

研究成果の刊行に関する一覧表

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Hotta A., Uda A., Fujita O., Tanabayashi K., and Yamada A	Preparation of Monoclonal Antibodies for Detection and Identification of <i>Francisella tularensis</i> .	Clin Vaccine Immunol	14	81-84	2007
Takayama-Ito M., Inoue KI., Shoji Y., Inoue S., Iijima T., Sakai T., Kurane I. and Morimoto K	A highly attenuated rabies virus HEP-Flury strain reverts to virulent by single amino acid substitution to arginine at position 333 in glycoprotein	Virus Res.	119	208-215	2006
Park C.-H., Kondo M., Inoue S., Noguchi A., Oyamada T., Yoshikawa H. and Yamada A	The Histopathogenesis of Paralytic Rabies in Six-Week-Old C57BL/6J Mice Following Inoculation of the CVS-11 Strain into the Right Triceps Surae Muscle.	J.Vet.Med.Sci	68	589-595	2006
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Original Article

Development of a Real-Time PCR Assay for Detection and Quantification of *Francisella tularensis*

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SUMMARY: The facultative intracellular bacterium, *Francisella tularensis*, is an etiological agent of tularemia and is also considered to be a potential biological threat agent due to its extreme infectivity. We established a real-time PCR assay using the LightCycler (LC) system to detect a *Francisella*-specific sequence of the outer membrane protein (*fopA*) gene. Twenty-five *F. tularensis* strains including 16 Japanese isolates were subjected to this LC-PCR assay, and were tested positive, whereas *Francisella philomiragia* and other bacteria species did not show any specific fluorescent signal. A linear response was observed using *F. tularensis* genomic DNAs of between 20 fg and 2 ng, corresponding to 1.2 to 1.2×10^5 bacteria. The newly established real-time PCR allows the detection of the *F. tularensis* genome specifically, sensitively, and rapidly. This assay may contribute to the standardization of the laboratory diagnosis of tularemia.

INTRODUCTION

Francisella tularensis, the causative agent of tularemia in humans and animals, is a small Gram-negative intracellular bacterium. Although *F. tularensis*, *F. novicida* and *F. philomiragia* are currently considered to be independent species of the genus *Francisella*, Ellis et al. (1) recently proposed that *F. novicida* should be classified as a subspecies of *F. tularensis* based on the nucleotide sequence of the 16S ribosomal DNA. In this study we regarded *F. novicida* as one of the subspecies of *F. tularensis* according to this proposition. Species and subspecies of *Francisella* differ with regard to their biochemical properties, geographic distribution and virulence in humans (1). Of the four *F. tularensis* subspecies, *F. tularensis* subsp. *holarctica* is widely distributed in a wide range of animal reservoir hosts throughout most of the Northern Hemisphere, including Japan. It is transmitted to humans by various routes, including: the direct handling of infectious carcasses; the ingestion of contaminated food, vegetation or water; being bitten by infected arthropod vectors; and the inhalation of infectious dust, soil or aerosols (1). The *F. tularensis* subsp. *tularensis* (also known as Type A) is mainly distributed in North America. This subspecies bacterium is extremely infectious, since as few as 10 organisms are capable of inducing disease in humans following intradermal inoculation or inhalation (2,3). It is also highly virulent in that case fatality rates as high as 30 to 60% were reported in untreated pneumonic and severe systemic forms of the disease (4). *F. tularensis* has been, therefore, considered to be a class A bioterrorism agent by the Centers for Disease Control and Prevention (CDC), Atlanta, Ga., USA (5).

The diagnosis of human cases of tularemia is usually accomplished by the demonstration of an antibody response to *F. tularensis* by tube- or micro-agglutination assay and enzyme-linked immunosorbent assay (ELISA) (6-8). Specific

antibody response in patient serum is detectable from 4 to 7 days after the onset of the disease by micro-agglutination assay (8). Furthermore, those immunological assays can be confounded by serum cross-reactivity with antigens of other genera of bacteria (genus *Brucella*, *Haemophilus* and *Yersinia*) (9). For the identification of *F. tularensis*, a conventional culture assay is usually conducted. It not only requires at least 2-4 days for adequate growth of the organism (10), but also is prone to showing false-negative results. Furthermore, the cultivation of *F. tularensis* poses a considerable risk of laboratory-acquired infection. Laboratory work should therefore be performed under biosafety level 3 (BSL-3) conditions. An immunochromatographic hand-held assay has been developed based on polyclonal and monoclonal antibodies against lipopolysaccharide of *F. tularensis* live vaccine strain (LVS), but the sensitivity was relatively low: 10^6 colony-forming units (CFU)/ml in phosphate-buffered saline (PBS) and 10^6 to 10^7 CFU/ml in spiked human sera (11). To overcome these problems, PCR assays targeting the 16S ribosomal RNA (12) or targeting genes encoding the outer membrane proteins such as *fopA* (13) and the 17-kDa major membrane lipoprotein (14-16) have been successfully used to detect *F. tularensis* DNA, but most of them are inferior to real-time PCR assay, both in sensitivity and rapidity (17-19).

The LightCycler (LC) technology enables real-time and high-speed detection of PCR products. It employs two hybridization probes labeled with fluorophore that allow the sequence-specific emission of fluorescence caused by the fluorescence resonance energy transfer (FRET) that occurs when the two probes anneal to the target DNA in close proximity (20). In this study, we describe a real-time PCR assay using the LC system for the specific detection of *F. tularensis* DNA using specific primers and hybridization probes targeting the *fopA* gene.

MATERIALS AND METHODS

Bacterial species and strains: All *Francisella* strains were isolated or collected by Ohara Research Laboratory (Fukushima, Japan) and kindly provided by Dr. Hiromi Fujita. Those strains were originated from various sources such as

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humans, animals, ticks and water (Table 1) (21). The bacteria were grown on Eugon agar (Becton, Dickinson and Company, Sparks, Md., USA) plates supplemented with 8% rabbit defibrinated blood (Nippon Biotest Laboratories, Inc., Tokyo, Japan) (22). All the *F. tularensis* strains, except LVS, were handled in the BSL-3 laboratory in the National Institute of Infectious Diseases (NIID), Tokyo.

To evaluate whether DNA amplification is specific for *F. tularensis*, 24 non-*Francisella* bacterial species (43 strains) were used (Table 2). Some bacteria were chosen because they either represent possible threat agents or were genetically related to these species. Others were selected because they were intracellular parasites with their life cycles similar to that of *Francisella*-bacteria. Eleven were obtained from the American Type Culture Collection (ATCC) (Rockville, Md., USA), and 6 were from the Biological Resource Center of the National Institute of Technology and Evaluation (NBRC) (Chiba, Japan). The others were stocked and/or maintained at NIID. The inactivated *Coxiella burnetii* cultures (23) and *Wolbachia pestis* (24) genomic DNA were kindly provided by Drs. Hideto Fukushi (Department of Veterinary Medicine, Faculty of Applied Biological Science, Gifu University, Gifu, Japan) and Tetsuhiko Sasaki (Department of Biological Science, Graduate School of Science, University of Tokyo, Tokyo, Japan), respectively.

Template DNA preparation: Cultivated bacterial cells were suspended in 200 μ l of PBS and inactivated at 95°C for 15 min. DNA was extracted with phenol-chloroform-isoamyl alcohol or with the SepaGene DNA Extraction Kit

(Sanko Junyaku Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. Purified DNA was dissolved in TE buffer (10 mM Tris-HCl [pH8.0], 1 mM EDTA [pH8.0], Nippon Gene, Tokyo, Japan) at a concentration of 10 ng/ml. All samples were aliquoted and stored at -20°C until use.

Construction of control plasmid DNA: The *fopA* amplicon (708-bp) was generated by PCR from *F. tularensis* LVS genomic DNA using the primer set reported by Higgins et al. (17) and subsequently cloned into pCR2.1 vector (Invitrogen, Darstadt, Germany). The recombinant plasmid, pCR-*fopA*, was amplified in the *Escherichia coli* DH5 α , and purified by the Qiagen plasmid mini kit (Qiagen, Hilden, Germany). The absorbance of the DNA solution was measured at 260 nm using a spectrophotometer (DU 530, Beckman, Fullerton, Calif., USA), and the concentration was calculated.

Oligonucleotide primers and hybridization probes: The primers and probes were designed based on the nucleotide sequence of *F. tularensis fopA* gene (GeneBank accession no. M93695) (25) as shown in Table 3. The expected size of the DNA fragment amplified using Ft-F and Ft-R primers was 249-bp. The two hybridization probes, Ft-Flu and Ft-LcR labeled with fluorescein (FL) or LightCycler Red640 (Lc-R) dye, respectively, were designed to bind neighbored on the target DNA. The primers and probes were synthesized by Nihon Gene Research Laboratories Inc. (Miyagi, Japan).

