Microbiology and Immunology

Microbiol Immunol 2016; 60: 168–176
doi: 10.1111/1348-0421.12363

ORIGINAL ARTICLE

Virulence of representative Japanese *Francisella tularensis* and immunologic consequences of infection in mice

Akitoyo Hotta[1], Osamu Fujita[1], Akihiko Uda[1], Yoshie Yamamoto[1], Neekun Sharma[1,2], Kiyoshi Tanabayashi[1], Akio Yamada[3] and Shigeru Morikawa[1,2]

[1]Department of Veterinary Science, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku, Tokyo 162-8640, [2]United Graduate School of Veterinary Science Gifu University, 1-1 Yanagido, Gifu 501-1193 and [3]Laboratory of Veterinary Public Health, Graduate School of Agricultural and Life Sciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo, Tokyo 113-8657, Japan

ABSTRACT

***Francisella tularensis*, which causes tularemia, is widely distributed in the Northern hemisphere. *F. tularensis* strains isolated in Japan are genetically unique from non-Japanese strains; however, their phenotypic properties have not been well studied. Thus, mice were infected with representative Japanese strains of *F. tularensis* and their virulence and mouse immune responses to them assessed. Of four representative Japanese strains, the Ebina, Jap and Tsuchiya strains were susceptible to H_2O_2 and did not grow well intracellularly. Only Yama strain grew intracellularly and was lethal to mice. Infection with Yama strain resulted in drastic increases in IFN-γ, CD4 and CD8 double-positive T cells and Th1 cells (CD3, CD4 and Tim3-positive cells), and a decrease in the ratio of CD8-positive CD4-negative T cells in mice. C57BL/6J mice that survived infection produced IgM antibodies to LPS and IgG2c antibodies to 43, 19 and 17 kDa proteinase K-sensitive components. These data are valuable for understanding the phenotypic properties of *F. tularensis* in Japan.**

Key words *Francisella tularensis*, immune response, Japan, virulence.

Francisella tularensis, which causes tularemia, is a highly infectious and virulent intracellular gram-negative bacterium that has three subspecies: *tularensis*, *holarctica*, and *mediasiatica*. Subspecies *tularensis* and *holarctica* are commonly associated with human disease. Subspecies *tularensis* is found only in North America and causes a severe acute disease that is often fatal (1). It is further separated into some distinct clades genetically and these clades have been shown to differ in their virulence in mice (2). Subspecies *holarctica*, found throughout the northern hemisphere, causes a milder and less frequently fatal disease. It is further divided into three biovars (I, II and *japonica*) based on susceptibility to erythromycin, biochemical properties, the isolated areas and genetic properties (1, 3).

In Japan, tularemia was first reported as "hare meat poisoning" in 1837 (4) Approximately 1400 cases of human tularemia have been reported since 1924, however the incidence has become extremely low since the 1990s (5). *F. tularensis* was first isolated in 1926 and more than 100 strains have now been isolated from humans, hares, ticks and shrew moles (6). The representative strains Ebina, Jap, Tsuchiya and Yama have been studied by several researchers (7, 8) and are discriminated as subspecies *holarctica* biovar *japonica*. Biovar *japonica* is considered to be an evolutionary intermediate between subspecies *tularensis* and *holarctica* biovar I and II (9); however, virulence has not been compared among subspecies of *holarctica*.

Correspondence
Akitoyo Hotta, Department of Veterinary Science, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku, Tokyo 162-8640, Japan. Tel: +81 3 5285 1111, fax: +81 3 5285 1179, email: ahotta@nih.go.jp

Received 23 November 2015; revised 19 January 2016; accepted 2 February 2016.

List of Abbreviations: dpi, days post inoculation; Cy, cyanin; CDM, Chamberlain's chemically defined minimal medium; GM-CSF, granulocyte-macrophage colony-stimulating factor; hpi, hours post-inoculation; i.d., intradermal; i.n., intranasal; i.p., intraperitoneal; LVS, live vaccine strain; MH, Mueller–Hinton; NIID, National Institute of Infectious Diseases; PE, phycoerythrin; WB, western blotting.

The virulence of *F. tularensis* has been mainly studied in Schu strain and LVS, as references for subspecies *tularensis* and *holarctica* biovar II, respectively; these strains are known to be less virulent than the wild-type strain (10). The virulence of other strains, including Japanese strains, has not been studied in detail. In this study, to further explore the virulence of subspecies *holarctica* biovar *japonica*, their ability to grow, resistance to oxidative stress and serum complement-mediated killing, and lethality to mice were compared. In addition, the immunologic consequences of infection in mice were analyzed.

MATERIALS AND METHODS

Bacteria strains

Five strains of *F. tularensis* subspecies *holarctica* biovar *japonica* (Ebina, Jap, Tsuchiya, Yama and Kato) were kindly provided by Dr. H. Fujita, Ohara Research Laboratory, Fukushima, Japan. Ebina, Jap, Tsuchiya and Yama strains were isolated more than 50 years ago (6) and are distributed in several laboratories (7, 9). Additionally, Kato strain, the most recent clinical isolate, was tested as a control wild-type strain. The origins and sources of these strains are shown in Table 1; however, their passage histories are unknown. The bacteria were cultured on chocolate Difco Eugon agar (Becton Dickinson, Sparks, MD, USA) with 8% (v/v) sheep blood and incubated at 37°C in a biosafety level 3 laboratory in the NIID.

Determination of bacterial growth curves in broth medium

Bacterial growths of each strain were evaluated in a broth of CDM (11) and cation-adjusted MH medium (Difco, Detroit, MI, USA) supplemented with 0.025% (w/v) ferric pyrophosphate, 0.1% (v/v) glucose, and 2% (v/v) IsoVitaleX (Becton Dickinson) (MH) (12). Bacteria cultured on chocolate Eugon agar plates for 48 hr were suspended in 10% (v/v) glycerol-containing CDM broth medium at an OD_{600} of 1.0. The suspension was inoculated into 3 mL of each broth medium in 16 mL screw cap tubes. The tubes were incubated at 37°C with shaking at 200 rpm and OD_{600} values measured.

