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NOTES

Preparation of Monoclonal Antibodies for Detection and Identification of *Francisella tularensis*▽

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Monoclonal antibodies (MAbs) against *Francisella tularensis* were obtained. Three MAbs specifically reacted with *F. tularensis*, while four MAbs reacted with other members of the genus *Francisella* as well. Fluorescent isothiocyanate-conjugated MAbs unequivocally stained bacterial cells in specimens from experimentally infected mice. Two MAbs agglutinated *F. tularensis* antigen in the agglutination tests. These MAbs should improve methods for detection and identification of *F. tularensis*.

Francisella tularensis is a gram-negative coccobacillus that causes tularemia in humans and animals. Tularemia is traditionally diagnosed by the isolation of *F. tularensis* or the detection of specific antibodies. Isolated bacteria were subsequently identified by slide agglutination or immunofluorescence tests using anti-*F. tularensis* immune serum. Specific antibodies are frequently detected by the microagglutination test (18) in most clinical laboratories. However, because such antibodies cross-react with other bacteria (3), there is a need for an improved method for the serodiagnosis of tularemia. Antigenic analysis of *F. tularensis* as well as other members of the genus is important because *Francisella novicida* and *Francisella philomiragia* have biochemical and genetic properties similar to those of *F. tularensis* (9), although they rarely cause tularemia-like diseases (13, 22). Monoclonal antibodies (MAbs) are a useful tool for analyzing the antigenic properties of bacteria (15) because they recognize a single epitope with high specificity. Although some MAbs against *F. tularensis* lipopolysaccharide (LPS) have been produced (5, 10), MAbs against other antigenic components are not available commercially. In this study, we obtained seven MAbs that recognize at least five different epitopes carried by *F. tularensis*. Four MAbs reacted with *F. novicida* and *F. philomiragia* as well. These MAbs can be used for antigenic analyses of *Francisella* organisms as well as for the diagnosis of tularemia and tularemia-like diseases.

Twenty-six *F. tularensis* strains (15 Japanese strains and 11 non-Japanese strains), the *F. novicida* U112 strain, and the *F. philomiragia* 029 strain were kindly provided by H. Fujita, Ohara Research Laboratory, Fukushima, Japan. Two *F. philomiragia* strains (ATCC 25017 and ATCC 25018), and *Brucella abortus*, *Brucella melitensis*, *Brucella suis*, *Escherichia coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae* subsp. *pneumoniae*, *Pasteurella aerogenes*, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis* were propagated in our laboratory. All *F. tularensis* strains were propagated on Difco Eugon agar (Becton, Dickinson and Company, Sparks, MD) with chocolatized 8% sheep blood in a biosafety level-3 laboratory. The MAb against *F. tularensis* LPS (FB11) (Biodesign International, Saco, ME) was used as a reference, and fluorescent isothiocyanate (FITC)-labeled antirabies virus monoclonal antibody (Fujirebio Diagnostics, Inc. Malvern, PA) was used as an isotype control. All animal experiments were approved by the animal research committee of the National Institute of Infectious Diseases.

Hybridoma clones secreting MAbs (M11D3, M11H7, M13B10, M14B11, M15C6, S11E7, and U22F2) were obtained by the fusion of mouse myeloma cells (P3-X63-Ag8.653) and spleen cells from BALB/c mice, which had been immunized with the formalin-inactivated *F. tularensis* GIEM Miura (Japanese) strain, the Schu (non-Japanese) strain, or the *F. novicida* U112 strain, as described elsewhere (14). Characteristics of the MAbs (Table 1) were based on MAbs obtained from hybridoma supernatant or mice ascitic fluids. Western blotting following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed that the MAbs recognized at least five different epitopes carried by *F. tularensis* LVS (Fig. 1). The banding patterns obtained with the Schu and GIEM Miura strains were not different from those obtained with the LVS strain (data not shown). MAb M14B11 stained ladder-like bands having molecular masses greater than 15 kDa. Identical ladder-like bands were obtained with MAbs M11H7 and M15C6 (data not shown). These three MAbs also reacted with purified LPS (Fig. 1), a major protective antigen of *F. tularensis* (17). On the other hand, MAb M11D3, M13B10, and S11E7 reactions produced single bands with molecular masses of 40, 17, and 10 kDa, respectively, while MAb U22F2 reactions produced 41- and 43-kDa bands (Fig. 1). These four MAbs did not react with proteinase K-digested antigen (data not shown), suggesting that the MAbs recognized protein components. *F. tularensis* proteins of 10, 17, 40, 41, and 43 kDa were found to be recognized by the sera from tularemia patients (4, 12). In addition, immunoreactive membrane components of *F. tularensis* might play important roles in both the invasion of host cells and escape from phagolysososmes (6, 11). Although it is

