch of the small intestine (Fig. 5f).

DISCUSSION

In the present study, we aimed to determine possible immunogens for a vaccine against *Y. ptb* by investigating the survival rate and serum antibody levels of mice inoculated with inactivated *Y. ptb* with strong expression of YadA, rYadA or PBS. Of note, the mice that received inactivated *Y. ptb* or rYadA had significantly elevated anti-*Y. ptb* antibody levels and all the mice that received rYadA survived. Moreover, according to the pathological findings, serum YadA-specific IgG prevented the spread of *Y. ptb* that invaded the body as a mechanism to prevent lethal lesions caused by *Y. ptb* infection. There were many cells targeted by *Y. ptb* in the organs, which adhered to them via YadA and resulted in pathogenicity. We believed that their activity could not lead to death because of the presence of YadA-specific IgG. However, rYadA could not induce mucosal immunity, so rYadA could not defend against *Y. ptb* invasion in the intestine.

In the pathogenesis of yersiniosis, the *Y. ptb* organisms infect the host via the oral route and adhere to the M cells of the intestinal Peyer's patches. They then infiltrate into the tissue and grow proliferously. Subsequent hematogenous spread to various organs results in the formation of necrotic lesions and sepsis, which is frequently lethal [18]. Pathogenic *Y. ptb* factors involved in the formation of these lesions include YadA and *Yersinia* outer membrane proteins (Yops) encoded by virulence plasmid DNA, and invasins and *Y. ptb*-derived mitogen encoded by chromosomal DNA. YadA, although not cytotoxic itself, has various functions in the pathogenesis of disease, such as enabling *Y. ptb* to adhere to target cells and inhibiting phagocytosis by host cells [2, 11].

Vaccine development for the control of yersiniosis has been studied previously. Vaccines that target the LcrV and invasins proteins related to the type III secretion system have been considered, but not yet put into practical use [6, 34, 35]. Furthermore, we previously obtained no effects with either formalin-inactivated *Y. ptb* or Yops used as immunogens in preliminary experiments (private communication). In another study reporting a recombinant vaccine for *Y. ptb*, Daniel *et al.* showed high survival rates while maintaining high safety using *Lactobacillus lactis* [9]. However, the survival rate was 55% to 80% despite the small number of *Y. ptb* bacteria in the challenge (10^3), and all mice that died were dead within 2 weeks after the challenge. In addition, about 10^6 *Y. ptb* were detected in the spleen and liver after the observation period. However, we did not perform long-term observations for the vaccine we developed and used fewer mice than Daniel *et al.* Therefore, while it could not be determined clearly, but the survival rate was 100% despite a 10^7 *Y. ptb* challenge and no *Y. ptb* were detected immunohistochemically in the spleen or liver, so it was considered to be superior in these points. YadA was used as an immunogen in this study, and subcutaneous administration (twice at an interval of 1 week) of inactivated *Y. ptb* with strong expression of YadA and rYadA yielded survival rates of 60% and 100%, respectively, with no observed side effects from the vaccination. In addition, although inactivated vaccines generally have low immunogenicity compared with live attenuated vaccines and require immune enhancement with the use of multiple doses or adjuvants [5, 7, 23], we found that inoculation twice with rYadA without an immune-enhancing agent appeared to generate a sufficient serum rYadA-specific IgG antibody titer to prevent lethal infection in mice. Surviving mice exhibited mild lesions, which mostly consisted of organizing tissue. We considered that rYadA could not defend in organisms from infecting and growing in the mucosa but could reduce hematogenous spread and the risk of sepsis, resulting in *Y. ptb* lesions that were not severe. These results suggest that subcutaneous administration of rYadA generates sufficient serum rYadA-specific IgG antibody in mice to prevent sepsis due to *Y. ptb*; therefore, rYadA may be useful as a vaccine immunogen. On the other hand, although there was no significant difference between inactivated *Y. ptb* and rYadA antibody titers, there was a difference in survival rate. And there were significant differences in the antibody titers of *Y. ptb* and YadA-HD. And therefore, two possibilities were considered. The first possibility is that immunity may have been enhanced by some action by some antigen present in recombinant *E. coli*, not present in *Y. ptb*. We found that antibodies to only the antigen of *Y. ptb* were insufficient as immunogens because dead mice appeared even the antibody titer of *Y. ptb* was high. The second possibility is that domains other than YadA-HD, membrane anchor, coiled coil segment, stalk or neck may have been important as immunogens. YadA-HD is the domain that first adheres to target cells [21, 29]. Therefore, we had an idea that many of the functions of YadA required YadA-HD, and it was highly useful as the immunogen, but that idea was different because rYadA-HD antibody titer was significant difference between rYadA administered group and inactivated *Y. ptb* administered group. Since the functions of each domain have not yet been reported, so we could not investigate and perform ELISA for domains other than YadA-HD, so we could not identify important antigens against *Y. ptb* infection. These antigens will be the subject of further studies.