Table 1. Francisella tularensis strains tested in this study

	Isolated			
Strain	Year	Source	Area (Pref.)	Comment
Jap	1926	Human	Fukushima	First isolate in Japan
Ebina	1950	Human	Miyagi	Only Japanese strain sequenced genome opened
Yama	1957	Ticks	Fukushima	Used as antigen for serological test in Japan
Tsuchiya	1958	Human	Fukushima	
Kato	1989	Human	Yamagata	Latest clinical isolate in Japan

Bactericidal assays

For the H_2O_2 susceptibility test, approximately 10^8 CFU of bacteria were inoculated into 0.01 mol/L PBS (Wako, Osaka, Japan) with or without 0.01% (v/v) H_2O_2 (Nacalai Tesque, Kyoto, Japan). The mixtures were incubated for 4 hr at room temperature without shaking (13). Thereafter, the bacterial CFU in each mixture was determined. For serum complement-mediated killing assay, bacteria were incubated with PBS containing either 50% (v/v) fresh or heat-inactivated (negative control) normal guinea pig sera for 1 hr at 37°C with slow agitation, after which viable bacteria were enumerated. *Escherichia coli* DH5α was used as a positive control for serum complement activity (14).

Cell culture infection

J774.1 murine macrophages-like cells (RCB0434) (RIKEN Bioresource Center, Ibaraki, Japan) were propagated in RPMI1640 medium (Wako) containing 10% (v/v) heat inactivated FBS at 37°C in 5% CO_2. The cells were grown on 24-well plates (1×10^5 cells per well) and inoculated with bacteria at an indicated MOI, this point being designated as time 0. After incubation for 1 hr, the cells were washed and 1 mL of fresh 10% FBS-RPMI1640 with gentamicin (10 μg/mL) added into the wells. After 2 and 24 hr of inoculation, the cells were washed twice with PBS and treated with 100 μL of 1% (w/v) saponin in distilled water for 5 minutes to lyse them. To measure viable bacteria, the lysed cells were serially diluted with saline and aliquots of each dilution cultured on Chocolate II agar (BD, Fukushima, Japan). After incubation for 3 days, bacterial CFU were determined. Bacterial enumeration was performed with triplicated samples in at least two independent experiments.

Inoculation of mice

Specific-pathogen-free 6-week-old male BALB/c (24–28 g body weight) and C57BL/6J mice (20–24 g) were purchased from Japan SLC (Shizuoka, Japan). All mice were housed in cages with free access to food and water. C57BL/6J mice were administered 10^2 CFU of bacteria/25 μL i.d. ($n = 7$ or 8/group). BALB/c mice were administered 10^0 or 10^2 CFU of bacteria/100 μL i.p.

or /20 μL i.n., respectively ($n = 3$/group). The inoculated doses were confirmed by plating at each inoculation. Signs of disease and the body weights of individual mice were monitored daily until 17 dpi; the mice that had survived were killed at 20 or 21 dpi. *F. tularensis* infection was confirmed by serological tests in the mice that had survived. For time course analyses, the mice that had been inoculated with 10^2 CFU of Yama by the i.p. route were killed at 48 and 96 hpi. The experiments were performed at animal biological safety level 3 in strict accordance with the Animal Experimentation Guidelines of NIID and the protocols were approved by the Institutional Animal Care and Use Committee of NIID (Permit Nos. 106049, 107077 and 111116).

Serological tests

Serum antibody to *F. tularensis* was analyzed by ELISA and WB. These assays were essentially performed as described previously (15, 16) with slight modifications. In each assay, sera from normal mice were used as a negative control. HRP-labeled anti-mouse IgG1, IgG2a, IgG2b, IgG2c, IgG3 (Southernbiotech, Birmingham, AL, USA), IgM (Santa Cruz Biotechnol, Santa Cruz, CA, USA), and IgA (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) antibodies were used for detecting antibody reactions. In the ELISA, 2,2′-azinobis (3-ethylbenthiazolinesulfonic acid) peroxidase substrate (Sigma-Aldrich, Tokyo, Japan) was used for color development. In WB analysis, Precision Plus Protein Prestained Standards (BioRad, Hercules, CA, USA) were used as molecular weight markers.

Flow cytometric analysis

Mouse blood leucocyte numbers were quantified by using a Flow cytometer Epics XL (Beckman-Coulter, Hialeah, FL, USA) and EXPO32 software (Beckman-Coulter) as described in the manufacturer's instructions. Blood samples were collected in sterile tubes containing EDTA and the number of cells counted with a hematology analyzer, pocH-100 (Sysmex; Kobe, Japan). After adding five volumes of sterilized distilled water to the blood, the mixture was suspended with an equal volume of 2 × PBS and then centrifuged. The blood cells in the pellet were stained for 1 hr at 4°C with the following monoclonal antibodies: R-PE-anti-Tim3 (clone 8b.2c12), PE-Cy7-anti-CD4 (clone GK1.5), PE-anti-CD4 (clone GK1.5), PE-Cy5-anti-CD3 (clone 145-2c11) and PE-Cy7-anti-CD8 (clone 53-6.7). All antibodies were purchased from eBioscience (San Diego, CA, USA). After staining, OptiLyse C (Beckman-Coulter) was added to fix lymphocytes. Analysis gates selected were morphologically based on side scatter and forward scatter settings, which are typically used for lymphocytic analysis, and then gated based on the mouse CD3 marker-positive cells to analyze the T-lymphocytic compartment. To control for background and establish thresholds for gating positive cells, appropriate isotype control antibodies were used. Results are expressed as percentage of gated cells.

Multiplex analysis of cytokines in mouse sera

Concentrations of GM-CSF, IFN-γ, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-17 and TNF-α in individual mouse sera (25 μL) were determined in duplicate by flow cytometry using a FlowCytomix mouse Th1/Th2 10plex bead kit against known standard curves and according to the manufacturer's instructions (Bender MedSystems, Austria, Vienna). Data were analyzed using a flow cytometer Epics XL and EXPO32 software as described in the manufacturer's instructions.