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TABLE 1. Summary of the characteristics of monoclonal antibodies

MAb	Immunized antigen[a]	Antigen reacted (kDa)[b]	Reaction against species (no. of strains tested)[c]			Agglutination activity[d]	Ig isotype[e]
			F. tularensis (26)	*F. novicida* (1)	*F. philomiragia* (3)		
M11D3	GIEM Miura	40	+	+	+	−	M
M11H7	GIEM Miura	>15[g]	+	−	−	−	G3
M13B10	GIEM Miura	17	+	+	+	−	G1
M14B11	GIEM Miura	>15[g]	+	−	−	+	G2a
M15C6	GIEM Miura	>15[g]	+	−	−	+	M
S11E7	Schu	10	+	+	+	−	G1
U22F2	U112	41, 43	+	+	−	−	G1
FB11[f]	15	>15[g]	+	−	−	−	G2a

[a] *Francisella* strains used for immunization of mice.
[b] Molecular mass of *F. tularensis* antigen appeared in Western blotting following SDS-PAGE.
[c] Determined by indirect fluorescence assay: +, positive; −, negative.
[d] Determined by microagglutination test: +, positive; −, negative.
[e] Determined with a mouse monoclonal antibody isotyping test kit (Serotec, Oxford, United Kingdom).
[f] Reference MAb purchased commercially.
[g] Ladder-like bands of molecular mass greater than 15 kDa.

unclear whether our MAbs recognize these essential components, they may help to analyze the pathogenicity of *F. tularensis*. We are presently attempting to determine the epitopes recognized by these MAbs.

All MAbs reacted with all Japanese and non-Japanese *F. tularensis* strains but did not react with *B. abortus*, *B. melitensis*, *B. suis*, *Y. enterocolitica*, *Y. pseudotuberculosis*, *E. coli*, *H. influenzae*, *K. pneumoniae* subsp. *pneumoniae* or *P. aerogenes* by indirect fluorescence assay. Since cross-reactions among *F. tularensis*, *Brucella* spp., and *Yersinia* spp. have been discussed by many researchers (3, 19), reactions of the MAbs against *B. abortus*, *Y. enterocolitica*, and *Y. pseudotuberculosis* were further analyzed by Western blotting. The results indicated that our MAbs did not react with the antigens of these three bacteria (data not shown). MAbs M11H7, M14B11, and M15C6 did not react with *F. novicida* or *F. philomiragia* (Fig. 1), indicating that these three MAbs were specific for *F. tularensis*. On the other hand, MAbs M11D3, M13B10, S11E7, and U22F2 appeared to recognize the conserved epitopes among *F. tularensis*, *F. novicida*, and *F. philomiragia* (Fig. 1). Since the antigens of *F. philomiragia* recognized by MAbs M13B10 and U22F2 migrated differently than those from *F. tularensis* and *F. novicida*, *F. philomiragia* seemed to be more distantly related to *F. tularensis* and *F. novicida*. This finding seems to be in good