A wide variety of animals is sensitive to the effects of *Y. ptb*, and lethal yersiniosis has been reported in species, such as the parakeet, maple butterfly, marmot, bat, toucan and squirrel monkey [8, 19, 27, 28, 30, 31]. In addition, cases of infection in humans have been reported in recent years [22, 38]. *Y. ptb* is carried by the pig, wild rodents and birds, making prevention of intrusion by

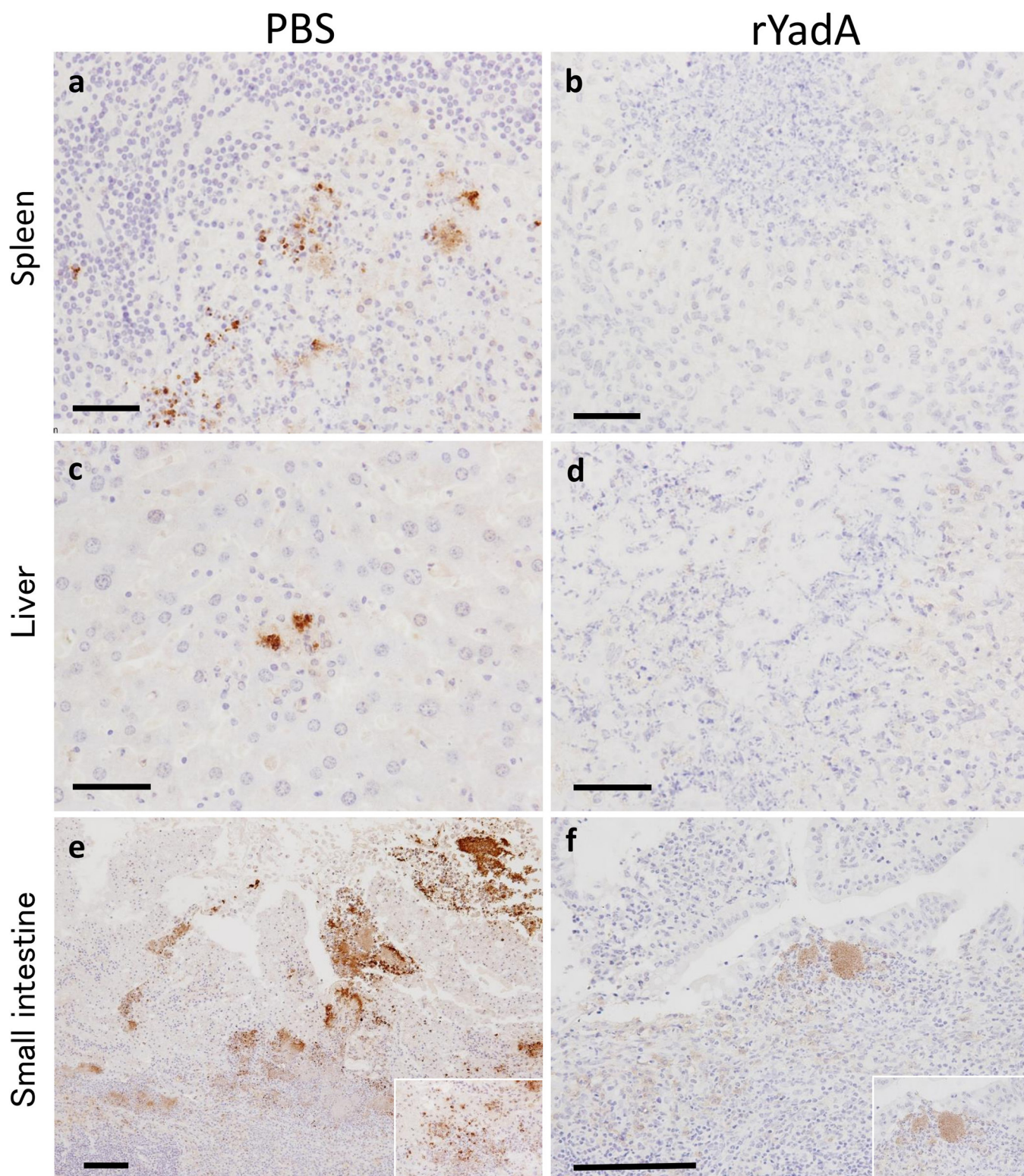


Fig. 5. Comparisons of immunohistochemical findings. a) The spleen of a non-surviving mouse from the group administered PBS. In severe necrotic foci, there are many *Y. ptb* positive for anti-*Y. ptb* O4 antibody (Bar=20 μ m). b) The spleen of a surviving mouse from the group administered rYadA. In infiltrates and necrotic foci, there are no *Y. ptb* positive for anti-*Y. ptb* O4 antibody (Bar=20 μ m). c) The liver of a non-surviving mouse from the group administered PBS. In infiltrates and severe necrotic foci, there are many *Y. ptb* positive for anti-*Y. ptb* O4 antibody (Bar=20 μ m). d) The liver of a surviving mouse from the group administered rYadA. In infiltrates, there is no *Y. ptb* positive for anti-*Y. ptb* O4 antibody (Bar=20 μ m). e) Peyer's patches in a non-surviving mouse from the group administered PBS. In necrotic foci of the intestinal epithelium, lamina propria and mucosal muscle layer, many *Y. ptb* are positive for anti-*Y. ptb* O4 antibody (Bar=100 μ m). f) Peyer's patches in a surviving mouse from the group administered rYadA. In infiltrates from the intestinal epithelium, lamina propria and mucosal muscle layer, there are mild-to-moderate numbers of *Y. ptb* positive for anti-*Y. ptb* O4 antibody (Bar=100 μ m).