LC-PCR assay and product detection: The amplification mixture consisting of 2 μ l of 10 \times reaction mix (LightCycler FastStart Master Hybridization Probes; Roche Diagnostics, Mannheim, Germany), 3 mM MgCl₂, 0.5 μ M of each

Table 1. List of strains of *F. tularensis* used in this study

Isolate	Origin	Year isolated	State	Country
<i>Francisella tularensis</i> subsp. <i>holarctica</i>				
1 Ebina	Human lymph node	1950	Miyagi	Japan
2 Yama	<i>Ixodes</i> sp.	1957	Fukushima	Japan
3 Naomatsu	Human lymph node	1968	Akita	Japan
4 Yato 96	<i>Lepus brachyurus</i>	1968	Akita	Japan
5 GIEM Miura	Human ulcer	1975	Miyagi	Japan
6 Yato 107	<i>Lepus brachyurus</i>	1979	Fukushima	Japan
7 Kikuchi	Human lymph node	1982	Fukushima	Japan
8 Ootake	<i>Heamaphysalis flava</i>	1982	Miyagi	Japan
9 Suzushichi	Human lymph node	1982	Yamagata	Japan
10 Mitsuo	Human ulcer	1983	Miyagi	Japan
11 Nikaido	Human lymph node	1984	Fukushima	Japan
12 Sami	Human lymph node	1980	Akita	Japan
13 Chiba	Human lymph node	1980	Aomori	Japan
14 Azumaya	Human lymph node	1981	Akita	Japan
15 Kokuchi	Human lymph node	1981	Yamagata	Japan
16 Kato	Human lymph node	1989	Yamagata	Japan
17 N9	<i>Microtus arvalis</i>	1948		Russia
18 N503	<i>Dermacentor pictus</i>	1949		Russia
19 Tungliao (TyH)	<i>Citellus</i> sp.	1957		China
20 N1915	<i>Lepus europaeus</i>	1962		Ukraine
21 Russian Vaccine (RV)	<i>F. tularensis</i> strain 15	unknown		Russia
22 Live Vaccine Strain (LVS)	RV	1961		
<i>Francisella tularensis</i> subsp. <i>tularensis</i>				
23 38 (P38)	Human lymph node	1920		USA
24 Schu	Human ulcer	1941	Ohio	USA
<i>Francisella tularensis</i> subsp. <i>novicida</i>				
25 U112	Water	1950	Utah	USA
<i>Francisella philomiragia</i>				
26 029 (Y-29)	Water	1960	Utah	USA

oligonucleotide primer (Ft-F and Ft-R), 0.2 μ M FL hybridization probe (Ft-Flu), 0.4 μ M LC-Red 640 probe (Ft-LcR), and 1 μ l of template DNA in a final volume of 20 μ l in the LC capillaries was placed in the LC instrument (Quick

Table 2. Summary of the results of the LC-PCR conducted on various bacteria strains unrelated to *Francisella*

	Species	Strain	Result of LC-PCR
1	<i>Bacillus anthracis</i>	PA I	-
2	<i>Bacillus anthracis</i>	PA II	-
3	<i>Bacillus cereus</i>	NBRC 3466	-
4	<i>Bacillus cereus</i>	NBRC 13494	-
5	<i>Bacillus cereus</i>	NBRC 15305	-
6	<i>Bacillus thuringensis</i>	NBRC 3951	-
7	<i>Bacillus thuringensis</i>	NBRC 13865	-
8	<i>Bacillus thuringensis</i>	NBRC 13866	-
9	<i>Bacillus subtilis</i>	2	-
10	<i>Bacillus subtilis</i>	3	-
11	<i>Bacillus subtilis</i>	52	-
12	<i>Bacillus subtilis</i>	62	-
13	<i>Bacillus subtilis</i>	80	-
14	<i>Borrelia afzelii</i>	P/Gau	-
15	<i>Borrelia burgdorferi</i>	B31 (ATCC 35210)	-
16	<i>Borrelia garinii</i>	FujiP2	-
17	<i>Borrelia garinii</i>	HP1	-
18	<i>Borrelia japonica</i>	612	-
19	<i>Brucella abortus</i> biovar 1	125	-
20	<i>Brucella canis</i>	QE13	-
21	<i>Brucella melitensis</i> biovar 1	16M	-
22	<i>Brucella suis</i> biovar 1	1330	-
23	<i>Coxiella burnetii</i>	Priscilla	-
24	<i>Coxiella burnetii</i>	Nine Mile	-
25	<i>Coxiella burnetii</i>	Ohio	-
26	<i>Escherichia coli</i>	DH5 α	-
27	<i>Haemophilus influenzae</i> Type B	ATCC 10211	-
28	<i>Klebsiella pneumoniae</i> subsp. <i>pneumoniae</i>	ATCC 13883	-
29	<i>Legionella feeleii</i>	ATCC35072	-
30	<i>Legionella longbeachae</i>	ATCC33462	-
31	<i>Legionella pneumophila</i>	80-045	-
32	<i>Listeria monocytogenes</i>	ATCC 15315	-
33	<i>Mycobacterium tuberculosis</i>	ATCC 27294	-
34	<i>Ochrobactrum anthropi</i>	ATCC 49187	-
35	<i>Pasteurella aerogenes</i>	ATCC 27883	-
36	<i>Proteus mirabilis</i>	KH492	-
37	<i>Pseudomonas aeruginosa</i>	KH683	-
38	<i>Staphylococcus aureus</i> subsp. <i>aureus</i>	ATCC 29247	-
39	<i>Streptococcus pneumoniae</i>	ATCC49619	-
40	<i>Wolbachia persica</i>	Ref. 24	-
41	<i>Yersinia pestis</i>	Yreka	-
42	<i>Yersinia pseudotuberculosis</i>	319 (2a+)	-
43	<i>Yersinia enterocolitica</i>	Pa 177 (O9:B2)	-

System 330; Roche Diagnostics), and initially incubated at 95°C for 10 min to denature the template DNA, and to activate the FastStart *taq* DNA polymerase. The amplification cycle was as follows: 40 cycles at 95°C for 10 sec, 60°C for 15 sec, and 72°C for 10 sec. The temperature transition rate was 20°C/sec. The intensity of fluorescence was monitored at the end of each extension step. After the DNA amplification cycles, melting curve analysis was performed to confirm that the obtained signals were caused by the specific amplicons. The LC software (version 3) produced the standard curve by measuring the crossing point of each standard and plotting them against the logarithmic values of construction.

Sensitivity of LC-PCR assay for *F. tularensis fopA* gene:

To assess the analytical sensitivity of the LC-PCR assay, 10-fold serial dilutions of purified plasmid DNA (pCR-*fopA*) or genomic DNA of 6 strains (LVS, Miura, N503, TyH, 38 and Schu strains) were tested in triplicate. We next determined the least number of bacteria detectable by the LC-PCR assay. Single colonies of LVS strain were picked from a fresh culture and suspended in PBS (pH 7.0), and 10-fold serial dilutions were made. The genomic DNA was extracted by the SepaGene DNA Extraction Kit from each bacterial suspension and was tested in triplicate with the LC-PCR assay. For the determination of CFU, 100 μ l of each dilution was spread evenly on an Eugon agar plate supplemented with 8% rabbit defibrinated blood in duplicate. The plates were incubated for 37°C for 72 h, and the number of colonies was counted.

RESULTS

Amplification of DNAs from all *F. tularensis* strains:

Oligonucleotide primers and hybridization probes were designed to specifically identify *F. tularensis* (Table 3). It was shown that the LC-PCR assay successfully detected 25 of the *F. tularensis* strains tested, including 16 Japanese isolates (Fig. 1). The specific signal became detectable at 28 to 30 cycles when 1 pg of genomic DNA was used. The melting-curve analysis showed that *F. tularensis*-specific *Tm* was approximately 68°C (Fig. 2B).

Sensitivity of the real-time PCR assay: A dilution series (1 fg to 2 ng/reaction) of genomic DNA from the *F. tularensis* LVS strain was tested by the LC-PCR assay. The result showed that significant signals were detected between 20 fg and 2 ng per 20 μ l of LC reaction but not at 10 fg or less (Fig. 2). When DNAs from 5 other strains (Miura, N503, TyH, 38 and Schu) were used as templates, similar detection limits were observed (data not shown). To determine whether the linear response was observed with regard to the concentration of the template DNA, known amounts of extracted DNAs were subjected to the LC-PCR. By analysis with LC software, a linear regression curve was obtained between 20 fg and 2 ng of the DNAs with an error of 0.0303 and a correlation coefficient at -1.00 (data not shown). To determine the precise copy number detectable by the LC-PCR, purified plasmid DNA

Table 3. Oligonucleotides used in this LC-PCR assay

Oligonucleotide	Sequence*	Target gene	Nucleotide position	Primer/Probe
Ft-F	5'-GGCAAATCTAGCAGGTCA-3'	<i>fopA</i>	824-841	primer
Ft-R	5'-GCTGTAGTCGCACCATTATC-3'	<i>fopA</i>	1052-1073	primer
Ft-Flu	5'-ATGGCAGAGCGGGTACTAACAATGATTG-[FL]-3'	<i>fopA</i>	961-987	probe
Ft-LcR	5'-[Red640]-TGCTGGTTTAACATGGTTCCTTTGGTGG-[Ph]-3'	<i>fopA</i>	989-1015	probe

*[FL], Fluorescein; [Red640], LightCycler(Lc)-Red 640-N-hydroxy-succinimide ester; [Ph], 3'-phosphorylation.