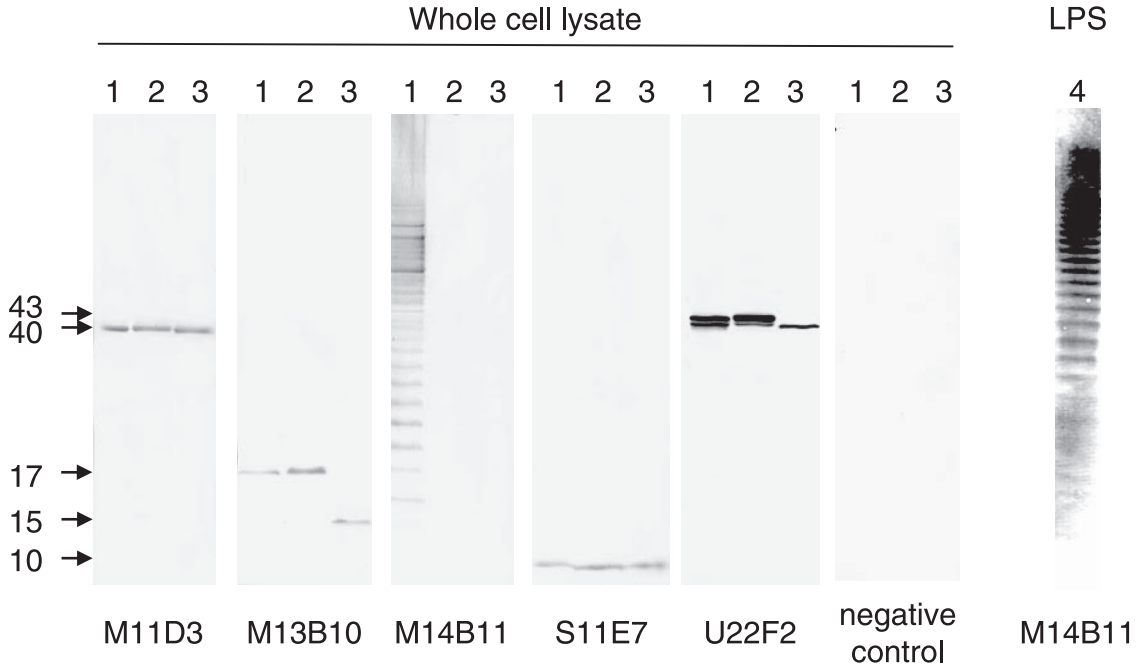

FIG. 1. Reactions of MAbs shown by Western blots following SDS-PAGE. Bacterial lysates from *F. tularensis* LVS, *F. novicida* U112, and *F. philomiragia* 029 (lanes 1 to 3, respectively) were reacted with MAbs M11D3, M13B10, M14B11, S11E7, and U22F2 and normal mouse serum (negative control). The reaction of MAb M14B11 against purified LPS from *F. tularensis* Schu (lane 4) is also shown. The positions of the molecular size markers are indicated (in kilodaltons).