Statistical analysis

Significance was assessed with GraphPad Prism ver. 6.0c Software (La Jolla, CA, USA). Numerical values for undetectable data in bacterial burden and cytokine measurement were input as half of the relevant detection limit. Graph data are presented as means ± SEM. Differences between groups were considered statistically significant for P values <0.01.

RESULTS

Growth in broth medium

Biological properties concerning virulence of *F. tularensis* were compared. Growth kinetics of each strain were measured in both CDM and MH broth media. Although Ebina and Yama strains and the reference strain Kato grew well in both CDM and MH between 4 and 16 hpi whereas Jap and Tsuchiya did not grow in CDM (Fig. 1a). Additionally, Jap and Tsuchiya strains were not able to multiply in CDM whereas the other strains multiplied approximately 100-fold during 24 hr of cultivation (data not shown).

Bactericidal activity

In the presence of 0.01% (v/v) of H_2O_2, more than 99.9% of organisms of Jap and Ebina strains were killed within 4 hr, whereas 16.8% and 67.1% of those of Tsuchiya and Yama strains, respectively, survived. On the other hand, Kato strain was not affected by this treatment. In contrast, bacterial numbers in the presence of 50% fresh or heat-treated serum were no different for the five

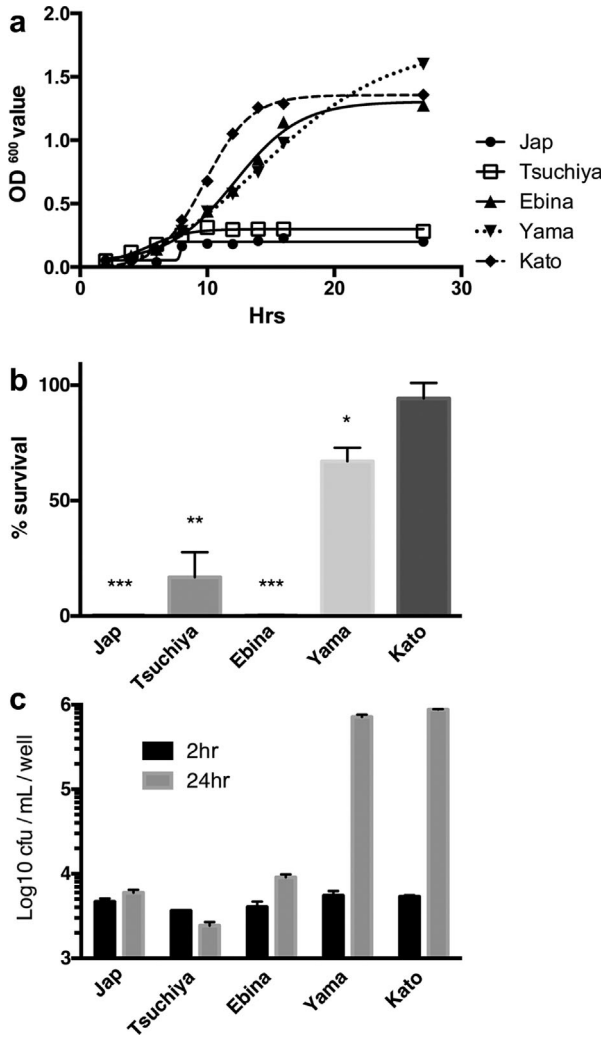

Fig. 1. Comparison of properties of Japanese *Francisella tularensis* strains. (a) Growth curve of each Japanese strain in CDM broth medium. Bacterial growth is shown by OD_{600} values of the culture at the indicated times after starting the culture. Boltzmann sigmoidal semi-logistic regression was used to fit the line from the data based on at least triplicate observations within a representative of three independent experiments. (b) Survival of Japanese strains after exposure to H_2O_2. Each strain was diluted to approximately 10^8 CFU/mL in PBS, and thereafter exposed to 0.01% of H_2O_2 for 4 hr at room temperature. Percent survival was calculated using the mean of the results obtained with duplicate samples and bars show the mean ± SEM (error bars) based on the average of survival rates of each strain from four experiments. *, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.0001$. (c) Intracellular growth of Japanese strains in J774.1 cells. Ebina, Jap, Tsuchiya, Yama and Kato strains were inoculated at MOI of 48, 12, 78, 48 and 36, respectively. The cells were lysed at 2 and 24 hpi and intracellular bacteria enumerated. Results are shown as mean ± SEM (error bars) based on at least triplicate observations within a representative of three independent experiments.

strains (data not shown), indicating that all the strains tested are resistant to serum complement-mediated killing.

Intracellular growth

Because growth in macrophages is known to correlate to some degree with virulence of *F. tularensis* (17), intracellular growth kinetics of each strain in J774.1 cells was analyzed. Yama and Kato strains replicated approximately 100-fold during 22 hr in the cells, whereas the other three strains (Ebina, Jap and Tsuchiya) did not. Figure 1c shows representative results obtained at an MOI of 10^1. These results indicate that Ebina, Jap and Tsuchiya strains cannot multiply intracellularly. Similar results were also observed when the bacteria were inoculated at MOIs of 10^0 and 10^2 (data not shown).

Comparison of virulence to mice by intradermal inoculation

Data from our *in vitro* experiments (Fig. 1) indicated that Jap, Tsuchiya and Ebina strains are less virulent than Yama strain. Because Ebina strain has been used as a standard Japanese strain and Yama has been used as an antigen for serological testing (Table 1), C57BL/6J mice were inoculated with Ebina and Yama strains by the i.d. route ($n = 8$/group) to compare their virulence. Four of seven mice inoculated with Yama strain (174 CFU) died or became moribund within 10 dpi (Fig. 2). The mice that survived lost body weight up to 10 dpi and recovered thereafter. All the mice inoculated with Ebina (200 CFU) that survived up to 20 dpi showed *F. tularensis*-specific antibody responses by ELISA (data not shown), indicating that they had been successfully infected with *F. tularensis*. On the other hand, all eight mice inoculated with reference strain Kato (300 CFU) died or became moribund within 7 dpi.