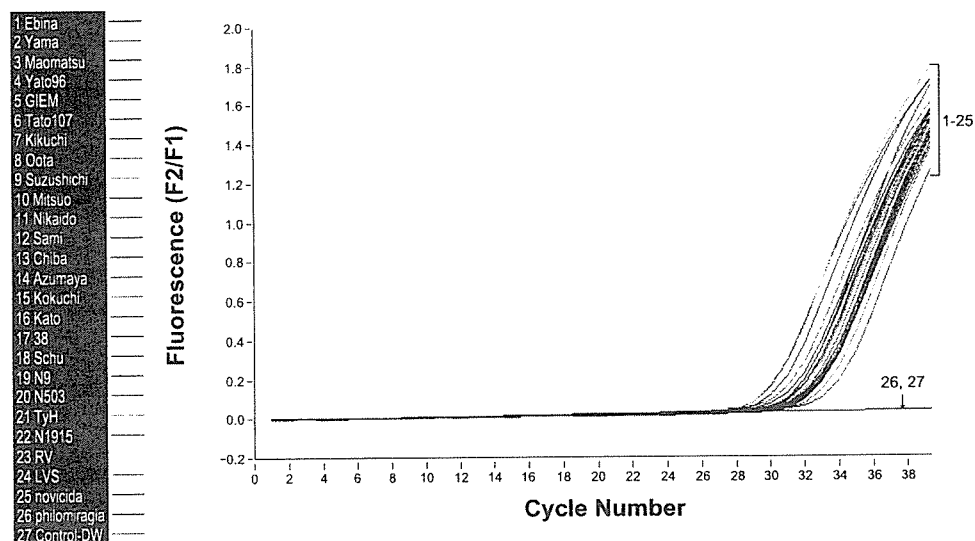


Fig. 1. Detection of the *fopA* gene by the LC-PCR assay. One pg of DNAs from 25 strains of *F. tularensis* and *F. philomiragia* was subjected to the LC-PCR.

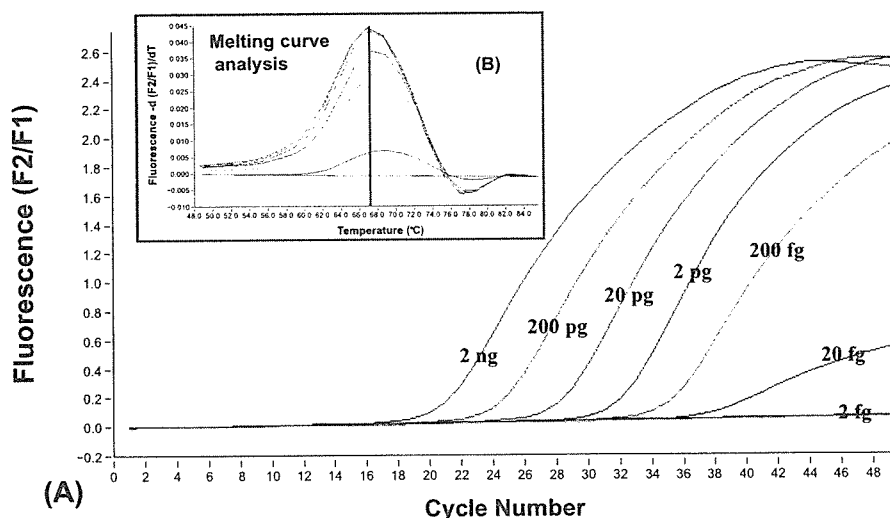


Fig. 2. Quantification analysis of the LC-PCR assay. (A) Twenty fg to 20 ng template DNA were used as template in the LC-PCR assay. (B) Melting-curve analysis of the above reactions.

containing 708-bp of *fopA* gene was subjected to the LC-PCR assay. Twenty femto grams of plasmid DNA corresponding to 10^1 copies of the gene were detected (Fig. 2). The 10-fold serially diluted *F. tularensis* (LVS) cell suspension was aliquoted into two parts. One part was processed for DNA extraction and LC-PCR, while the other was used for counting CFU. A significant fluorescent signal was detected in the LC-PCR assay when the DNAs from 1.2 CFU of the cell suspension were amplified by the LC-PCR (data not shown).

Specificity of the real-time PCR assay: To evaluate the specificity of the LC-PCR assay targeting the *F. tularensis fopA* gene, 1 ng of purified DNA from each of 43 non-*Francisella* organisms was tested. No significantly elevated signal was observed with any of the tested bacterial DNA (Table 2). The absence of amplified DNA was confirmed by agarose gel electrophoresis of LC-PCR products derived from non-*Francisella* organisms (data not shown). It is noteworthy that DNA obtained from *F. philomiragia* did not give rise to any positive response. These results indicated that this LC-PCR assay was highly specific for *F. tularensis*.

DISCUSSION

The development of a highly sensitive and specific PCR assay alleviates the problems associated with microorganisms that are found in low densities in tissue or tissue fluids, and that are difficult to cultivate. In recent years, real-time PCR has emerged as a valuable tool for the rapid identification of various microbes (26). The rapid-cycle real-time PCR method, LC, which is highly sensitive and specific for the detection and quantification of infectious agents, has been applied (27-33) to the diagnosis of a variety of pathogens. This report describes the development of a real-time PCR assay employing two primers for amplification and two hybridization probes for the specific detection of the *F. tularensis* DNA sequence in a rapid, accurate, and quantitative manner.

To establish the LC-PCR assay, we selected a specific primer pair and two independent hybridization probes that were derived from the nucleotide sequences of the *fopA* gene of *F. tularensis*. As shown in Fig. 2A, the limit of detection per reaction was 20 fg of the *F. tularensis* genomic DNA,

which corresponded to 10 copies of the *fopA* gene or 1.2 CFU of bacterial cells. The identification of *F. tularensis* can be achieved within 1 h using our standard protocol. The LC-PCR assay established in this study showed a level of sensitivity that was similar to or higher than the previously reported methods employing TaqMan technology for the detection of the *fopA* gene of *F. tularensis* (17-19). Our method is highly specific, since 37 non-*Francisella* organisms representing diverse genera did not give rise to positive responses (Table 2). Moreover, our LC-PCR assay could detect the *fopA* gene of *F. tularensis* but not that of *F. philomiragia*. Previous reports (17,19) showed that the real-time PCR assays targeting the *fopA* gene from *F. tularensis* could not discriminate it from that of *F. philomiragia*. Because the sequences of TaqMan probes used in the previous reports were highly conserved between *F. tularensis* and *F. philomiragia* (25 of 27 and 27 of 29 nucleotides, respectively), the probes could detect amplified DNAs from both *F. tularensis* and *F. philomiragia*. In contrast, two 27-mer oligonucleotides used as hybridization probes in our LC-PCR assay system contained 4 or 5 nucleotide differences when compared to the *F. philomiragia* sequence. Moreover, most of the differences were located near the 5'-end region of the second probe, Ft-LcR, indicating that hybridization of the probes to the amplified DNA from *F. philomiragia* did not take place efficiently under the conditions employed here. The involvement of two hybridization probes appeared to improve the specificity of the assay. The LC-PCR, therefore, can be used for the discrimination of *F. tularensis* from *F. philomiragia*.

Human cases of tularemia caused by either *F. tularensis* subsp. *tularensis* or *holarctica* have been reported in the United States, Spain, Sweden, Turkey, Bulgaria, and Kosovo in recent decades (34-40). Although more than 40 cases of tularemia were annually reported in Japan for 20 years after World War II, less than 10 cases per year have been reported since 1966 (41). No tularemia case was reported in 2000 or thereafter (Dr. H. Fujita, personal communication). The reason for the decline in the prevalence of tularemia has been discussed. The lifestyles of Japanese farmers and hunters who used to catch and cook wild animals as an important source of nutrition in endemic areas have changed tremendously (42).

Although tularemia is a rare disease in Japan, the possible introduction of the disease from foreign countries in which it is endemic as well as the intentional release of the bacteria should be taken into consideration (43). In recognition of the importance of tularemia, the Ministry of Health, Labour, and Welfare of Japan decided to add "tularemia" to the list of notifiable diseases in Japan beginning in 2003. The LC-PCR assay established in this study could be a powerful tool for conducting passive surveillance of tularemia through laboratory diagnosis.

