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Protective effects of oral immunization with formalin-inactivated whole-cell *Citrobacter rodentium* on *Citrobacter rodentium* infection in mice

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ABSTRACT

Evaluation of the efficacy of vaccine candidates that prevent enteropathogenic and enterohemorrhagic *Escherichia coli* (EPEC/EHEC) infection in mouse models is difficult due to their limited pathogenicity in mice. *Citrobacter rodentium*, a murine pathogenic bacterium that shares its infection strategy and virulence genes with EPEC/EHEC, has been used as a model pathogen to develop novel vaccine strategies or platforms for these bacteria. However, there are few reports on the comparative effectiveness of novel vaccine platforms as no *C. rodentium* vaccines have yet been prepared by standard methods such as bacteria attenuation or inactivation. In this study, we investigated the protective effect of the oral administration of formalin-inactivated *C. rodentium* (Fo-CR) on *C. rodentium* infection in two mouse strains, C57BL/6 and C3H/HeN, as these strains have different degrees of susceptibility to infection. In C57BL/6 mice, administration of Fo-CR induced significant *C. rodentium*-specific mucosal and systemic antibody responses, promoted bacterial clearance from the gut and inhibited colonic hyperplasia. Furthermore, in C3H/HeN mice, the administration followed by lethal *C. rodentium* infection induced significantly high avidity serum IgG specific to *C. rodentium* and inhibited death, body weight loss, and bacterial invasion to visceral organs. In conclusion, the oral administration of Fo-CR resulted in the protection of mice from *C. rodentium* infection, indicating that it serves as a reference method for evaluating the efficacy of novel oral vaccine candidates or platforms.

1. Introduction

Enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) infections are the major cause of diarrheal diseases (Kaper et al., 2004; Torres, 2017). These pathogenic *E. coli* strains intimately adhere to gut epithelial cells using the type III secretion system (T3SS) and form attaching and effacing (A/E) lesions resulting in diarrhea (Lewis et al., 2015). Moreover, EHEC is also responsible for severe clinical complications, such as hemolytic uremic syndrome (HUS) (Freedman et al., 2016). In the case of EHEC infection, treatments based on antibiotics have been associated with an increased risk of HUS due to toxin production as a consequence of the antibiotics-induced SOS response (Kimmitt et al., 2000). Therefore, it is presumed that a vaccine that prevents EPEC/EHEC from colonizing the intestinal tract would be the most effective strategy for preventing the diseases caused by these pathogens (Mayr et al., 2005). However, there are no licensed vaccines against EPEC/EHEC at present.

The virulence of EPEC/EHEC can be largely attributed to its toxins, T3SS proteins, and surface fimbrial and afimbrial adhesins (Garcia-Angulo et al., 2013; McWilliams and Torres, 2014). Therefore, these virulence factors have been studied as potential vaccine antigens for the neutralization of toxins or for the disruption of bacteria-host cell interactions at the intestinal epithelia to prevent colonization in the intestinal tract (Ferreira Oliveira et al., 2012; Gao et al., 2011; Mejías et al., 2016; Rojas-Lopez et al., 2018; Toledo et al., 2011). However, it is difficult to investigate the protective efficacy of vaccination in mouse models, as mice are inherently resistant to EPEC/EHEC infection (Mundy et al., 2006; Mundy et al., 2005). *Citrobacter rodentium* is a

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murine pathogenic bacterium that causes diarrhea and colonic epithelial hyperplasia in mice (Collins et al., 2014). *C. rodentium* shares its infection strategy and virulence genes with EPEC/EHEC (Collins et al., 2014). Therefore, numerous studies have evaluated the efficacy of oral vaccine strategies against EPEC/EHEC infection using *C. rodentium* infection models (Garcia-Angulo and Kalita, 2014), such as the use of antigen-expressing recombinant non-pathogenic lactic acid bacteria as novel live vectors for mucosal delivery of the antigens (Ferreira et al., 2011). However, there are few data on the comparative effectiveness of these novel vaccine platforms and vaccines prepared by standard methods, such as live attenuated or inactivated vaccines, as no *C. rodentium* vaccines have yet been prepared by these methods.

In this study, we investigated the protective effects of the oral administration of inactivated whole-cell antigens, which were prepared by the formalin treatment of *C. rodentium*, on the *C. rodentium* infection in two strains of mice, C57BL/6 and C3H/HeN, that have different susceptibilities to *C. rodentium* infection. We demonstrated that the oral administration of the formalin-inactivated *C. rodentium* had significant protective effects against infection.

2. Materials and methods

2.1. Bacterial strains and culture conditions

C. rodentium ATCC51459 (American Type Culture Collection (ATCC), Manassas, VA) was grown in LB medium or in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) at 37 °C. Determination of fecal shedding of *C. rodentium* was performed using MacConkey agar plates (NISSUI PHARMACEUTICAL, Japan). When required, antibiotics were added at the following concentrations, chloramphenicol (10 μg/ml) and tetracycline (3 μg/ml), into the medium containing agar that was sterilized by autoclaving and allowed to cool to 50 °C. After mixing the medium by swirling, plates were poured.