Virulence of Yama strain by intraperitoneal and intranasal inoculations

Among the four representative strains, only Yama strain was found to be virulent to mice. Thus, the virulence of Yama was further analyzed by i.p. and i.n. inoculations. Inoculation of 10^2 CFU of the bacteria was completely lethal to mice by both routes, whereas inoculation of 10^0 CFU was partially lethal (Fig. 3). Mice inoculated with 130 CFU of the bacteria by the i.p. route died or became moribund at 5–6 dpi. Two mice that had been inoculated with 3 CFU by the i.n. route and survived were sero-negative at 20 dpi (data not shown), indicating that infection had not been achieved in these mice. On the other hand, one mouse that recovered from weight

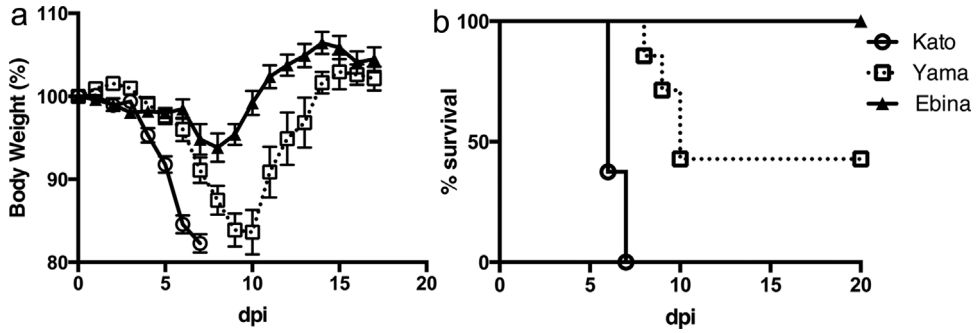

Fig. 2. Body weight and survival rate of mice infected with Japanese *Francisella tularensis* strains. C57BL/6J mice ($n = 7$ or 8) were inoculated i.d. with 10^2 CFU of Ebina, Yama or Kato strains. (a) The graph shows the means ± SEM (error bars) of body weight relative to day 0, starting with seven or eight mice per group at the beginning of the experiment. (b) Kaplan–Meier curve showing survival of mice.

loss following 1 CFU inoculated i.p. was sero-positive at 20 dpi (data not shown).

Antibody responses in the mice that survived after infection

Antibody responses of the infected C57BL/6J mice were analyzed by WB. A mixture of sera obtained from the mice that had been inoculated by the i.d. route with Yama ($n = 3$), reacted with whole cell lysates of the Yama strain. Among five anti-mouse IgG subclass antibodies tested, the strongest reactions were observed with anti-mouse IgG2c antibody, which reacted with whole cell lysate with bands appearing as 43, 19 and 17 kDa. No reactions were detected when the antigens were pre-treated with proteinase K, indicating that the IgG antibodies reacted with bacterial protein. IgM antibodies reacted in a ladder-like banding pattern (Fig. 4) that was not affected by pre-treatment with proteinase K, suggesting that the IgM antibodies recognize LPS antigen (data not shown). Similar reactions were also observed for a mixture of serum from mice that had survived inoculation with Ebina ($n = 8$) (data not shown). Although insufficient samples were available for analysis of significance, the dominant antibodies in the serum of the BALB/c mouse that survived ($n = 1$) were IgG2a and IgG3 (data not shown).

Bacteriological examination of Yama strain-infected mice

To determine cytokine and peripheral lymphocyte responses, the three mice inoculated with 10^2 CFU of Yama by the i.p. route were analyzed (Fig. 3). None of their organs showed macroscopic evidence of damages until 48 hpi, but at 96 hpi, enlargement of the spleen, discoloration of the liver, and peritoneal fibrous adhesions were observed in all the infected mice (data not shown). The bacteria were detected at 48 hpi in the spleen but not in blood. At 96 hpi, increased numbers of bacteria were observed in both the spleen and blood (Fig. 5a).

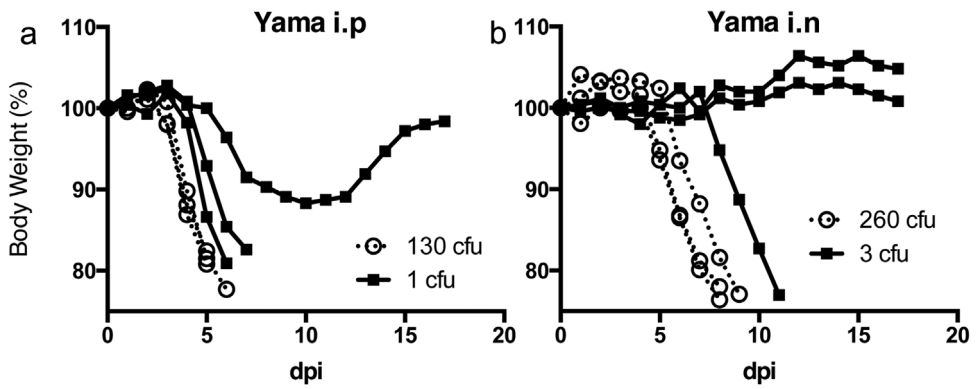

Fig. 3. Body weight of mice infected with *Francisella tularensis* Yama strain. BALB/c mice ($n = 3$) were inoculated with 10^0 and 10^2 CFU of bacteria i.p. or i.n. Relative body weights of individual mice are shown in both graphs.