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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 chocolate 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 phagolysosomes (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
			<i>F. tularensis</i> (26)	<i>F. novicida</i> (1)	<i>F. philomiragia</i> (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 fur-

ther 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

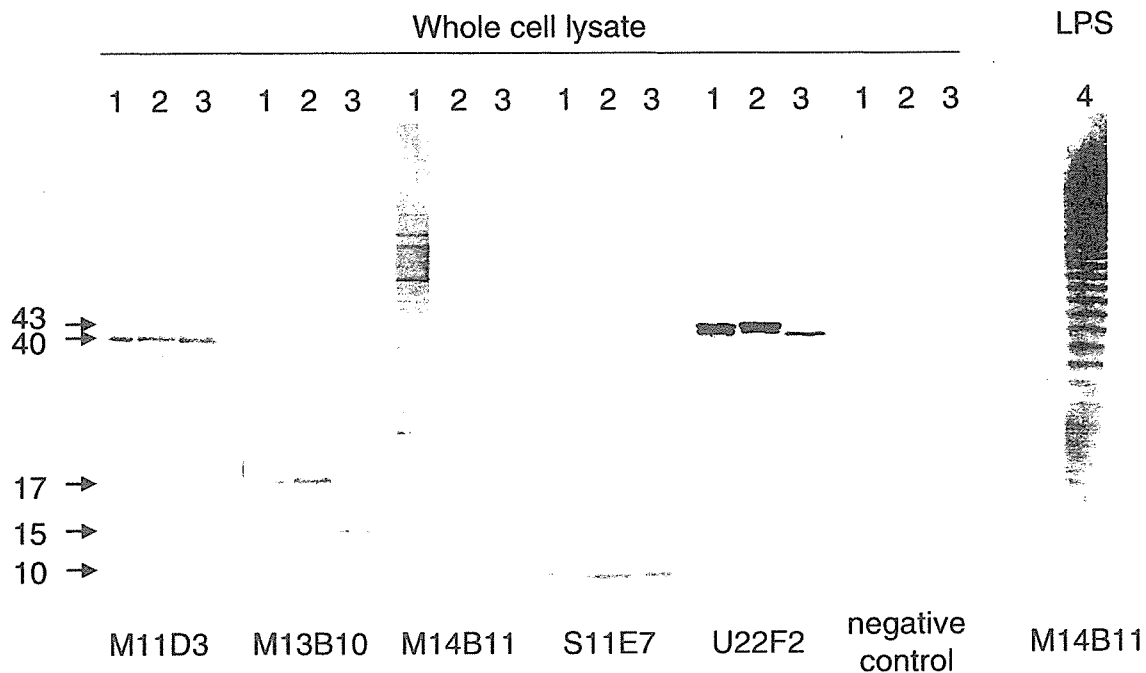


FIG. 1. Reactions of MAbs shown by Western blots following SDS-PAGE. Bacterial lysates from *F. tularensis* LVS, *F. novicida* U112, and *F. philomiragia* 029 (lanes 1 to 3, respectively) were reacted with MAbs M11D3, M13B10, M14B11, S11E7, and U22F2 and normal mouse serum (negative control). The reaction 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).

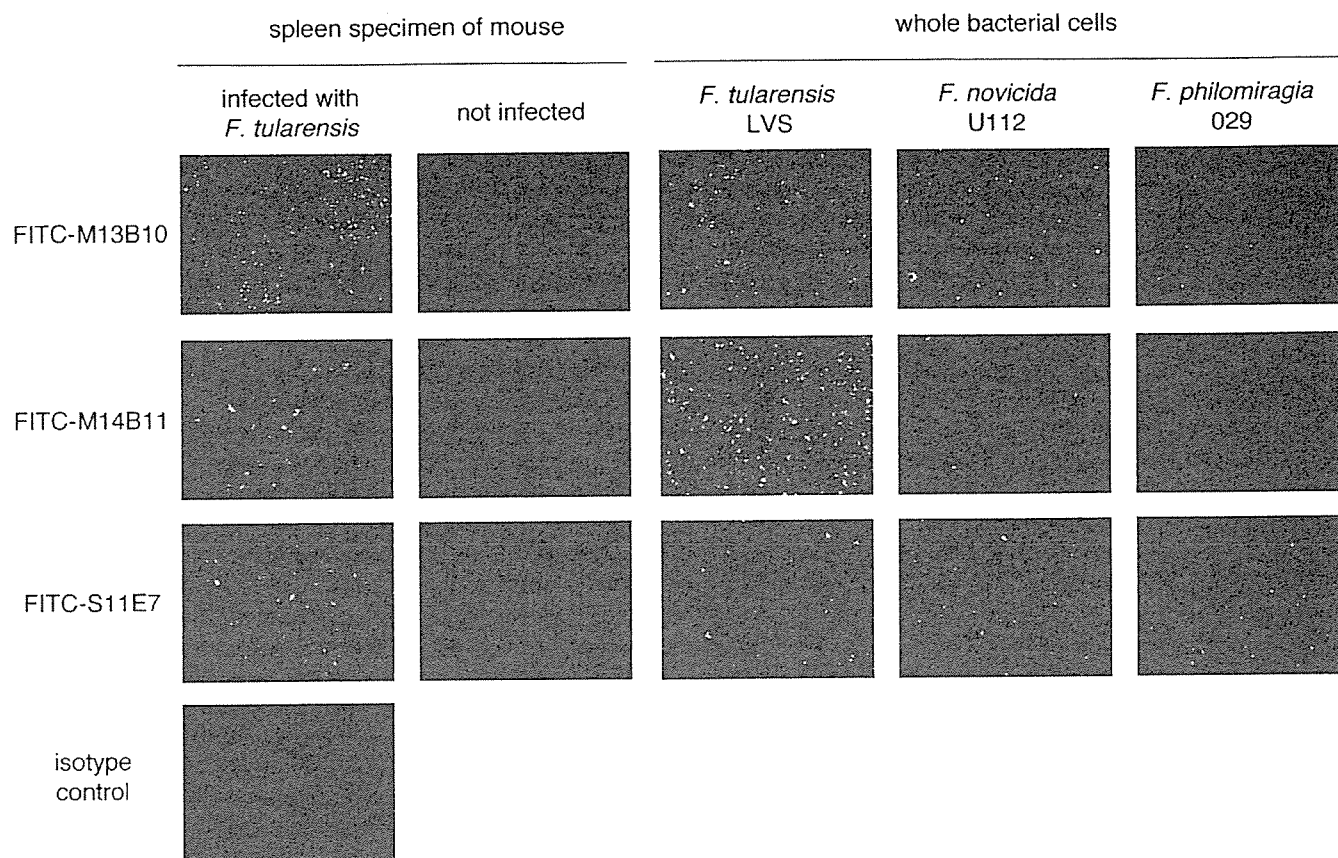


FIG. 2. Reactions of MAbs shown by DFA. FITC-labeled MAbs M13B10, M14B11, and S11E7 were reacted with the impression smears of the spleens from a mouse infected with *F. tularensis* Yama and an uninfected mouse and whole-bacteria cells of *F. tularensis* LVS, *F. novicida* U112, and *F. philomiragia* 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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A highly attenuated rabies virus HEP-Flury strain reverts to virulent by single amino acid substitution to arginine at position 333 in glycoprotein

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Abstract

An amino acid at position 333 in the glycoprotein of several fixed rabies virus strains is responsible for the pathogenicity in adult mice. Substitution of arginine at this position largely reduces the viral pathogenicity in adult mice. Attenuation by this single amino acid substitution has been established by using escape mutants selected by monoclonal antibodies and point-mutated virus generated by reverse-genetics. A highly attenuated HEP-Flury strain, which was selected by serial passages in cell cultures, has glutamine at this position. In this study, a point-mutated rHEP³³³R virus, having arginine at position 333, was generated and examined for the responsibility of this substitution in rabies pathogenicity. The rHEP³³³R acquired an ability to spread and propagate in mouse brain but the parental rHEP did not. The pathogenicity of rHEP³³³R to adult mice by intracerebral inoculation largely increased. We confirmed that an arginine at position 333 contributed to reversion of the pathogenicity in a highly attenuated HEP-Flury strain.

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Keywords: Rabies virus; Glycoprotein; Pathogenicity

1. Introduction

Rabies virus targets the central nervous system (CNS) and causes fatal encephalitis in almost all species of mammals. Rabies virus belongs to the genus *Lyssavirus* of the family Rhabdoviridae and has an unsegmented negative-sense RNA as the viral genome (Tordo et al., 1986). The genome is about 12 kb in length and encodes five genes for the structural proteins; N, P, M, G, and L. The G protein of rabies virus forms spike projection on the virus particle (Gaudin et al., 1992). Attachment of the G protein with a cellular receptor contributes to retrograde axonal transport and viral distribution in the brain (Etessami et

al., 2000; Mazarakis et al., 2001; Yan et al., 2002). Furthermore, the G protein is a main target for virus-neutralizing antibodies (VNAs) (Wiktor et al., 1973; Cox et al., 1977; Perrin et al., 1985). Thus, the G protein has been considered to be a major contributor in pathogenicity of rabies virus.

Various monoclonal antibodies (mAbs) against the G protein have been used to map epitopes and to characterize rabies virus pathogenicity. MAb-escape mutants of fixed rabies virus strains with amino acid substitution of glutamine, isoleucine, glycine, methionine, or serine for arginine or lysine at position 333 of the G protein, have lost their virulence in adult mice after intracerebral inoculation (Dietzschold et al., 1983; Seif et al., 1985; Tuffereau et al., 1989). From these results, it was concluded that a positively charged amino acid at position 333 in the G protein was critical for the pathogenicity of fixed strains. It has been also reported that the amino acid substitution at position 333 affected

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virus invasiveness into the CNS via peripheral routes (Kucera et al., 1985; Coulon et al., 1989) and a rate of cell-to-cell spread of the virus in cultured cells (Dietzschold et al., 1985). However, the mechanism of this attenuation has not been fully elucidated.