2.2. Generation of luciferase-expressing C. rodentium

A DNA fragment used for the generation of luciferase-expressing *C. rodentium* was constructed as follows (Supplemental Fig. 1A). The promoter region of the ampicillin resistance gene (*Amp*^{r}), the full-length NanoLuc gene, and the 5′ and 3′ ends of the *C. rodentium xylE* gene were amplified by PCR from pBluescript, pNL1.1 (Promega, Madison, WI), and *C. rodentium* genome DNA, respectively. Primers used for the PCR amplification are listed in Table 1. The PCR fragments were digested with restriction enzymes and assembled into pSTV28 (Takara), generating pSTV28–3'xylE-Amp^{r}P-Nluc-5'xylE. A DNA fragment for homologous recombination, consisting of the 5′ end of the *xylE* gene, the NanoLuc gene, the *Amp*^{r} promoter region, the chloramphenicol resistance cassette, and the 3′ end of the *xylE* gene, was amplified by PCR with pfu DNA polymerase (Agilent Technologies) from pSTV28–3'xylE-Amp^{r}P-Nluc-5'xylE using the following primers; Hind III-N-XylE-Fw and Sac II-C-XylE-Rv. *C. rodentium* harboring pRedET was incubated with 0.4% (w/v) L-arabinose to express Red recombinase for 1 h at 37 °C, used for electroporation of the prepared DNA fragment, plated onto the LB agar, and incubated overnight at 37 °C. Integration of the DNA fragment for the expression of the NanoLuc gene into the correct genomic location was confirmed by sequence analysis. After several passages in liquid LB to remove pRedET from the strain, a chloramphenicol-resistant, tetracycline-sensitive, and luciferase activity-positive clone was selected (designated luc$^+$ CR). The luciferase activities of luc$^+$ CR in vitro and in vivo were proportionate in the range of 2.4×10^2 to 10^7 colony-forming units (CFUs) (correlation coefficient $R^2 = 0.99$) (Supplemental Fig. 1B and 1C). Bacterial growth rate in the LB broth and the formation of A/E lesions on murine colonic epithelial cell line CMT-93 cells (CCL-223, ATCC) for luc$^+$ CR were comparable to those of the wild-type *C. rodentium* (Supplemental Fig. 1D and 1E), suggesting that the insertion of the luciferase gene cassette did not affect the virulence of the bacteria.

Table 1
primers used in this study.

Primer	Sequence
Sal1-Apm^r promoter-Fw	5′-CCCGTCGACGCTCATCAGCTCAGTATTGC-3′
BamH1-Apm^r promoter-Rv	5′-CCCGGATCCACTCTTCCTTTTTCAATATT-3′
Sma1-NanoLuc-Fw	5′-CCCCCCGGGCCACCATGGTCTTCACACTC-3′
EcoR1-NanoLuc-Rv	5′-CCCGAATTCTTACGCCAGAATGCGTTCG-3′
Nhe1-C-xylE-Fw	5′-CCCGCTAGCCCGTCTTTCAGCAGTTTGTC-3′
Sac2-C-xylE-Rv	5′-TTGCCGCGGATGGCGTTGGGGAAGATCTC-3′
Hind3-N-xylE-Fw	5′-CCCAAGCTTCTATTGCAGCAACCGCTTTGG-3′
Pst1-N-xylE-Rv	5′-CCCCTGCAGACGCAGTACACCAGCAGCTG-3′

2.3. Animal studies

Specific pathogen-free, 5-week-old, female C57BL/6 (for non-lethal challenge) or C3H/HeN (for lethal challenge) mice were purchased from Japan SLC (Hamamatsu, Japan) and housed in individual ventilated cages with free access to food and water, according to treatment. All animal experiments were approved by the institutional Animal Care and Use Committee.

2.4. Inactivation of C. rodentium

To prepare inactivated *C. rodentium*, *C. rodentium* was cultured with 5 ml of LB broth for 16 h at 37 °C with shaking (120 rpm), and the culture was then added to 300 ml of fresh LB broth (1/100) and incubated for a further 6 h at 37 °C with shaking (120 rpm). The culture was centrifuged at 2,000 ×*g* for 20 min at 4 °C and discarded the supernatants. The pelleted bacteria were then washed twice (2,000 ×g, 20 min, 4 °C) with 200 ml of ice-cold sterile phosphate-buffered saline pH 7.4 (PBS) and resuspended with ice-cold sterile PBS to 1×10^9 CFU/ml. To prepare formalin-inactivated *C. rodentium* (Fo-CR), 37% formalin was added to the suspension (final concentration 3%, v/v) and the suspension was incubated for 16 h at 4 °C. To prepare UV-inactivated *C. rodentium* (UV-CR), the suspension was irradiated with 5.0 J/cm^2 of UV light for 30 min using a UV linker (Model 2260, WAKENYAKU Co. Ltd., Japan). To prepare heat-inactivated *C. rodentium* (H-CR), the suspension was boiled in a water bath for 30 min. The inactivated-CRs (Fo-CR, UV-CR, and H-CR) were then washed twice with ice-cold sterile PBS and resuspended with sterile PBS to prepare inocula containing 1×10^9 inactivated bacteria/ml. Inactivation of *C. rodentium* viability was confirmed by culturing on LB agar plates.