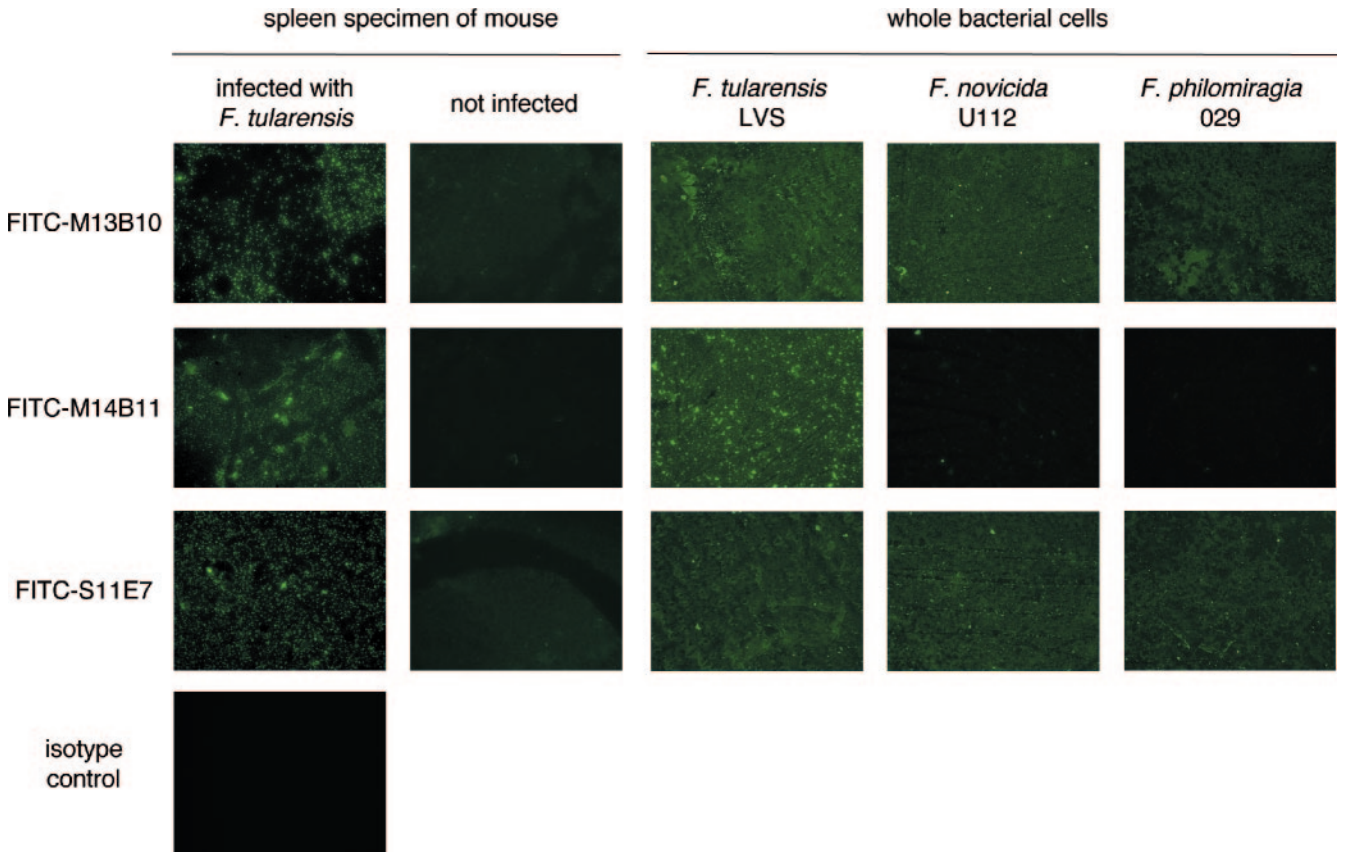

FIG. 2. Reactions of MAbs shown by DFA. FITC-labeled MAbs M13B10, M14B11, and S11E7 were reacted with the impression smears of the spleens from a mouse infected with *F. tularensis* Yama and an uninfected mouse and whole-bacteria cells of *F. tularensis* LVS, *F. novicida* U112, and *F. philomiragia* 029. FITC-labeled antirabies virus monoclonal antibody was used as an isotype control.

agreement with the view that *F. novicida* should be classified as a subspecies of *F. tularensis* (9, 13, 20). Although the numbers of strains tested were limited, it should be possible to use the MAbs to differentiate among *Francisella* species. Unusual *Francisella* organisms, including symbionts of ticks, have been found worldwide (2, 21). Although the antigenic properties of these unusual *Francisella* organisms are mostly unknown, our MAbs might help to characterize the relationships among the different *Francisella* organisms.