There are many fixed rabies virus strains with different levels of pathogenicity. Highly pathogenic strains such as CVS or Nishigahara kill adult mice by either intracerebral or peripheral infection. These strains exhibit high *in vivo* neuroinvasiveness; an ability of the virus to invade the CNS from a peripheral route of infection, and also high *in vitro* neurotropism; susceptibility of the virus to neuronal versus non-neuronal cells (Morimoto et al., 1998, 2000; Ito et al., 2001). Other pathogenic strains such as ERA, PV or LEP-Flury cause fatal encephalitis by intracerebral infection but not by peripheral infection, indicating that these viruses exhibit less *in vivo* neuroinvasiveness and higher *in vitro* neurotropism. Avirulent strains such as HEP-Flury or RC-HL do not cause fatal infection by either intracerebral or peripheral inoculations, suggesting that these viruses exhibit less *in vivo* neuroinvasiveness and lower *in vitro* neurotropism. The avirulent RC-HL strain, which is derived from virulent Nishigahara strain through serial passages in cell cultures, has arginine at position 333 in the G protein (Ito et al., 1994). Recently, it has been reported that regions other than position 333 in the G protein were responsible for the pathogenicity in the RC-HL strain and the arginine at position 333 did not participate in the pathogenic shift during attenuation of the RC-HL strain (Takayama-Ito et al., 2004). These data suggest that the responsibility of amino acid at position 333 for the pathogenicity depends on the strain and structure of the glycoprotein.

A highly attenuated HEP-Flury, high-egg-passage Flury strain which was derived from rabid human isolate by serial passages through brains of chicken, chick embryos, and cultured cells (Koprowski et al., 1954). The HEP-Flury strain causes transient weight loss and mild ruffled fur but does not cause death in adult mice even after intracerebral inoculation. As expected, the HEP-Flury strain has glutamine at position 333 (Morimoto et al., 1989). The LEP-Flury, low-egg-passage Flury strain, has arginine at that position and causes fatal infection after intracerebral inoculation. There are eight amino acid differences including position 333 between G proteins of these two strains (unpublished data). Thus, it is not clear about the extent to which an amino acid substitution at position 333 is responsible for the pathogenic shift during attenuation of the HEP-Flury strain.

In this study, in order to address whether the HEP-Flury strain increases its pathogenicity by a single amino acid change at position 333, we have generated a mutant virus, rHEP³³³R having a single amino acid substitution of arginine for glutamine at position 333, and characterized its growth properties *in vitro* and *in vivo*, neuroinvasiveness, its ability to induce VNA, and pathogenicity.

2. Materials and methods

2.1. Cells and viruses

Mouse neuroblastoma (NA) cells were maintained in Eagle's minimum essential medium (MEM) (SIGMA) containing 10%

fetal bovine serum (FBS) (GIBCO). Baby hamster kidney (BHK-21) cells were grown in Dulbecco's modified Eagle's MEM (SIGMA) supplemented with 10% FBS. A recombinant HEP-Flury (rHEP) virus was rescued from the full genome plasmid of HEP-Flury strain and propagated in NA or BHK-21 cells (K. Inoue et al., 2003). A highly pathogenic CVS strain was propagated in suckling mouse brains.

2.2. Rescue of mutant rHEP³³³R virus

For making a mutant rHEP³³³R, full-length genome plasmid pHEP³³³R with the genomic backbone of HEP-Flury strain was constructed in which arginine at position 333 in the G protein was encoded instead of glutamine (Fig. 1A). The rHEP³³³R virus was rescued using the method as described before (K. Inoue et al., 2003). Briefly, BHK-21 cells were grown overnight and transfected with 2.0 μ g of full-length genome plasmid (pHEP³³³R), 0.5 μ g of pH-N, 0.25 μ g of pH-P, 0.1 μ g of pH-L, and 0.15 μ g of pH-G using TransIT LT-1 (Panvera) according to the manufacturer's protocol. After 16 h, the cells were washed once and maintained for 2 days in Dulbecco's modified Eagle's MEM supplemented with 10% FBS. The culture medium was transferred to NA cells and incubated further 3 days. The rescued viruses were propagated in NA cells and stored at -80°C until use. To confirm nucleotide sequence of the rescued virus, sequencing of the G gene using ABI PRISM 310 Genetic Analyzer (Applied Biosystems) was performed.

2.3. Virus titration

Viral titers were determined by direct fluorescent test using NA cells. NA cells in 96-well plate were inoculated with serial 10-fold dilution of virus and incubated at 37°C for 2 days. Cells were fixed with 80% acetone for 20 min and stained with FITC-labeled anti-rabies mAb (Centcor). Antigen-positive foci were counted under a fluorescent microscope (OLIMPUS) and calculated as focus forming unit (ffu) per millilitre.

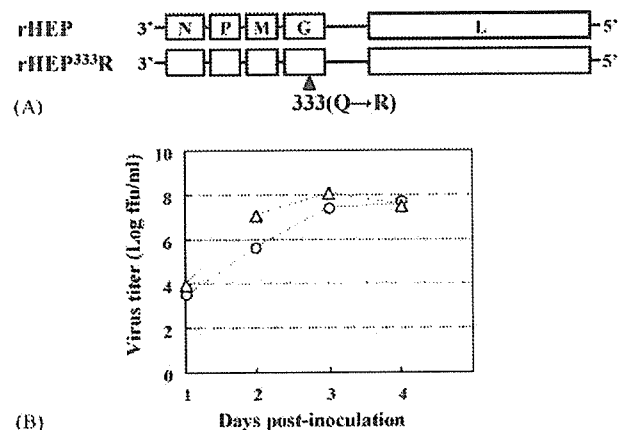


Fig. 1. (A) Schematic representation of genomes of rHEP and rHEP³³³R. (B) The virus growth curves of rHEP (open circle) and rHEP³³³R (open triangle) in neuroblastoma NA cells. NA cells were infected with either virus at an m.o.i. of 0.01. Virus titers in the cultured medium of the infected cells were determined by focus forming assay.

2.4. Inoculation of virus to mice and collection of mouse brains

The animal studies have been approved by the Committee for Animal Experimentation of National Institute of Infectious Diseases. Six-week-old ICR mice (Japan SLC Inc.) were inoculated with 10^5 ffu/0.03 ml of rHEP or rHEP³³³R by intracerebral inoculation under diethyl ether anesthesia, and the mice were observed for 14 days for death or clinical signs of rabies. To determine 50% lethal dose (LD₅₀) of each virus in adult mice, groups of six ICR mice were intracerebrally inoculated with 0.03 ml of serial 10-fold dilution of rHEP or rHEP³³³R. The LD₅₀ of each virus was calculated by the method of Reed and Muench (1938). To determine the virus growth in mouse brains, the virus-infected C57BL/6 mice were anesthetized with diethyl ether, and the brains were removed and weighted. The brains were homogenized in nine-fold volume of phosphate-buffered saline (–) [PBS (–)], and the homogenates were centrifuged at $4000 \times g$ for 10 min at 4 °C. Virus titers of the supernatant were determined by direct fluorescent test as describe above and were expressed as ffu per total of brain.

2.5. Anti-rabies virus-neutralizing antibodies (VNAs) in mouse sera

Sera of the mice inoculated with rHEP or rHEP³³³R were collected for determination of VNA titers against rabies virus. The VNA titers were determined by a modified rapid fluorescent focus inhibition test (Wiktor et al., 1984). Briefly, mouse sera were diluted into 10-fold with Eagle's MEM containing 0.2% bovine serum albumin, and heated at 56 °C for 30 min to inactivate complement. Serial four-fold dilutions of the sera were prepared and equal volume of virus suspension of rHEP or rHEP³³³R were added into each well. After incubation at 37 °C for 1 h, 100 µl of sera and virus suspension mixture was transferred into NA cells in 96-well plate. After 2 days incubation at 37 °C, the direct fluorescent test was carried out. VNA titers were defined as the highest serum dilution that neutralized 50% of the challenge virus and normalized to international unit (IU) using the World Health Organization (WHO) anti-rabies antibody standard.

2.6. RNA extraction and RT-PCR

RT-PCR for detecting rabies virus genomic RNA was performed with total RNAs extracted from mock-infected or infected C57BL/6 mouse brains. Total RNAs were extracted with ISOGEN (Nippongene) according to the manufacture's protocol. The cDNAs of rabies genomic RNAs were synthesized using sense primer [N5-a: 5'-ATG GAT GCC GAC AAG ATT GT-3'] with AMV Reverse Transcriptase XL (Takara Bio Inc.), and the N gene of viruses was amplified using N5-a primer and antisense primer [N3-a: 5'-CCC ACT CTG ATT GCC GAA TA-3']. PCR amplifications were carried out in a volume of 25 µl with Ex-Taq (Takara Bio Inc.). The mixture was denatured at 94 °C for 5 min, and then subjected to 30 cycles of reactions at 94 °C for 30 s, at 50 °C for 30 s, and at 72 °C for 1 min, and

the following reaction at 72 °C for 7 min. For an internal control, glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene was amplified by using G3PDH sense primer [5'-TGC CAA GGC TGT GGG CAA GGT CAT-3'] and antisense primer [5'-AAC AGG GTG GTG GAC CTC ATG GCC-3']. Sizes of the PCR products of N gene and G3PDH were 684 and 348 bp, respectively.