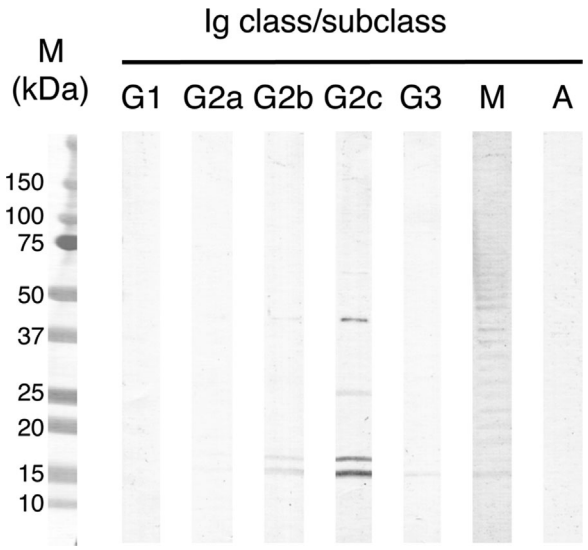

Fig. 4. Ig class/subclass in mice reactive with *Francisella tularensis* antigen. Western blot prepared with a mixture of sera from mice ($n = 3$) that had survived for 20 days after the i.d. inoculation of 174 CFU of *F. tularensis* Yama strain of antibody production of IgG (1, 2a, 2b, 2c, and 3), IgM, and IgA. HRP-labeled anti-mouse antibodies for detecting the reactions are indicated above each strip. M, molecular weight marker.

Cytokines quantification in sera of Yama strain infected mice

Among 10 cytokines analyzed, IL-6 responses varied among the three mice, serum concentrations being 176.6, 815.9 and 2147.3 pg/mL, respectively. On the other hand, there were significant increases in IFN-γ ($P < 0.0001$), serum concentrations being 619.2, 879.7 and 972.1 pg/mL, respectively, at 96 hpi (Fig. 5b). The other eight cytokines did not increased within their detectable ranges. The detection limits for each cytokine assayed were as follows: 1.7 pg/mL for IFN-γ, 6.1 pg/mL for IL-1α, 7.9 pg/mL for IL-4, 7.6 pg/mL for IL-5, 14.9 pg/mL for IL-6, 5.0 pg/mL for GM-CSF, 3.4 pg/mL for TNF-α, 208.1 pg/mL for IL-2, 137.8 pg/mL for IL-17 and 335.1 pg/mL for IL-10.

T cell numbers in blood of Yama strain infected mice

In flow cytometric analyses, no significant changes in CD3-positive lymphocyte counts in blood were observed at 48 hpi; however, increases in population of CD4 and CD8 double-positive T cells and Th1 cells (CD4 and Tim3-positive cells) and a decrease in CD8-positive CD4-negative T cells were observed at 96 hpi. Figure 5c shows subset percentages for the indicated cells among the gated CD3-positive lymphocytes. Although at 48 hpi the populations of CD4 and CD8 double-positive T cells in the infected and control mice were <2.1%, they had increased significantly at 96 hpi to 16.9%, 20.2% and 25.4%, respectively ($P = 0.0001$). Similarly, the Th1 cell population increased significantly from <3.1% to >27.8% ($P < 0.0001$), whereas the CD8-positive CD4-negative T cell population decreased from >24.6% to <1.1% ($P < 0.0001$). At all timepoints, the dominant lymphocytes in the blood were CD4-positive CD8-negative T cells

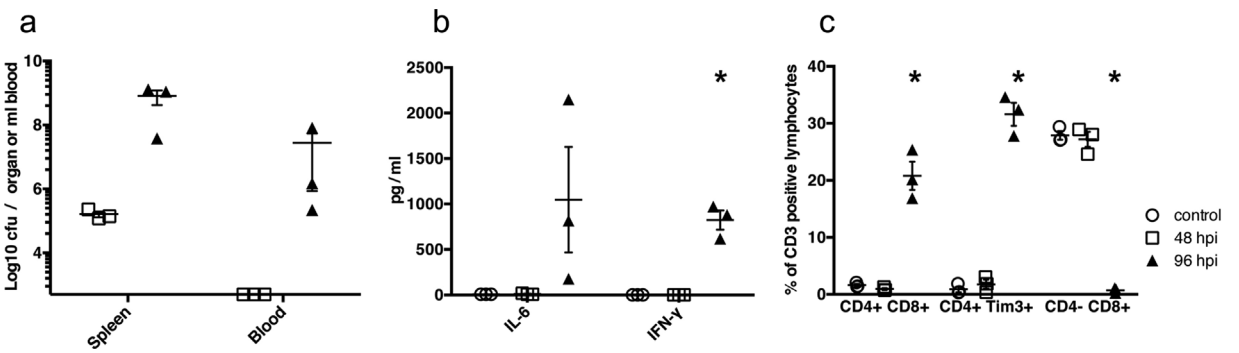

Fig. 5. Growth of bacteria and immunological responses of mice inoculated with *Francisella tularensis* Yama strain. BALB/c mice ($n = 3$/group) were inoculated i.p. with 328 CFU of *F. tularensis* Yama. The mice were euthanized at 48 and 96 hpi, and the spleen and blood were collected. A group of saline-inoculated mice (n = 3) euthanized at 96 hpi were served as a negative control. (A) Bacterial burdens in the spleen and blood were determined and are expressed as total CFU per spleen or blood. Samples from control mice were not determined. Bacterial burden in blood at 48 hpi were below the detection limit (100 CFU). (B) Cytokine responses in sera of the inoculated mice were analyzed. Data shown are for IL-6 and IFN-γ but the other eight cytokines measured, including GM-CSF, IL-1α, IL-2, IL-4, IL-5, IL-10, IL-17, and TNF-α, are not shown. Quantities of IL-6 in sera of negative control mice and two mice in the 48 hpi group were below the detection limit (14.9 pg/ml). Quantities of IFN-γ in sera of negative control mice, and of mice at 48 hpi were below the detection limit (1.7 pg/ml). (C) Flow cytometric analysis of cell population of gated CD3-positive T lymphocytes in the blood of mice. Results for individual mice are shown in all three graphs. The data below the detection limit are not shown on the graphs. Bars indicate mean ± SEM. Statistical significance was determined by one-way ANOVA (*$p \leq 0.0001$).

(ranging 62.1–68.9%). Additionally, the population of CD4 and CD8 double-negative T cells did not change significantly (range 4.1–8.2%).