F. tularensis antigen was agglutinated by MAbs M14B11 and M15C6 in both the microagglutination and the slide agglutination tests (Table 1). In the slide agglutination test, a solution containing MAb M14B11 (0.2 mg/ml of purified immunoglobulin G [IgG]) agglutinated an equal volume of *F. tularensis* whole-cell suspension (an optical density at 600 nm of 1.8), while solutions containing MAb M11H7 or FB11 (in excess of 0.8 mg/ml of purified IgG) did not show any agglutination at all (data not shown). Thus, *F. tularensis* could be rapidly identified by a simple slide agglutination test using MAb M14B11.

We next determined whether the MAbs could be used to identify *F. tularensis* in the tissue of infected animals by using a direct immunofluorescent assay (DFA). IgG MAbs purified with a protein G Sepharose column (Amersham Biosciences AB, Uppsala, Sweden) were conjugated with FITC with a Fluoro *Taq* FITC conjugation kit column (Sigma-Aldrich Co., St. Louis, MO) according to the manufacturer's protocol. When impression smears of the spleens from mice infected with the Yama strain were reacted with FITC-labeled MAbs M14B11, M13B10, and S11E7, bacterial cells were readily identified by fluorescence microscopy. FITC-labeled MAbs M13B10 and S11E7 also stained bacterial cells of *F. novicida* and *F. philomiragia* (Fig. 2). These results suggest that FITC-labeled MAbs can be used to detect and identify *Francisella* organisms from clinical samples.

Tularemia has been considered to be a disease confined to the northern hemisphere and most frequently in Scandinavia, North America, Japan, and Russia (7). However, it has emerged in other geographic locations recently (16). The prevalence and distribution of *F. tularensis* have received much attention because of fears that the organisms could be used as a bioterrorism agent. Furthermore, *F. tularensis* is associated with protozoa (1) and might reside in the environment in a viable but nonculturable form (8). Therefore, it would be very useful to have a method for detecting *F. tularensis* in environmental samples such as soil and water. The MAbs obtained here appear to be ideal tools for identifying not only *F. tularensis* but also *F. novicida* and *F. philomiragia* for ecological and epidemiological studies as well as for antigenic analyses of *Francisella* organisms, the pathogens of tularemia or tularemia-like diseases.