2.5. Immunization

C57BL/6 or C3H/HeN mice were orally administrated weekly for 4 weeks with 200 μl of the inactivated CR (2×10^8 inactivated bacteria per dose). Mice were fasted for 6 h before and 1 h after the immunization, and cimetidine was intraperitoneally administrated 15 min before the immunization.

2.6. Non-lethal C. rodentium infection

To prepare *C. rodentium* inocula, luciferase-expressing *C. rodentium* was cultured with LB broth for 16 h at 37 °C with shaking (120 rpm), and the culture was then added to fresh LB broth (1/100) and incubated for a further 6 h at 37 °C with shaking (120 rpm). The culture was washed twice with ice-cold sterile PBS, and then the bacteria were resuspended with sterile PBS to prepare inoculum containing 2.5×10^9 CFU/ml. C57BL/6 mice were inoculated with 200 μl of the luciferase-expressing *C. rodentium* suspension by oral gavage using a disposable feeding tube (Cat. No. 4202, FUCHIGAMI, Japan) under

nonanesthesia or left uninfected (n = 6 in each group). Fresh fecal pellets were collected at days 5, 9, 13, 16, and 19 for determination of *C. rodentium* loads in the feces. Fecal pellets were collected at day −44 (pre-immune), day −1 (post-immune), days 7 and 20 post infection. Fecal pellets were homogenized using a disposable pestle attached to a portable grinder (Cat. No. CG-4A, Funakoshi, Japan) in PBS at 25 mg/ml, centrifuged (20,000 ×*g*, 10 min, 4 °C), and the supernatants were collected as fecal extracts. Blood specimens were drawn from the tail vein at day −44 (pre-immune), day −1 (post-immune), days 7 and 20 post infection, and sera were prepared from the blood specimens by centrifugation (800 ×*g*, 20 min, 4 °C) after clotting. The fecal extracts and sera were stored at −30 °C until use for measurement of *C. rodentium*-specific antibody titers in the following experiments.

2.7. Evaluation of colonic hyperplasia

To evaluate colonic hyperplasia, the terminal 4-cm portion of each mouse colon was weighed. Further, the same tissues were fixed in 4% paraformaldehyde in PBS for 24 h at 4 °C, and then transferred to a 20% sucrose solution for 24 h. The fixed colon was then cut vertically into two parts of equal length, embedded in OCT media (Sakura Finetek Japan Co. Ltd., Japan), and flash frozen with liquid nitrogen. Ten micrometer-thick sections were cut and rinsed twice with PBS and once in PBS containing 2% bovine serum albumin (PBS/2% BSA). Sections were stained with PBS/2% BSA containing rabbit anti-O152 antibody (1:200, Denka Seiken Co., Ltd. Japan) for 1 h at room temperature. After washing with PBS, the sections were stained with a secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG, 1:200, Life Technologies), Hoechst33342 (2 μg/ml final concentration) and Actistain555 (1:200, Cytoskeleton, Inc.) for 1 h. Sections were rinsed 3 times with PBS and mounted with ProLong Gold Antifade Mountant (Thermo Fisher Scientific). Images of randomly selected sections were acquired using a Zeiss LSM700 Laser scanning microscope and analyzed using the accompanying software (Zen 2012).

2.8. Determination of fecal shedding of luciferase-expressing C. rodentium by luciferase assay

Freshly collected fecal pellets from mice infected with luciferase-expressing *C. rodentium* were suspended in sterile PBS at 10 mg/ml, then the debris were removed by brief centrifugation and the supernatants was centrifuged (3,000 ×*g*, 10 min, 4 °C) to obtain bacterial pellets. The pelleted bacteria were lysed with Passive Lysis Buffer (Promega). The bacterial lysate was then mixed with an equal amount of Nano-Glo substrate (Promega) and the luciferase activity was measured using the GloMax-Multi Detection System (Promega).

2.9. Lethal C. rodentium infection

To prepare *C. rodentium* inocula, *C. rodentium* was cultured with 5 ml of LB broth for 16 h at 37 °C with shaking (120 rpm), and the culture was then added to 300 ml of fresh LB broth (1/100) and incubated for a further 6 h at 37 °C with shaking (120 rpm). The culture was centrifuged at 2,000 ×*g* for 20 min at 4 °C and discarded the supernatants. The pelleted bacteria were then washed twice (2,000 ×g, 20 min, 4 °C) with 200 ml of ice-cold sterile PBS, and the bacteria were then resuspended with sterile PBS to prepare inoculum containing 1×10^9 CFU/ml. C3H/HeN mice were inoculated with 200 μl of the *C. rodentium* suspension by oral gavage or were left uninfected. Fresh fecal pellets were collected at days 5, 8, and 12 for determination of the *C. rodentium* load in the feces. Fecal pellets and blood were also collected at day −29 (pre-immune), day −1 (post-immune), days 7 and 14 post infection. Fecal extracts and sera were prepared as described in the Materials and Methods section and were used for measurement of *C. rodentium*-specific antibody titers. Mice were sacrificed at day 14 post infection, and the livers and spleens were collected to determine bacterial invasion to these visceral organs.