carrier animals very difficult in animal breeding facilities [17, 33]. In addition, once an animal in the facility is infected, it may serve as a source of infection for all the animals in the breeding facility, resulting in an epidemic. Therefore, there is an urgent need to establish a means to prevent infection. As mentioned previously, results with the rYadA vaccine in mice suggest that this immunogen may be used to generate sufficient antibody levels to prevent sepsis due to *Y. ptb*. In addition, it is not necessary to handle pathogenic bacteria when producing the rYadA vaccine because of the use of recombinant *E. coli* and it is possible to produce the vaccine in a relatively short time, thereby facilitating stability and mass culture. Therefore, use of rYadA as a vaccine immunogen may contribute greatly to the prevention of yersiniosis. However, in the study by Zhang *et al.*, although the survival rate of the mice was good, they reported that *Y. ptb* was detected in internal organs after the observation period, so the mice were possibly carrier animals [41]. In our study, *Y. ptb* was not detected in the spleen or liver by immunohistochemistry, but the immunohistochemical method has low detection sensitivity. Therefore, subclinical infection due to vaccine administration is conceivable. It is necessary to conduct a long-term investigation in the future, determine the amount of *Y. ptb* in the feces and determine the colonization mechanism.

In conclusion, the present study results revealed that rYadA may be a useful immunogen for vaccine development as a valid defense method against yersiniosis caused by *Y. ptb*. In this study, it was possible to confer a high protective effect against sepsis due to *Y. ptb* in the sensitive mouse by inoculation with an rYadA vaccine. This is a first step in the prevention of *Y. ptb* infection and may be considered a breakthrough result that could be applied to vaccine production in this country for the prevention of yersiniosis in a variety of animal species.

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