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REFERENCES

1. **Abd, H., T. Johansson, I. Golovliov, G. Sandström, and M. Forsman.** 2003. Survival and growth of *Francisella tularensis* in *Acanthamoeba castellanii*. Appl. Environ. Microbiol. **69:**600–606.
2. **Barns, S. M., C. C. Grow, R. T. Okinaka, P. Keim, and C. R. Kuske.** 2005. Detection of diverse new *Francisella*-like bacteria in environmental samples. Appl. Environ. Microbiol. **71:**5494–5500.
3. **Behan, K. A., and G. C. Klein.** 1982. Reduction of *Brucella* species and *Francisella tularensis* cross-reacting agglutinins by dithiothreitol. J. Clin. Microbiol. **16:**756–757.
4. **Bevanger, L., J. A. Maeland, and A. I. Naess.** 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:**922–926.
5. **Bhatti, A. R., J. P. Wong, and D. E. Woods.** 1993. Production and partial characterization of hybridoma clones secreting monoclonal antibodies against *Francisella tularensis*. Hybridoma **12:**197–202.
6. **Clemens, D. L., B.-Y. Lee, and M. A. Horwitz.** 2005. *Francisella tularensis* enters macrophages via a novel process involving pseudopod loops. Infect. Immun. **73:**5892–5902.
7. **Ellis, J., P. C. Oyston, M. Green, and R. W. Titball.** 2002. Tularemia. Clin. Microbiol. Rev. **15:**631–646.
8. **Forsman, M., E. W. Henningson, E. Larsson, T. Johansson, and G. Sandström.** 2000. *Francisella tularensis* does not manifest virulence in viable but non-culturable state. FEMS Microbiol. Ecol. **31:**217–224.
9. **Forsman, M., G. Sandström, and A. Sjöstedt.** 1994. Analysis of 16S ribosomal DNA sequences of *Francisella* strains and utilization for determination of the phylogeny of the genus and for identification of strains by PCR. Int. J. Syst. Bacteriol. **44:**38–46.
10. **Fulop, M. J., T. Webber, R. J. Manchee, and D. C. Kelly.** 1991. Production and characterization of monoclonal antibodies directed against the lipopolysaccharide of *Francisella tularensis*. J. Clin. Microbiol. **29:**1407–1412.
11. **Golovliov, I., V. Baranov, Z. Krocova, H. Kovarova, and A. Sjöstedt.** 2003. An attenuated strain of the facultative intracellular bacterium *Francisella tularensis* can escape the phagosome of monocytic cells. Infect. Immun. **71:**5940–5950.
12. **Havlasova, J., L. Hernychova, P. Halada, V. Pellantova, J. Krejsek, J. Stulik, A. Macela, P. R. Jungblut, P. Larsson, and M. Forsman.** 2002. Mapping of immunoreactive antigens of *Francisella tularensis* live vaccine strain. Proteomics **2:**857–867.
13. **Hollis, D. G., R. E. Weaver, A. G. Steigerwalt, J. D. Wenger, C. W. Moss, and D. J. Brenner.** 1989. *Francisella philomiragia* comb. nov. (formerly *Yersinia philomiragia*) and *Francisella tularensis* biogroup novicida (formerly *Francisella novicida*) associated with human disease. J. Clin. Microbiol. **27:**1601–1608.
14. **Hotta, A., M. Kawamura, H. To, M. Andoh, T. Yamaguchi, H. Fukushi, and K. Hirai.** 2002. Phase variation analysis of *Coxiella burnetii* during serial passage in cell culture by use of monoclonal antibodies. Infect. Immun. **70:**4747–4749.
15. **Hotta, A., G. Q. Zhang, M. Andoh, T. Yamaguchi, H. Fukushi, and K. Hirai.** 2004. Use of monoclonal antibodies for analyses of *Coxiella burnetii* major antigens. J. Vet. Med. Sci. **66:**1289–1291.
16. **Petersen, J. M., and M. E. Schriefer.** 2005. Tularemia: emergence/re-emergence. Vet. Res. **36:**455–467.
17. **Sandström, G., A. Sjöstedt, T. Johansson, K. Kuoppa, and J. C. Williams.** 1992. Immunogenicity and toxicity of lipopolysaccharide from *Francisella tularensis* LVS. FEMS Microbiol. Immunol. **5:**201–210.
18. **Sato, T., H. Fujita, Y. Ohara, and M. Homma.** 1990. Microagglutination test for early and specific serodiagnosis of tularemia. J. Clin. Microbiol. **28:**2372–2374.
19. **Schmitt, P., W. Splettstosser, M. Porsch-Ozcurumez, E. J. Finke, and R. Grunow.** 2005. A novel screening ELISA and a confirmatory Western blot useful for diagnosis and epidemiological studies of tularemia. Epidemiol. Infect. **133:**759–766.
20. **Sjöstedt, A. B.** 2005. Family XVII. *FRANCISELLAE*, genus I. Francisella *Dorofe'ev 1947*, *176*^{AL}, p. 200–210. *In* D. J. Brenner, N. R. Krieg, J. T. Staley, and G. M. Garrity (ed.), The proteobacteria, part B. Bergey's manual of systematic bacteriology, 2nd ed., vol. 2. Springer-Verlag, New York, NY.
21. **Sun, L. V., G. A. Scoles, D. Fish, and S. L. O'Neill.** 2000. *Francisella*-like endosymbionts of ticks. J. Invertebr. Pathol. **76:**301–303.
22. **Whipp, M. J., J. M. Davis, G. Lum, J. de Boer, Y. Zhou, S. W. Bearden, J. M. Petersen, M. C. Chu, and G. Hogg.** 2003. Characterization of a *novicida*-like subspecies of *Francisella tularensis* isolated in Australia. J. Med. Microbiol. **52:**839–842.