DISCUSSION

Because cases of tularemia in humans are rare and manifested by relatively mild symptoms in Japan and *F. tularensis* subspecies *holarctica* biovar *japonica* is considered to be unique from isolates from other areas (7, 18), phenotypic assessment of *F. tularensis* in Japan is interesting. The aim of this study was to determine the virulence of representative Japanese strains.

To begin with, we compared ability to grow in broth medium and in J774.1 cells and resistance against oxidative stress and serum complement-mediated killing between the selected Japanese strains. We found that Jap and Tsuchiya strains are not able to grow in CDM (Fig. 1a), whereas these strains can grow efficiently in MH (data not shown). In the preliminary experiments, both Jap and Tsuchiya strains grew well in tryptic soy and BHI-based modified broth medium (19) and in MH. These three broth mediums are nutrient-rich compared with CDM (11). Thus, beef extract and casein, major constituents of MH, may be necessary for the growth of Jap and Tsuchiya strains. According to Carlson *et al*., one major difference between CDM and MH is the presence of a high level of spermine in the CDM (20), a polyamines that is produced only by eukaryotes (21). It is likely that spermine affects the growth of Jap and Tsuchiya strains and that this indicates environmental differences.

H_2O_2 susceptibility tests showed that Jap and Ebina strains are susceptible to oxidative stress, whereas Tsuchiya and Yama strains are partially resistant (Fig. 1b). It is possible that the susceptible strains are unable to express enzymes that metabolize and neutralize reactive oxygen species, such as catalase (22), glutathione peroxidase (23) and MoxR ATPase (24). H_2O_2 is a bactericidal molecule that can be produced by macrophages. Given that successful intracellular growth of *F. tularensis* depends on the ability to escape from the host cell endosome, Jap and Ebina strains may not grow intracellularly. This hypothesis was confirmed by the bacterial burden in J774.1 cells at 2 and 24 hpi (Fig. 1c). Taken together, the results of *in vitro* experiments suggest that three of the four representative strains are less virulent than the reference strain Kato. Although we did not test Jap and Tsuchiya strains, these strains would also be less virulent than Yama strain. Growth in CDM and H_2O_2 resistance may be one of the markers of virulence in Japanese *F. tularensis*.

Results of infection of mice suggest that *F. tularensis* Yama strain injected into the abdominal cavity invades the spleen within 48 hpi and then spreads to blood by 96 hpi (Fig. 5a). During bacterial spreading, we observed changes in blood cytokine concentrations and cell numbers. Although the range of IL-6 concentrations in mice killed at 96 hpi was broad, this difference did not correspond with other obtained in each mouse, such as bacterial burden in the spleen or blood. Increases in IFN-γ concentrations in blood have also been reported in mice infected with Korean *F. tularensis* (25) and subspecies *tularensis* prototype strain Schu (26). Given that Th1 cells secrete IFN-γ to fight intracellular pathogens, we consider the correlation between increases in IFN-γ and Th1 cell (CD4- and Tim3-positive cells) populations at 96 hpi (Fig. 5c) unsurprising.

Our finding of increased numbers of CD4 and CD8 double-positive T cells in blood (Fig. 5c) may be related to Chen *et al*.'s reported observation of depletion of CD4 and CD8 double-positive T cells in the thymuses of infected mice (27). Similarly, the decrease in CD8-positive CD4-negative T cells (Fig. 5c) may be attributable to recruitment of the cells observed by Rasmussen *et al*. (28). They reported an increase in CD3- and CD8-positive cells in the spleens of infected mice. Because we did not assess leukocyte populations in organs in this study, further studies are needed to better understand the circulation of leukocytes in infected mice. It seems likely that CD8-positive CD4-negative T cells are required to control *F. tularensis* infection and that CD4 and CD8 double-positive T cell counts may increase in blood to counteract the depletion of CD8-positive CD4-negative T cells.

The antibody response to *F. tularensis* infection in mice has not yet been well analyzed. Our WB results indicate that the mice that survived produced IgG antibodies against bacterial protein components and IgM antibodies against LPS (Fig. 4). Similar reaction patterns have been reported in mice immunized with membrane protein fraction antigens (29) and with catanionic surfactant vesicle vaccine (30), demonstrating their usefulness for both prophylactic and therapeutic purposes. IgM antibodies to *F. tularensis* LPS are reportedly important for the rapid generation of protective immunity (31). *F. tularensis* 43 kDa of FopA outer membrane protein (32), 19 kDa of FopB outer membrane protein (33) and 17 kDa of Tul4 lipoprotein (34) are known to be recognized by the humoral response to LVS (35, 33). The predominant reaction of IgG2c antibodies in the early stage of infection (Fig. 4) indicates induction of Th-1-mediated immune response in C57BL/6J mice (36, 37). Further analyses of the kinetics of antibody responses in mice are of interest.

Taken together, our results support those obtained in vaccinated mice by several researchers. Host–pathogen interactions of *F. tularensis* in Japan may not be different from those of *F. tularensis* from other areas.

Generally, *F. tularensis* is known to proliferate efficiently in CDM and cultured cells and to be resistant to 0.01% H_2O_2. However, we found that these properties are not the same in the four Japanese strains tested in this study. These differences may be attributable to mutation caused by serial passages in artificial media because such culture conditions are known to affect the characteristics of *F. tularensis* (38, 39). Ebina strain is the only Japanese strain whose whole genome sequence has been determined (40) and recovery of virulence by serial passages in mice will be possible as reported (41); analyzing other fresh Japanese isolates is also important. Recently, Molins *et al.* discussed the necessity of careful consideration of strain selection because subspecies *tularensis* Schu strain is less virulent than other subspecies of *tularensis* strains (10). Similarly, we propose a reconsideration regarding representative Japanese *F. tularensis* strain selection. Further studies comparing a fresh isolate and its serially *in vitro*-passaged mutants are now in progress.

ACKNOWLEDGMENTS

The authors thank Dr. H. Fujita (Ohara Research Laboratory, Ohara Research Laboratory, Fukushima, Japan) for his help in providing the bacterial strains. This study was supported by a Health Science Research Grant from the Ministry of Health, Labour and Welfare of Japan (H23-Shinkou-Wakate-022) and the Research Program on Emerging and Re-emerging Infectious Diseases from the Japan Agency for Medical Research and Development, AMED.