2.7. Immunohistochemical analysis

Six-week-old C57BL/6 mice were anesthetized by diethyl ether and were inoculated intracerebrally with 10^5 ffu per 0.03 ml of rHEP or rHEP³³³R. After 4 days post-inoculation (dpi), mice were deeply anesthetized by etherisation, and the brains were perfused with 10% sucrose in PBS and fixed in 4% formaldehyde containing 10% sucrose. After perfusion, the brains were removed and fixed in 4% formaldehyde and 30% sucrose in PBS (–) for 1 day. The brains were stored in 30% sucrose in PBS (–) until use. Samples were frozen and serially sectioned in the coronal plane at a thickness of 50 µm. To identify virus-infected neurons, free-floating tissue sections were processed according to the avidin-biotin-peroxidase method (Vectastain; Vector Laboratories) with anti-rabies N antibody as the first antibody (S. Inoue et al., 2003). The reacted tissue sections were mounted on gelatin-coated glass slides, air-dried, and coverslipped. The slides were observed under a bright field microscope (OLIMPUS).

Adult (12–16-week-old) male Wistar rats weighing 230–260 g were first deeply anesthetized by intraperitoneal injection with pentobarbital (50 mg/kg). They were kept anesthetized by injecting 0.2 ml of (50 mg/ml) ketamine every 2–3 h. The rat was mounted in a stereotaxic frame, and the skull was exposed. Burr holes were made for electrode access according to coordinates by Paxinos and Watson (1986). The animals were inoculated with 10^4 ffu/0.5 µl of rHEP or rHEP³³³R including pontamine sky blue (as a marker for the injection site) at hippocampus dentate gyrus region. To inject virus into the hippocampal layers, a grass-microelectrode glued to a Hamilton syringe was used. An injection site was identified by recording evoked field potentials with stimulation of the perforant pathway. Thirty-six hours after the inoculation, brains of the infected rats were removed and used for immunohistochemical studies as described above.

3. Results

3.1. Growth property of rHEP and rHEP³³³R in cultured cells

Single point mutated rHEP³³³R virus was made by using reverse genetics, in which glutamine was substituted with arginine at position 333 in the G protein (Fig. 1A). The growth kinetics of the parental rHEP and rHEP³³³R viruses in mouse neuroblastoma NA cells were shown in Fig. 1B. There were no significant differences in growth in NA cell cultures between rHEP³³³R and rHEP. The single amino acid substitution in the surface G protein did not affect growth property of these two

Table 1
In vitro neurotropism of rHEP and rHEP³³³R

Strain	Titer (ffu/ml ^a)		In vitro neurotropism index ^b
	NA	BHK	
rHEP	1.0×10^8	1.0×10^8	0.0
rHEP ³³³ R	3.5×10^8	1.1×10^7	1.5
LEP-Flury	1.0×10^7	2.0×10^5	1.7
CVS	1.5×10^8	4.5×10^6	1.5

^a Each virus was titrated in NA or BHK-21 cells.

^b The in vitro neurotropism index is expressed with the logarithm of the titer in NA cells minus the logarithm of the titer in BHK-21 cells.

strains. In addition, the pH threshold for low pH-dependent fusion activity, which was responsible for the entry of virus from endosome to cytoplasm of cell, was not affected by the single amino acid change. These two viruses induced the fusion in BHK-21 cells when the cells were exposed below pH 5.8 (data not shown). Table 1 shows the in vitro neurotropism index of rabies strains, which is indicated as infectivity ratio of the virus in NA cells versus in BHK-21 cells (Morimoto et al., 1998). The value of ratio 1.0 indicates that the virus has 10 times stronger infectivity to neuronal cells than that to non-neuronal cells. Pathogenic rabies strains commonly exhibited higher in vitro neurotropism than attenuated strains did. The index of the pathogenic strain was generally over 1.0, whereas the index of the attenuated strain decreased to be around 0 (Morimoto et al., 1998, 2000; Ito et al., 2001). The indices of rHEP and rHEP³³³R showed 0 and 1.5, respectively. The index of rHEP³³³R increased similar to those of pathogenic LEP-Flury and CVS strains.

3.2. Pathogenicity of rHEP³³³R to adult mice

In order to determine the pathogenicity of rHEP and rHEP³³³R in adult mice, 10^5 ffu of each virus were inoculated intracerebrally. The rHEP-infected mice showed mild symptoms with slight reduction of body weight (Fig. 2A). On the other hand, the mice inoculated with rHEP³³³R showed clinical symptoms indicative of the CNS disease, such as paralysis and hyperactivity. All of the mice inoculated with rHEP³³³R died within 11 days post-infection (dpi). This result clearly shows that the rHEP³³³R increased its pathogenicity in adult mice by a single amino acid change. The LD₅₀ of rHEP³³³R was determined, and it was calculated that 154 ffu of rHEP³³³R was required for one LD₅₀. The rHEP did not cause death even when 10^6 ffu of the virus were inoculated. Thus, the ffu required for one LD₅₀ was calculated as more than 10^6 ffu. Highly pathogenic CVS strain required 1.5 ffu for one LD₅₀ (data not shown), indicating that rHEP³³³R was 100-times less virulent than the CVS strain. Peripheral pathogenicity was assessed with intramuscular inoculation of mice with rHEP, rHEP³³³R, or CVS strain. None of the mice inoculated intramuscularly with either rHEP or rHEP³³³R died, although one of the six mice inoculated with rHEP³³³R exhibited hind leg paralysis (Fig. 2B). The CVS strain exhibited high in vivo neuroinvasiveness. The in vivo neuroinvasiveness was not increased by the shift to arginine at position 333 in the G protein of HEP-Flury strain.

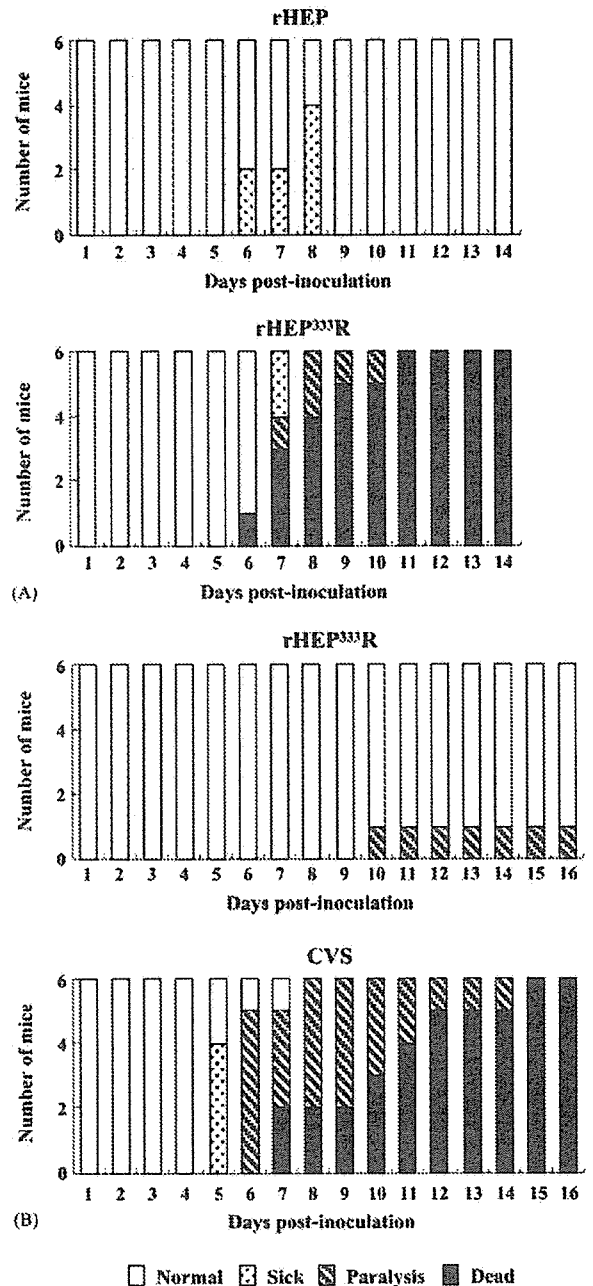


Fig. 2. Mortality and morbidity of mice infected with rabies virus. Six ICR mice were inoculated intracerebrally with 10^5 ffu of rHEP or rHEP³³³R (A) and were inoculated intramuscularly with 10^7 ffu of rHEP³³³R or 10^6 ffu of CVS (B). The infected mice were observed for clinical signs of rabies for 14 days (A) or 16 days (B).