2.10. Determination of fecal shedding of C. rodentium by agar plate culture

Freshly collected fecal pellets were suspended in sterile PBS, then the debris was removed by flash centrifugation for 5 s (Cat. No. FACTF001, FAVORGEN BIOTECH CORP., Taiwan), and the supernatants were plated onto MacConkey agar plates after serial dilutions with PBS and incubated for 24 h at 37 °C before enumeration of bacterial CFU.

2.11. Determination of C. rodentium in the spleen and liver

The livers and spleens obtained from infected mice at day 14 were minced with scissors and suspended in 600 μl and 200 μl of PBS, respectively. The bacterial CFU was then determined by culturing on MacConkey agar plates as described above.

2.12. Measurement of C. rodentium-reactive antibodies by ELISA

For measurement of the *C. rodentium*-specific antibodies by ELISA, 96-well plates were coated with *C. rodentium* as follows: *C. rodentium* was cultured with LB broth for 16 h at 37 °C with shaking (120 rpm), and the culture was then added to fresh LB broth (1/100) and incubated for a further 6 h at 37 °C with shaking (120 rpm). Bacteria were harvested by centrifugation (3,000 ×*g*, 10 min, room temperature), washed twice with PBS containing 0.1% NaN_3 and resuspended to a density of 1×10^8 CFU/ml in PBS containing 0.1% NaN_3. Wells of 96-well plate (Maxisoap, Nunc, Roskilde, Denmark) were incubated with 100 μl of the bacterial suspension overnight at 4 °C to bind *C. rodentium* to the wells. The supernatants were discarded, and the bacteria were then fixed with 0.15% glutaraldehyde (in 0.15 M phosphate buffer, pH 7.0) for 5 min at rt. After washing with PBS containing 0.1% tween 20, endogenous peroxidase was inactivated by incubation with PBS containing 1.0% (w/v) BSA and 0.04% (w/v) H_2O_2 for 1 h at 37 °C. Fecal extracts (1:5) or sera (1:100) from individual mice that were prepared as described in the Materials and Methods section and diluted with PBS containing 1% BSA were added, and incubated for 1 h at 37 °C. Horseradish peroxidase (HRP)-conjugated polyclonal goat anti-mouse IgG or IgA antibodies (Southern Biotech, Birmingham, AL) were added to the wells and incubated for 1 h at 37 °C. Plates were developed using *o*-phenylenediamine substrate for 30 min at 37 °C, and OD_{492} values were obtained.

To determine the avidity index, sera (1:100) were added to duplicate wells coated with *C. rodentium*. After 1 h incubation at 37 °C, the wells were rinsed once with PBS containing 0.1% Tween 20. Half of the wells were then incubated with PBS containing 4 M urea, and the other half were incubated with PBS without urea for 10 min at room temperature. After two additional washes with PBS containing 0.1% Tween 20, normal ELISA procedure was performed as described above. The avidity index, expressed as a percentage, was calculated based on the ratio between the OD values with and without the urea treatment.

2.13. Statistical analysis

Statistical analyses were performed using GraphPad Prism 7(Graph Pad Software Inc.). Data analysis of body weight change, the number of *C. rodentium* in the feces and organs, colon weights and antibody titers were performed by one-way ANOVA with Tukey's post hoc test. Survival rate was analyzed by Log-rank (Mantel-cox) test. p values of < 0.05 were considered statistically significant.