DISCLOSURE

The authors have no conflicts of interest to declare.

REFERENCES

1. Ellis J., Oyston P.C., Green M., Titball R.W. (2002) Tularemia. *Clin Microbiol Rev*, **15**: 631–46.
2. Molins C.R., Delorey M.J., Yockey B.M., Young J.W., Sheldon S. W., Reese S.M., Schriefer M.E., Petersen J.M. (2010) Virulence differences among *Francisella tularensis* subsp. *tularensis* clades in mice. *PLoS ONE* **5**: e10205.
3. Sjostedt A. (2007) Tularemia: History, epidemiology, pathogen physiology, and clinical manifestations. *Ann N Y Acad Sci* **1105**: 1–29.
4. Ohara H., Ichikawa K. (1962) The distribution of Yato-byo and its clinical observations. *Ann Rep Ohara Hosp (in Japanese)* **11**: 1–22.
5. Ohara Y., Sato T., Homma M. (1996) Epidemiological analysis of tularemia in Japan (yato-byo). *FEMS Immunol Med Microbiol* **13**: 185–9.
6. Fujita H. (1994) Short historical review of the isolates of tularemia agent in the early years of tularemia research in Japan with list of stock cultures of *Francisella tularensis* and other selected species in Ohara Research Laboratory. *Ann Rep Ohara Hosp* **37**: 5–12 (in Japanese).
7. Broekhuijsen M., Larsson P., Johansson A., Bystrom M., Eriksson U., Larsson E., Prior R.G., Sjostedt A., Titball R.W., Forsman M. (2003) Genome-wide DNA microarray analysis of *Francisella tularensis* strains demonstrates extensive genetic conservation within the species but identifies regions that are unique to the highly virulent *F. tularensis* subsp. *tularensis*. *J Clin Microbiol* **41**: 2924–31.
8. Thomas R., Johansson A., Neeson B., Isherwood K., Sjostedt A., Ellis J., Titball R.W. (2003) Discrimination of human pathogenic subspecies of *Francisella tularensis* by using restriction fragment length polymorphism. *J Clin Microbiol* **41**: 50–7.
9. Svensson K., Larsson P., Johansson D., Bystrom M., Forsman M., Johansson A. (2005) Evolution of subspecies of *Francisella tularensis*. *J Bacteriol* **187**: 3903–8.
10. Molins C.R., Delorey M.J., Yockey B.M., Young J.W., Belisle J. T., Schriefer M.E., Petersen J.M. (2014) Virulence difference between the prototypic Schu S4 strain (A1a) and *Francisella tularensis* A1a, A1b, A2 and type B strains in a murine model of infection. *BMC Infect Dis* **14**: 67.
11. Chamberlain R.E. (1965) Evaluation of live tularemia vaccine prepared in a chemically defined medium. *Appl Microbiol* **13**: 232–5.
12. Baker C.N., Hollis D.G., Thornsberry C. (1985) Antimicrobial susceptibility testing of *Francisella tularensis* with a modified Mueller-Hinton broth. *J Clin Microbiol* **22**: 212–5.
13. Lindgren H., Honn M., Salomonsson E., Kuoppa K., Forsberg A., Sjostedt A. (2011) Iron content differs between *Francisella tularensis* subspecies *tularensis* and subspecies *holarctica* strains and correlates to their susceptibility to H(2)O (2)-induced killing. *Infect Immun* **79**: 1218–24.
14. Clay C.D., Soni S., Gunn J.S., Schlesinger L.S. (2008) Evasion of complement-mediated lysis and complement C3 deposition are regulated by *Francisella tularensis* lipopolysaccharide O antigen. *J Immunol* **181**: 5568–78.
15. Hotta A., Tanabayashi K., Yamamoto Y., Fujita O., Uda A., Mizoguchi T., Yamada A. (2012) Seroprevalence of tularemia in wild bears and hares in Japan. *Zoonoses Public Health* **59**: 89–95.
16. Sharma N., Hotta A., Yamamoto Y., Fujita O., Uda A., Morikawa S., Yamada A., Tanabayashi K. (2013) Detection of *Francisella tularensis*-specific antibodies in patients with tularemia by a novel competitive enzyme-linked immunosorbent assay. *Clin Vaccine Immunol* **20**: 9–16.
17. Fujita H., Watanabe Y., Sato T., Ohara Y., Homma M. (1993) The entry and intracellular multiplication of *Francisella tularensis* in cultured cells: Its correlation with virulence in experimental mice. *Microbiol Immunol* **37**: 837–42.
18. Johansson A., Ibrahim A., Goransson I., Eriksson U., Gurycova D., Clarridge J.E. 3rd, Sjostedt A. (2000) Evaluation of PCR-based methods for discrimination of *Francisella* species and subspecies and development of a specific PCR that distinguishes the two major subspecies of *Francisella tularensis*. *J Clin Microbiol* **38**: 4180–5.
19. Mc Gann P., Rozak D.A., Nikolich M.P., Bowden R.A., Lindler L.E., Wolcott M.J., Lathigra R. (2010) A novel brain