3.3. Virus propagation in mouse brains and VNA in the mouse sera

Next, the in vivo properties of rHEP and rHEP³³³R were compared for virus titer, genome replication, antigen distribution, and VNA titer (Fig. 3). Virus titer of the rHEP was not detected during the observation period, whereas virus titer of the rHEP³³³R was $10^{4.65}$ ffu per brain at 4 dpi and increased to a

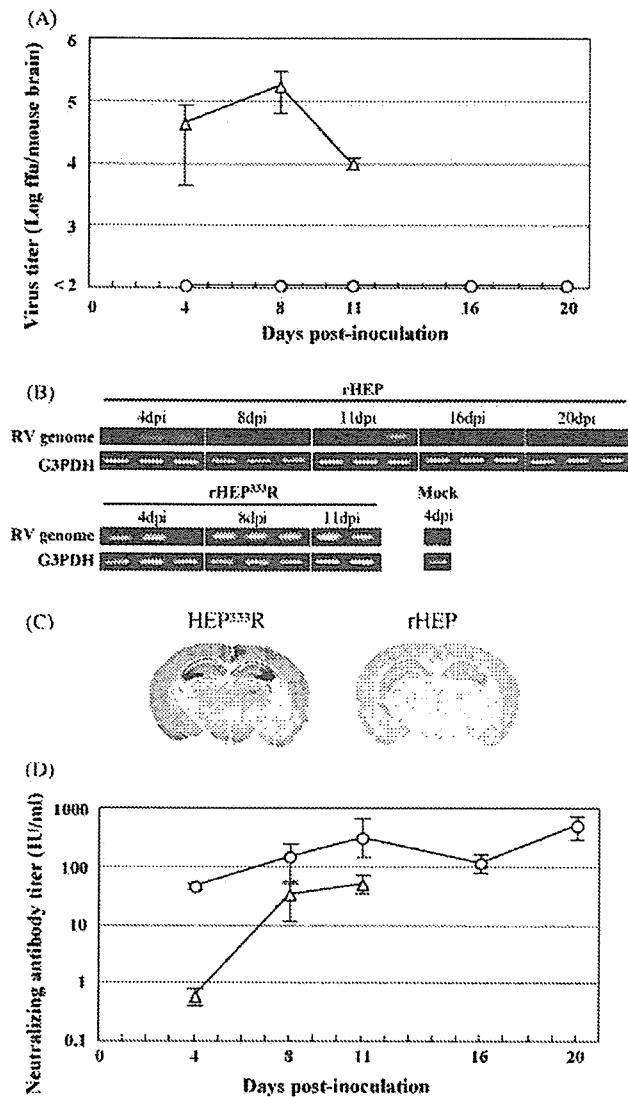


Fig. 3. Virus propagation of rHEP and rHEP³³³R in infected mouse brains: (A) virus titer of rHEP and rHEP³³³R in the infected mouse brains. (B) RT-PCR analysis of viral genome in the infected mouse brains. (C) Immunohistochemical analysis for rabies virus N protein. (D) Anti-rabies neutralizing antibody titers in sera of the infected mice. C57BL/6 mice were infected intracerebrally with 10^5 ffu of rHEP (open circle) or rHEP³³³R (open triangle). Brains and sera of the infected mice were collected at 4, 8, 11, 16, and 20 dpi. The virus titers were determined by focus forming assay, and shown as titers in total of brain suspension. The RT-PCR analysis was performed with rabies genomic RNA and G3PDH mRNA in the 10% mouse brain emulsions. At 4 days after inoculation, the mice were perfused, and the brains were subjected for immunohistochemistry with anti-rabies N antibody. Left side, rHEP³³³R; right side, rHEP. The titers of VNA were expressed as geometric means \pm standard errors ($n=3$).

peak of $10^{5.25}$ ffu per brain at 8 dpi (Fig. 3A). All mice inoculated with rHEP³³³R were dead within 11 dpi.

The viral genomic RNA in the mouse brains was examined by an RT-PCR analysis (Fig. 3B). The band specific for rabies N gene amplified from the genomic sense RNA was detected in the brains inoculated with rHEP³³³R at 4, 8, and 11 dpi. In contrast, weak signal of N gene in the brains inoculated with rHEP was detected at 4 dpi, and disappeared at 8 dpi, while weak signal

remained in one of the three brains at 11 dpi. The infectious rHEP viruses were not detected in the brains during the observation period through 20 dpi (Fig. 3A).

The propagation of virus in the brain was also investigated by immunohistochemical analysis for rabies virus N protein. The brain inoculated intracerebrally with either rHEP or rHEP³³³R was removed at 4 dpi and stained with anti-rabies N antibody. As shown in Fig. 3C, antigen-positive cells were presented mainly in the CA3 region of hippocampus of the rHEP³³³R-infected mouse brain, whereas no apparent antigen-positive cells were detected in the rHEP-infected mouse brain.

VNAs play an important role in protection of rabies infection. Titers of the VNA in sera of the mice inoculated with either rHEP³³³R or rHEP were compared (Fig. 3D). Rapid induction of the antibody response was detected in sera of the rHEP-infected mice. The titers were 45 IU/ml at 4 dpi, and increased to 500 IU/ml at 11 dpi. The titers remained high level during the observation period though 20 dpi. On the other hand, titers of the VNA in sera of the mice inoculated with rHEP³³³R were low (0.6 IU/ml) at 4 dpi. Although the titers increased to 34 IU/ml at 8 dpi, all of the mice infected with rHEP³³³R died at 11 dpi. The VNA could not overcome propagation of the rHEP³³³R at the late stage of infection.

3.4. Neuronal transport of virus in rat brain

A standard intracerebral inoculation with large inoculum of virus breaks blood–brain barrier and stimulates immune responses in the host. To exclude a possibility that the rapid induction of VNA might prohibit spread of the rHEP strain, stereotaxic inoculation of the viruses into hippocampus dentate gyrus region of the rat brain was performed, which induced much less immune responses (Yang and Jackson, 1992). The spread of the virus was examined at the early stage of infection (36 hpi), and differences of axonal transport in the CNS were assessed (Fig. 4). In inoculation with the rHEP³³³R, spread of antigen-positive cells was observed in the hippocampus CA1 and CA3 regions (Fig. 4D), and lateral entorhinal cortex (LEA) and medial entorhinal cortex (MEA) regions (Fig. 4E, F, respectively). Neurons in LEA and MEA are known to project into hippocampus. In contrast, no antigen-positive cells were detected around the injected site and in LEA and MEA regions of the rHEP-inoculated rat brain. These observations supported that rHEP³³³R gained an ability of axonal transport in the CNS as shown in Kelly and Strick (2000).

4. Discussion

The G protein of rabies virus is related to cell attachment (Lentz et al., 1982; Thoulouze et al., 1998; Tuffereau et al., 1998; Yan et al., 2002), induction of apoptosis (Jackson and Rossiter, 1997; Morimoto et al., 1999; Prehaud et al., 2003), and immune responses (Wiktor et al., 1973; Cox et al., 1977; Perrin et al., 1985). Therefore, the G protein plays the most important role in its pathogenicity. An amino acid at position 333 in the G protein has been shown to be critical to the pathogenicity in adult mice (Dietzschold et al., 1983; Seif et al., 1985; Tuffereau et al.,

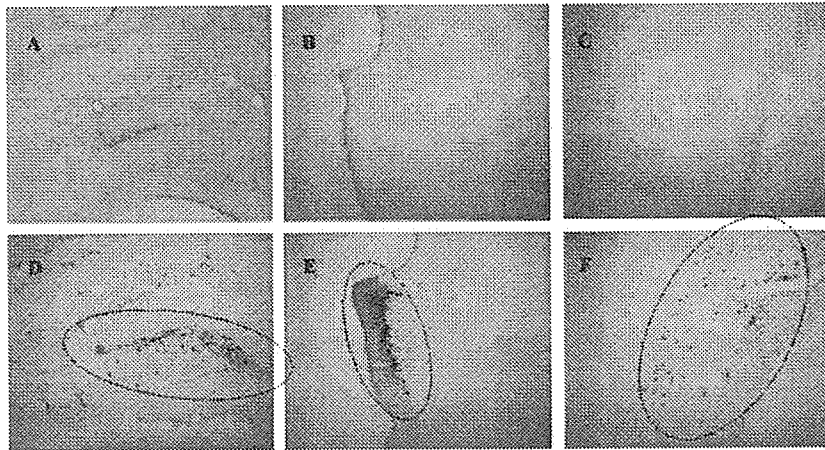


Fig. 4. Retrograde axonal transport of rHEP³³³R in virus-infected rat brains. Wistar rats were infected with 10^4 ffu of rHEP (A–C) or rHEP³³³R (D–F). The viruses were infected with the stereotaxic inoculation at hippocampus dentate gyrus region. At 36 h after inoculation, the rats were sacrificed for immunohistochemistry with anti-rabies N antibody. Brown signals indicated the rabies virus-antigen positive cells. Pontamine sky blue diffused around the injection site. (A, D) hippocampus region around the injection site. (B, E) lateral entorhinal cortex (LEA), (C, F) medial entorhinal cortex (MEA) projects to the injection site.

1989). However, it has been reported that the other positions were also responsible for the pathogenic shift (Takayama-Ito et al., 2004). This study revealed that an arginine at position 333 increased pathogenicity of a highly attenuated HEP-Flury strain. The rHEP³³³R spread through retrograde axonal transport and propagated efficiently in the CNS, while the parental rHEP did not.

As cellular receptors of rabies virus, a nicotinic acetylcholine receptor (Lentz et al., 1982), a neural cell adhesion molecule (NCAM) (Thoulouze et al., 1998), and a low-affinity neurotrophin receptor p75NTR (Tuffereau et al., 1998) have been reported. An ability of the G protein to bind p75NTR was dependent on the presence of lysine or arginine at positions 330 or 333 (Tuffereau et al., 1998). Thus, this molecule might be responsible for an ability of rHEP³³³R to spread and propagate in the CNS. As shown in Table 1, a significant difference of in vitro neurotropism was found between rHEP and rHEP³³³R, suggesting that the arginine at position 333 altered binding property of the virus into cell species. Pathogenic viruses commonly exhibited higher in vitro neurotropism (Morimoto et al., 1998, 2000; Ito et al., 2001). It might indicate that the receptor usage of rabies virus was shifted to more neurotropic state. CVS strain are able to invade the CNS from a peripheral site and killed mice, whereas other less pathogenic strains such as PV, ERA or rHEP³³³R do not kill mice and rarely induce flaccid hind leg paralysis by a peripheral route of inoculation (Smith, 1981; Galelli et al., 2000). In this regard, it was shown that the arginine at position 333 is not responsible for in vivo neuroinvasiveness of HEP-Flury strain.