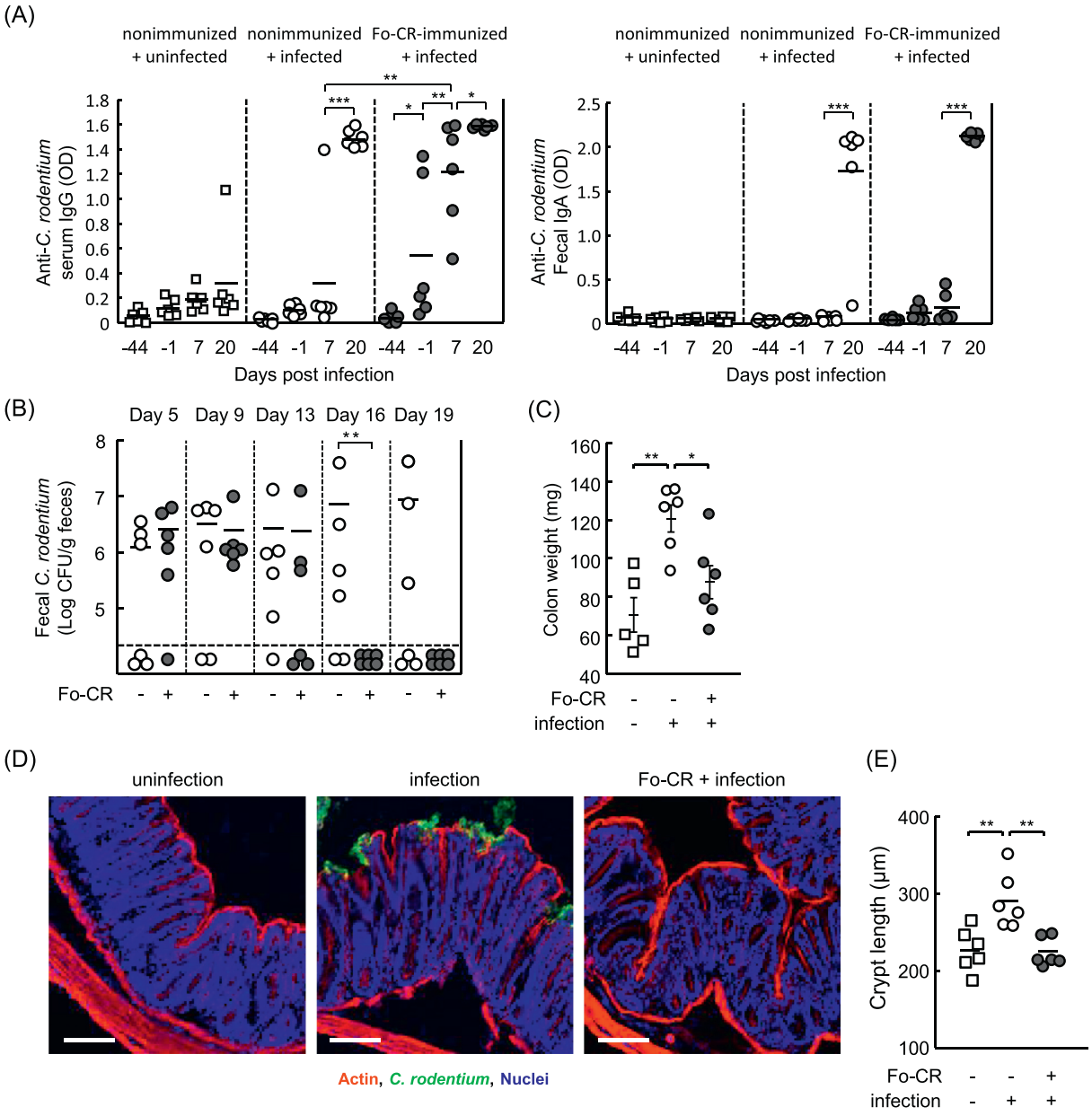

Fig. 1. Protective effects of the oral administration of formalin-inactivated *C. rodentium* (Fo-CR) on a murine model of non-lethal *C. rodentium* infection. C57BL/6 mice that were orally administrated with Fo-CR weekly for 4 times (n = 6) or nonimmunized (n = 6) were orally infected with 5×10^8 CFU of luc$^+$ CR. An additional 6 mice were left nonimmunized and uninfected (control). Each symbol in the panels represents data obtained from one mouse. (A) *C. rodentium*-specific serum IgG (left panel) and fecal IgA (right panel) titers of mice without any treatment (nonimmunized + uninfected), nonimmunized mice with infection (non-immunized + infected), and Fo-CR-immunized mice with infection (Fo-CR-immunized + infected) at days −44, −1, 7 and 20 post infection were determined by ELISA. (B) Numbers of *C. rodentium* in freshly isolated fecal pellets obtained from the nonimmunized + infected (open circles) or Fo-CR-immunized + infected mice (closed circles in grey) were quantified over a time course of 19 days by luciferase assay. Horizontal dotted lines indicate the detection limit. (C) Weights of the terminal 4 cm of the colons of uninfected control mice, nonimmunized mice and Fo-CR-immunized mice infected with *C. rodentium* were measured at 20 days post infection. Means ± SEMs are shown. (D) Cross sections of the terminal portions of the colons that were stained for nuclei (blue), F-actin (red), and *C. rodentium* O-152 (green) of uninfected control mice, nonimmunized mice and Fo-CR-immunized mice infected with *C. rodentium* analyzed on day 20 post infection (n = 6/group). A representative fluorescence image for each group is shown. Original magnification: ×20. Scale bars: 100 μm. (E) Quantitative analysis of colonic crypt length of nonimmunized + uninfected, nonimmunized + infected, and Fo-CR-immunized + infected mice. Means are shown. *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$ by one-way ANOVA followed by Tukey's test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Protective effects of the oral administration of formalin-inactivated C. rodentium (Fo-CR) on a murine model of non-lethal C. rodentium infection