heart infusion broth supports the study of common *Francisella tularensis* serotypes. *J Microbiol Methods* **80**: 164–71.
20. Carlson P.E. Jr., Horzempa J., O'dee D.M., Robinson C.M., Neophytou P., Labrinidis A., Nau G.J. (2009) Global transcriptional response to spermine, a component of the intramacrophage environment, reveals regulation of *Francisella* gene expression through insertion sequence elements. *J Bacteriol* **191**: 6855–64.
21. Igarashi K., Kashiwagi K. (2000) Polyamines: Mysterious modulators of cellular functions. *Biochem Biophys Res Commun* **271**: 559–64.
22. Lindgren H., Shen H., Zingmark C., Golovliov I., Conlan W., Sjostedt A. (2007) Resistance of *Francisella tularensis* strains against reactive nitrogen and oxygen species with special reference to the role of KatG. *Infect Immun* **75**: 1303–9.
23. Binesse J., Lindgren H., Lindgren L., Conlan W., Sjostedt A. (2015) Roles of reactive oxygen species-degrading enzymes of *Francisella tularensis* SCHU S4. *Infect Immun* **83**: 2255–63.
24. Dieppedale J., Sobral D., Dupuis M., Dubail I., Klimentova J., Stulik J., Postic G., Frapy E., Meibom K.L., Barel M., Charbit A. (2011) Identification of a putative chaperone involved in stress resistance and virulence in *Francisella tularensis*. *Infect Immun* **79**: 1428–39.
25. Kim E.J., Park S.H., Choi Y.S., Shim S.K., Park M.Y., Park M.S., Hwang K.J. (2008) Cytokine response in Balb/c mice infected with *Francisella tularensis* LVS and the Pohang isolate. *J Vet Sci* **9**: 309–15.
26. Huntley J.F., Conley P.G., Rasko D.A., Hagman K.E., Apicella M.A., Norgard M.V. (2008) Native outer membrane proteins protect mice against pulmonary challenge with virulent type A *Francisella tularensis*. *Infect Immun* **76**: 3664–71.
27. Chen W., Kuolee R., Austin J.W., Shen H., Che Y., Conlan J.W. (2005) Low dose aerosol infection of mice with virulent type A *Francisella tularensis* induces severe thymus atrophy and CD4+CD8+ thymocyte depletion. *Microb Pathog* **39**: 189–96.
28. Rasmussen J.W., Tam J.W., Okan N.A., Mena P., Furie M.B., Thanassi D.G., Benach J.L., Van Der Velden A.W. (2012) Phenotypic, morphological, and functional heterogeneity of splenic immature myeloid cells in the host response to tularemia. *Infect Immun* **80**: 2371–81.
29. Sutherland M.D., Goodyear A.W., Troyer R.M., Chandler J.C., Dow S.W., Belisle J.T. (2012) Post-exposure immunization against *Francisella tularensis* membrane proteins augments protective efficacy of gentamicin in a mouse model of pneumonic tularemia. *Vaccine* **30**: 4977–82.
30. Richard K., Mann B.J., Stocker L., Barry E.M., Qin A., Cole L.E., Hurley M.T., Ernst R.K., Michalek S.M., Stein D.C., Deshong P., Vogel S.N. (2014) Novel catanionic surfactant vesicle vaccines protect against *Francisella tularensis* LVS and confer significant partial protection against *F. tularensis* Schu S4 strain. *Clin Vaccine Immunol* **21**: 212–26.
31. Del Barrio L., Sahoo M., Lantier L., Reynolds J.M., Ceballos-Olvera I., Re F. (2015) Production of anti-LPS IgM by B1a B Cells depends on IL-1beta and is protective against lung infection with *Francisella tularensis* LVS. *PLoS Pathog* **11**: e1004706.
32. Bevanger L., Maeland J.A., Naess A.I. (1989) Competitive enzyme immunoassay for antibodies to a 43,000-molecular-weight *Francisella tularensis* outer membrane protein for the diagnosis of tularemia. *J Clin Microbiol* **27**: 922–6.
33. Yu J.J., Goluguri T., Guentzel M.N., Chambers J.P., Murthy A. K., Klose K.E., Forsthuber T.G., Arulanandam B.P. (2010) *Francisella tularensis* T-cell antigen identification using humanized HLA-DR4 transgenic mice. *Clin Vaccine Immunol* **17**: 215–22.
34. Sjöstedt A., Sandstrom G., Tarnvik A., Jaurin B. (1989) Molecular cloning and expression of a T-cell stimulating membrane protein of *Francisella tularensis*. *Microb Pathog* **6**: 403–14.
35. Sjöstedt A., Tarnvik A., Sandstrom G. (1991) The T-cell-stimulating 17-kilodalton protein of *Francisella tularensis* LVS is a lipoprotein. *Infect Immun* **59**: 3163–8.
36. Elzer P.H., Jacobson R.H., Nielsen K.H., Douglas J.T., Winter A. J. (1994) BALB/c mice infected with *Brucella abortus* express protracted polyclonal responses of both IgG2a and IgG3 isotypes. *Immunol Lett* **42**: 145–50.
37. Martin R.M., Lew A.M. (1998) Is IgG2a a good Th1 marker in mice? *Immunol Today* **19**: 49.
38. Hazlett K.R., Caldon S.D., Mcarthur D.G., Cirillo K.A., Kirimanjeswara G.S., Magguilli M.L., Malik M., Shah A., Broderick S., Golovliov I., Metzger D.W., Rajan K., Sellati T.J., Loegering D.J. (2008) Adaptation of *Francisella tularensis* to the mammalian environment is governed by cues which can be mimicked in vitro. *Infect Immun* **76**: 4479–88.
39. Soni S., Ernst R.K., Muszynski A., Mohapatra N.P., Perry, M.B., Vinogradov E., Carlson R.W., Gunn J.S. (2010) *Francisella tularensis* blue-gray phase variation involves structural modifications of lipopolysaccharide o-antigen, core and lipid a and affects intramacrophage survival and vaccine efficacy. *Front Microbiol* **1**: 129.
40. Champion M.D., Zeng Q., Nix E.B., Nano F.E., Keim P., Kodira C.D., Borowsky M., Young S., Koehrsen M., Engels R., Pearson M., Howarth C., Larson L., White J., Alvarado L., Forsman M., Bearden S.W., Sjostedt A., Titball R., Michell S.L., Birren B., Galagan J. (2009) Comparative genomic characterization of *Francisella tularensis* strains belonging to low and high virulence subspecies. *PLoS Pathog* **5**: e1000459.
41. Uda A., Sekizuka T., Tanabayashi K., Fujita O., Kuroda M., Hotta A., Sugiura N., Sharma N., Morikawa S., Yamada A. (2014) Role of pathogenicity determinant protein C (PdpC) in determining the virulence of the *Francisella tularensis* subspecies *tularensis* SCHU. *PLoS ONE* **9**: e89075.