The single amino acid substitution at position 333 dramatically altered the ability of the rHEP strain to spread in the CNS through retrograde axonal transport (Fig. 4). Yan et al. (2002) have reported that mutants with glutamine at position 333 in CVS and its derivative strains (CVS-N2c, CVS-B2c) did not change the distribution pattern of the virus spread in the CNS, however, that the same mutant of SN-10 strain lost an ability of the virus to spread in the CNS. It is, therefore, likely that involvement of

the amino acid at position 333 for viral spread in the CNS was dependent on the strains. The attenuation of HEP-Flury strain was similar to the attenuation by replacement of the arginine in SN-10 strain.

Intracerebral injection of virus disturbs the immune state of the CNS and stimulates the immune responses. However, stereotaxic inoculation stimulates much less induction of immune responses. Yang and Jackson (1992) have indicated these avirulent mutants of the CVS strain lost virulence after intracerebral inoculation but remained neurovirulent after stereotaxic inoculation. Therefore, they suggested a balance between the spreading efficiency of the virus in the CNS and the level of immune stimulation in the host would determine the pathogenicity (Yang and Jackson, 1992). The avirulent HEP-Flury (rHEP) and the SN-10³³³Gln mutant were incapable to spread in the CNS even after stereotaxic inoculation. In this case, therefore, it was excluded that the rapid induction of VNAs could prevent the rHEP to propagate in the early stage of infection.

There are two proposed models for spread of rabies virus; one is into contiguous cells by neuron-to-neuron transmission, and the other is into noncontiguous cells by free virions (Dietzschold et al., 1985). The neuron-to-neuron transmission through synapse junction would be preferentially utilized in the CNS. In the case of HEP-Flury or SN-10 strain, arginine at position 333 may be critical for the neuron-to-neuron transmission.

VNA is the most important factor in clearance of rabies virus from the CNS (Dietzschold et al., 1992; Hooper et al., 1998). The mice inoculated with rHEP exhibited rapid induction of VNAs without efficient propagation of the virus in the brain. On the other hand, efficient virus propagation was detected in the mouse brains infected with rHEP³³³R, and the induction of VNAs against rHEP³³³R was slower than that of rHEP. The rapid spread of rHEP³³³R in neuron-to-neuron through connecting synapses could retard the immune responses at the early stage of infection. A high level of virus propagation and massive distribution of rHEP³³³R might impair the rapid immune responses

in the CNS. The presence of VNAs at the late stage of infection reduced the infectious rHEP³³³R, but did not clear the virus completely, and the mice did not overcome death from rabies (Fig. 3). Several researchers (Coulon et al., 1982; Marcovistz et al., 1994; Irwin et al., 1999) have observed that the VNA response was faster and higher in avirulent mutant RV194-2 or AvO1 strain infected mice compared to mice infected with CVS strain. These data suggest immune responses of the host against rabies virus were different between pathogenic and avirulent strains.

The G proteins of avirulent viruses induced apoptosis more strongly than those of highly pathogenic viruses in the infected neuron (Morimoto et al., 1999; Prehaud et al., 2003), and the apoptosis consequently evoked higher immune response (Pulmanousahakul et al., 2001; Faber et al., 2002). Resident cells in the CNS might differently induce innate immunity and the following immune responses in an abortive infection or a productive infection. The rHEP having lower in vitro neurotropism might more easily attach to immunocompetent nonneuronal cells than the rHEP³³³R having higher in vitro neurotropism. In addition, it has been reported that suppression of T cell-mediated immune response was caused by infection with pathogenic rabies viruses (Wiktor et al., 1977; Perrin et al., 1996) and might be due to an induction of apoptosis in T cells (Baloul et al., 2004). However, this immune suppression against rabies infection remains to be clarified. Further studies analyzing the differences in immune response induced by rHEP and rHEP³³³R infections are needed to elucidate why rHEP infection induced the VNA so rapidly and efficiently or how rHEP³³³R infection retarded the induction of immune response although the virus had been propagated in the CNS.

In conclusion, we demonstrated here direct evidences that virulence the of the HEP-Flury strain was gained by a single point mutation to arginine at position 333 in the G protein and that the amino acid was responsible for a change of in vitro neurotropism and an ability of the virus to spread by retrograde axonal transport and propagate in the CNS.

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The Histopathogenesis of Paralytic Rabies in Six-Week-Old C57BL/6J Mice Following Inoculation of the CVS-11 Strain into the Right Triceps Surae Muscle

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ABSTRACT. A fatal encephalomyelitis was developed after intracerebral and hind limb inoculation of 6-week-old C57BL/6J mice by the inoculation of fixed rabies virus (CVS-11 strain), intracerebrally and into hind. After the intracerebral inoculation, virus antigens were detected in the cerebral cortex and hippocampus at 2 days postinoculation (PI), and later spread centrifugally to thalamus, brain stem, cerebellum, spinal cord and spinal ganglia. At 4 days PI, severe apoptosis and DNA fragmentation were observed in the hippocampus and cerebral cortex. All mice infected intracerebrally were dead without limb paralysis at from 10 to 11 days PI. In contrast, mice infected with virus intramuscularly were persistently observed virus antigens in the myocytes at the site of inoculation from 2 days PI. At 4 days PI, the antigens were demonstrated in the spinal dorsal root ganglia, spinal cord and muscle spindles without their detection in the cerebrum and hippocampus. There were no apoptosis in the spinal cord and dorsal root ganglia, however hind limb paralysis was found in all infected mice. Hind limb paralysis was progressed to quadriparalysis, and mice were dead from 11 to 13 days PI. From 4 days PI, necrosis of neuron was observed in the the spinal and dorsal ganglia with infiltration of lymphocyte. This study suggested that the necrosis of spinal neurons was more important to cause the paralysis of hind limb rather than the severe cerebral infection and apoptosis in C57BL/6J mice infected with CVS-11 strain. The virus primarily replicated in the muscles was ascended the spinal cord via afferent fibers and retrogradely invaded the cerebrum, and with subsequent spread to muscle spindles.

KEY WORDS: C57BL/6J mouse, encephalomyelitis, pathogenesis, rabies (CVS-11).

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Rabies is an ancient disease that is still endemic in many parts of the world. Rabies virus is a neurotropic virus that causes fatal encephalomyelitis in humans and animals [23]; however, the precise invasion routes from the periphery to the central nervous system (CNS) are unknown. It was hypothesized that replication of virus in skeletal muscle might be necessary to generate sufficient virus for entry into the peripheral nervous system [4, 5]. Other studies indicate that the virus can enter nerve terminals directly without replication in muscle [3, 9, 15, 18, 20, 30]. Anatomical analyses of neuronal nuclei infected by rabies virus following peripheral inoculation have demonstrated that the virus propagates in the CNS across chains of synaptically connected neurons [1, 19, 27, 29].

The Challenge Virus Standard (CVS) strain of fixed virus is a pathogenic virus that causes fetal acute myeloencephalitis. In several studies, it was reported that apoptosis plays an important role in pathogenesis of CVS virus infected animals [23]. Apoptosis is considered individual cell death, as it does not induce damage to neighboring cells. In contrast, necrotic cell death, which occurs in response to acute cellular damage, is characterized by cellular alterations that differ from those characteristic of apoptosis, including cell swelling, random degradation of chromatin, and eventually cell lysis [7, 21]. Intracerebrally inoculated rabies virus (CVS strain) caused strong apoptosis in the cerebral cortex and hippocampus in suckling and adult ICR mice [16, 22], while the Purkinje cells of the mouse and rat embryonic spinal motor neurons were more resistant to apoptotic cell

death [10, 16]. These reports suggest that various neuronal cell types have susceptibility to rabies virus but that the mechanism of induction of cell death differs among neurons after infection.

In this study, fixed CVS-11 strain was inoculated into the cerebrum and peripheral muscles of adult C57BL/6J mice, and the primary target cells and the sequential involvement of major regions during infection in the peripheral tissues and CNS were investigated. In addition, the patterns of neuronal cell death with time courses in the brain and spinal cord of infected mice were compared.

MATERIAL AND METHODS

Virus, animals, and inoculations: The CVS-11 strain of fixed rabies virus, which was obtained from Dr. C. E. Rupprecht (Rabies Section, Viral and Rickettsia Zoonoses Branch, Center for Disease Control and Prevention, Atlanta, GA, U.S.A.), was replicated grown in mouse A/J (H-2a) neuroblastoma cells as previously described [27]. Thirty nine 6-week-old C57BL/6J mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). Intracerebrally (right forebrain), 18 mice were inoculated with viral doses of 10^5 plaque-forming units of the CVS-11 strain suspended in phosphate-buffered saline (PBS). Whereas, intramuscularly (right triceps surae muscle), 21 mice were inoculated viral doses of 10^5 and 10^7 plaque-forming units of the CVS-11 strain suspended in phosphate-buffered saline (PBS). Two groups of 2 uninfected control mice were respectively inoc-