We tested the protective effects of formalin-inactivated *C. rodentium* (Fo-CR) on a non-lethal *C. rodentium* infection model using C57BL/6 mice. Mice were orally administrated with Fo-CR weekly for 4 weeks, then challenged with luciferase-expressing *C. rodentium*. As described in the Materials and Methods section, this strain showed an almost perfect correlation between the luciferase activities and CFU as well as demonstrating virulence similar to that of the original strain (Supplemental Fig. 1). *C. rodentium*-specific serum IgG was weakly but significantly ($p < 0.05$) raised after the Fo-CR treatment (day −1) and

increased within 7 days post infection in the Fo-CR-treated mice (Fig. 1A). In contrast, the increase in *C. rodentium*-specific systemic IgG after infection in the untreated mice took 20 days (Fig. 1A). Although it is not significant, *C. rodentium*-specific mucosal IgA responses were induced slightly after the Fo-CR treatment and further increased in the early phase of the infection in two out of six of the Fo-CR-treated mice. However, no *C. rodentium*-specific mucosal IgA responses were observed in any of the untreated mice up to 20 days post infection. These results indicated that the Fo-CR treatment primed the anti-*C. rodentium* immune responses.

To observe whether the primed immune responses act on rapid bacterial clearance, the duration of bacterial fecal shedding was analyzed. The duration of bacterial shedding in the Fo-CR-treated mice was significantly shorter than that in the untreated mice, with shedding no longer observed in any Fo-CR-treated mice by day 16 ($p < 0.01$). In contrast, half of the untreated mice shed the bacteria throughout the experiment (Fig. 1B). Furthermore, we assessed the pathological effect of the Fo-CR treatment on infection-induced colonic hyperplasia. Colon weights were increased significantly ($p < 0.01$) in the untreated, but not in the Fo-CR-treated mice after *C. rodentium* infection, in comparison with the uninfected control mice (Fig. 1C). Microscopic analysis revealed colonization of considerable amounts of *C. rodentium* on the colonic epithelia in 2 out of 6 untreated mice at day 20 post infection but not in any of the Fo-CR treated mice (Fig. 1D). Colonic crypt length was significantly ($p < 0.01$) increased in the untreated mice after *C. rodentium* infection, but those of Fo-CR-treated mice were comparable with the uninfected controls (Fig. 1E). These results indicate that the Fo-CR treatment promoted clearance of *C. rodentium* from the gut and inhibited inflammation caused by *C. rodentium* colonization.

3.2. Protective effect of the oral administration of inactivated C. rodentium on a murine model of lethal C. rodentium infection

Next, we tested whether the oral administration of Fo-CR could also protect C3H/HeN mice from a lethal *C. rodentium* challenge. Infection of the C3H/He strain results in a high mortality rate because of fatal infectious diarrhea (Papapietro et al., 2013). Protection was evaluated based on survival, body weight loss, fecal shedding, and bacterial invasion to visceral organs. In untreated mice, 67.5% of mice succumbed to the infection by day 14 post infection, whereas all Fo-CR-treated mice survived throughout the experimental period (Fig. 2A). In addition, the Fo-CR-treatment significantly ($p < 0.05$) inhibited body weight loss (Fig. 2B). The number of *C. rodentium* in the feces of the Fo-CR-treated mice tended to be lower than that in the untreated mice (Fig. 2C). To investigate the effect of Fo-CR treatment on *C. rodentium* invasion across the intestinal epithelial barrier, the number of viable *C. rodentium* in the livers and spleens were counted. We found that the Fo-CR treatment significantly ($p < 0.01$ for liver and $p < 0.05$ for spleen) reduced the number of viable *C. rodentium* in these organs (Fig. 2D), suggesting that invasion of the bacteria across the intestinal epithelial barrier was partially inhibited in the Fo-CR-treated mice. We also analyzed *C. rodentium*-specific fecal IgA and serum IgG responses. Although, the Fo-CR treatment failed to induce significant fecal IgA and serum IgG responses even after the final dose of immunization, the Fo-CR-treated mice showed significantly ($p < 0.05$) higher levels of *C. rodentium*-specific fecal IgA titers at 1-week post infection than the untreated mice (Fig. 2E). *C. rodentium*-specific serum IgG levels in the untreated and Fo-CR-treated mice were comparable (Fig. 2E). The avidity index, as measured by ELISA, of *C. rodentium*-specific serum IgG in the Fo-CR-treated mice was significantly ($p < 0.01$) higher than that in the untreated mice (Fig. 2F), suggesting that the Fo-CR treatment had a priming effect. These results demonstrated that the oral administration of Fo-CR also provided the effective protection against lethal *C. rodentium* infection in C3H/HeN mice.

To characterize the protective antigens of inactivated *C. rodentium*, the protective effects of inactivated CR prepared by UV treatment and heating were tested. C3H/HeN mice were orally administrated with UV-inactivated CR (UV-CR) weekly for 4 times and then infected with *C. rodentium*. The UV-CR treatment did not protect mice from succumbing to *C. rodentium* infection (Supplement Fig. 2A). In addition, the number of *C. rodentium* in the feces was comparable between the UV-treated and untreated mice (Supplement Fig. 2B). Similarly, heat-inactivated CR treatment did not inhibit body weight loss, fecal shedding of bacteria or bacterial invasion to the visceral organs (Supplemental Fig. 3A, 3B, and 3C). These results suggest that the protective antigens of inactivated whole-cell *C. rodentium* are components that are unstable under heating and their conformational structures, which could be stabilized by formalin-, but not UV-, treatment, are important for the protective effect of oral immunization with the inactivated whole-cell *C. rodentium*.

4. Discussion

C. rodentium infection models have been used as a tool to assess the proof of concept of anti-EHEC/EPEC vaccine candidates as *C. rodentium* shares the same mechanism of infection and many virulence genes with EHEC/EPEC. Recently, several novel vaccine platforms have been reported, such as the use of antigen-expressing recombinant non-pathogenic lactic acid bacteria as live vectors for the mucosal delivery of antigens (Ferreira et al., 2011). However, as there is little information available on the comparative effectiveness of these novel mucosal vaccine platforms with vaccines prepared by standard or conventional methods, the true efficacies of these vaccines remain unclear. As one conventional method, inactivated Enterotoxigenic *E. coli* (ETEC) vaccine consisting of formalin-inactivated *E. coli* that expresses most common colonization factor antigens and recombinantly produces the cholera toxin B subunit was examined in human trials (Åhrén et al., 1998; Jertborn et al., 1998). Sur et al. also reported the protective efficacy of an inactivated whole-cell oral cholera vaccine in a human trial (Sur et al., 2011). These reports suggested that oral vaccination with an inactivated whole-cell bacterium has a certain protective efficacy. Therefore, we prepared formalin-inactivated whole *C. rodentium*, investigated the effects of this vaccine, and demonstrated its protective effect on non-lethal and lethal *C. rodentium* infection in mouse models.

In the non-lethal infection model, oral Fo-CR administration significantly induced mucosal and systemic antibody responses specific to *C. rodentium,* promoted bacterial clearance, and inhibited colonic hyperplasia. In addition, in the lethal infection model, the Fo-CR treatment showed significant protective effects, including inhibition of death, body weight loss, and invasion of the bacteria across the epithelial barrier to the visceral organs. In contrast to C57BL/6 mice, *C. rodentium*-specific antibody responses were not detected in the C3H/HeN mice immunized with Fo-CR before challenge. However, the Fo-CR-treated mice showed higher *C. rodentium*-specific mucosal IgA titers and serum IgG avidity than did the untreated mice after challenge. These results suggest that vaccination with Fo-CR has significant priming effect. Although numerous studies set their research endpoints on antigen-specific IgG and IgA antibody responses without challenge experiments, the protective effects of vaccination cannot be predicted by antibody responses alone, particularly in infectious diseases for which vaccination strategies have not been established.

As the T3SS components are required for intimate attachment of *C. rodentium* to colonic epithelial cells, they are thought to be promising antigens for vaccination (Rojas-Lopez et al., 2018). Previous reports demonstrated that antibodies, especially IgG, specific for the T3SS components induced by *C. rodentium* infection play an important role in the clearance of the bacteria (Kamada et al., 2015; Maaser et al., 2004). However, our culture conditions for the preparation of inactivated *C. rodentium* did not induce expression of the T3SS components as such expression requires culturing of the bacteria in media containing bicarbonate, such as DMEM (Crepin et al., 2016; Yang et al., 2010). Therefore, the protective antigens in Fo-CR that induce protective

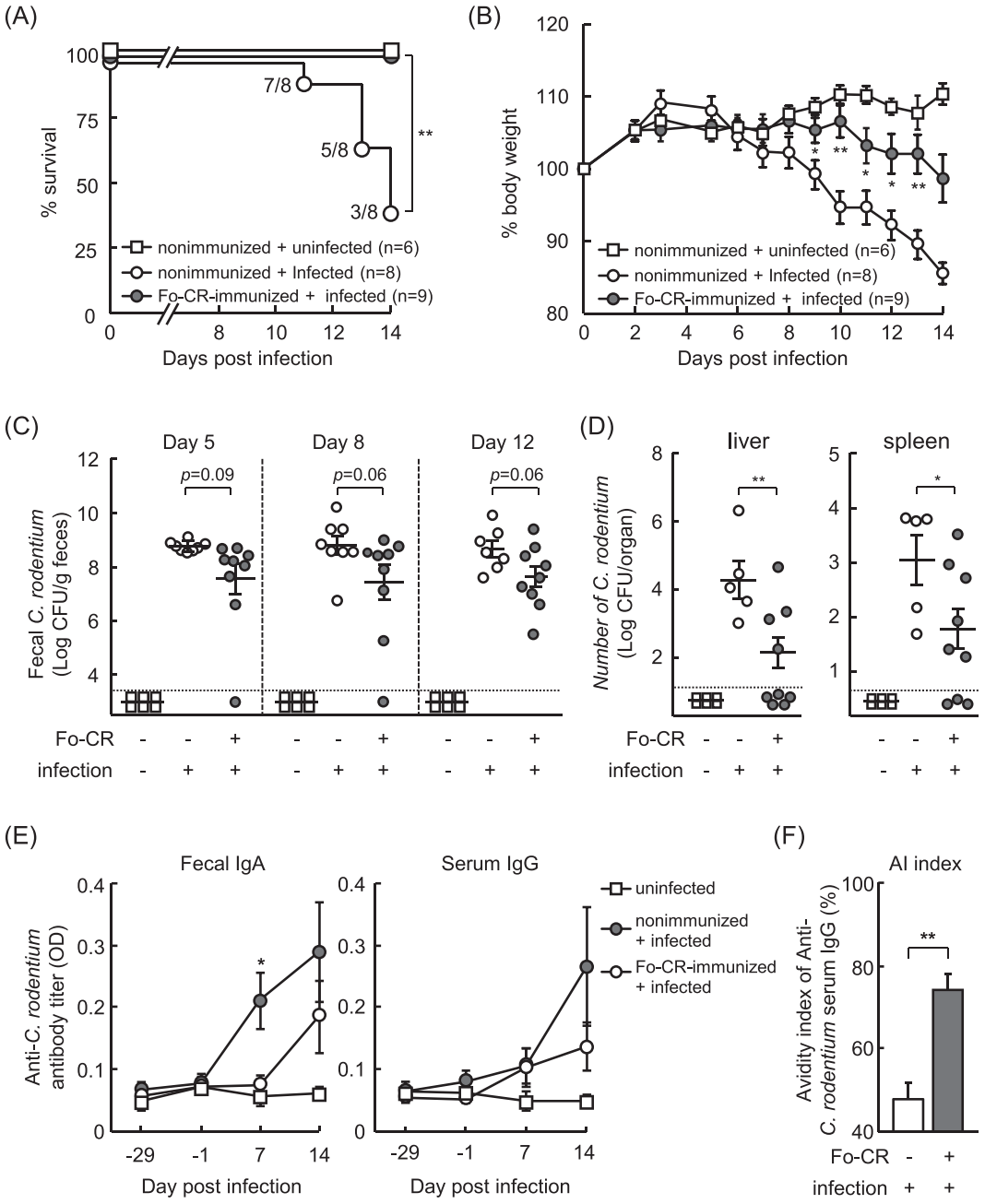

Fig. 2. Protective effects of the oral administration of formalin-inactivated *C. rodentium* on a murine model of lethal *C. rodentium* infection. C3H/HeN mice orally administrated with Fo-CR weekly for 4 weeks or nonimmunized were infected with 2×10^8 CFU of *C. rodentium*. (A) Survival curves of mice without any treatment (nonimmunized + uninfected, n = 6), nonimmunized mice with infection (nonimmunized + infected, n = 8), and Fo-CR- immunized mice with infection (Fo-CR-immunized + infected, n = 9)(up to day 14 post infection). (B) Body weights of the mice shown in the panel A (n = 3 to 9). Means ± SEMs are shown. (C) Numbers of *C. rodentium* in freshly isolated fecal pellets obtained from the mice shown in the panel A were quantified at days 5, 8, and 12 post infection. Each symbol in the panels C and D represents data obtained from one mouse. Means ± SEMs are shown. Horizontal dotted lines indicate the detection limit. (D) Bacterial loads in the liver and spleen of the mice shown in the panel A at day 14 post infection. Means ± SEMs are shown. Horizontal dotted lines indicate detection limit. (E) *C. rodentium*-specific fecal IgA and serum IgG titers of uninfected control mice and nonimmunized or Fo-CR-immunized mice at days −29, −1, 7 and 14 post infection were determined by ELISA. Means ± SEMs are shown. (F) Avidity index, as measured by ELISA, of *C. rodentium*-specific serum IgG of nonimmunized + infected (n = 3) and Fo-CR-immunized + infected (n = 5) mice at day 14 post infection. Means ± SEMs are shown. Results of two independent experiments were combined and shown. *; $p < 0.05$, **; $p < 0.01$ by Log-rank test (survival) and one-way ANOVA followed by Tukey's test (body weights, *C. rodentium* loads, antibody titers).

immune responses are considered to be component(s) other than the T3SS components. We compared the protective effect of inactivated CR prepared by three different methods (formalin, UV, or heating) to investigate the nature of the antigens responsible for the protective effects of inactivated CRs and found that only formalin-treated CR have protective effects on *C. rodentium* infection. These results suggest that the protective antigens contained in inactivated CR are proteins, but not carbohydrates, such as lipopolysaccharides, as the antigens were unstable under heating and their conformational structures are critical for their protective effects. Future analysis to clarify the protective mechanisms of Fo-CR will help the development of effective oral EPEC/EHEC vaccines.

In conclusion, oral vaccination with Fo-CR can serve as a reference method to screen oral vaccine candidates or novel oral vaccine

platforms and evaluate their efficacies in *C. rodentium* infection models.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical statement

All animal experiments were approved by the institutional Animal Care and Use Committee (Approval numbers for the vaccination and infection experiments: 2012-163, 2014-044, 2014-150, and 2017-242).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.02.016.

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