TABLE 4
Continued

No. isolates						Nucleotide no. in the E region	957	967	969	984	993	996	1011	1028	1035	1055	1059	1068	1071	1077	1004	1006	1116	1120	1134	1149	1152	1164	1176	1185	1194	1212	1218	1233	1263	1323	1341	1389	1398	1425	1429	1434	1480
2005	2007	2008	2009	Cluster	Subcluster	Consensus	T	G	C	T	T	T	T	C	A	G	G	A	A	C	G	T	C	A	C	C	G	G	T	C	A	C	T	G	T	T	T	A	C	A	G	G	C
1				A	A-1												A					Y																					
1							C										A									R																
1																	R										R															
1																											R															
1																	A																									
1																	A						T																			
1																	R						Y																			
1																																										
2																																										
2											Y																															
2																								G			A															
2																	A																									
5																																										
	1			A	A-2																						A															
	1																										A								C							
	2													T													A									G						
	2													T													A															
	9																										A															
		2																									A															
		2																G									A						A									
		3																									A															
			1							C										T							A															
			2																	T							A															
		1		A	A-3																						A				G											
		1																									A				T	G										
		1						R																			A					G										
		1																									A					G	R									
		1											C														A					G										
		2																							T		A					G										
		2																									A					G										
		2														A					A						A					G										
		24																									A					G										
	3			B						C													T		T				T	C		C									T
	1			C					T	C		C			G				G			T					C	T	G	T	C			A					A	T
	2									C		C			G				G			T					C	T	G	T	C			A					A	T

*Y = C or T; W = A or T; R = A or G. Isolates that had superimposed signals in the nucleotide sequence are indicated in gray.

TABLE 5

Amino acid sequence differences within envelope (E) gene among strains of Japanese encephalitis virus isolated, Toyama Prefecture, Japan*

No. isolates						Amino acid no. in the E region	161	181	231	281	312	323	343	365	366	374	477
2005	2007	2008	2009	Cluster	Subcluster	Consensus	A	G	T	T	K	V	A	S	S	M	D
1				A	A-1										S or P		
1																	
1																	
1																	
1																	
1																	
1																	
2																	
2																	
2																V	
2																	
5																	
	1			A	A-2						K or E						
	1																
	2									A			V				
	2									A			V				
	9																
		2							M								
		2						S									
		3															
			1														
			2														
		1		A	A-3												
		1															
		1										V or I					
		1															D or N
		1															
		2															
		2															
		2					T							N			
		24															
	3			B													
	1			C													
	2																

*Isolates that have amino acid differences from another isolate(s) are indicated in gray.

2007 had an additional nine-nucleotide deletion (nucleotide no. 34–43) similar to Sw/Kagawa/35/2004. These isolates were found to constitute the clusters C and C′ by phylogenetic analysis of the E and C/prM genes, as indicated in Figure 4A and B. All the isolates in 2008 and 2009 had a novel additional deletion of seven nucleotides (nucleotide no. 44–51), although they were divided into two different subclusters (A-2 and A-3) according to the E gene (Table 4) and subclusters A′-1 and A′-3 according to the C/prM gene (Table 6).

On the basis of these results, changes in JEV in Toyama during 2005–2009 are summarized in Figure 6. In 2005, strains of "A′-1 (C/prM)/A-1 (E)/Ishikawa type (3′UTR)" predominated. In 2007, strains of "A′-1/A-2/Ishikawa type" and "A′-2/A-2/Ishikawa type" emerged. Minor strains of

TABLE 6

Nucleotide sequence differences within capsid (C)/premembrane (prM) gene among strains of Japanese encephalitis virus isolated, Toyama Prefecture, Japan

No. isolates						Nucleotide no.	C region				prM region										
2005	2007	2008	2009	Cluster	Subcluster	Consensus	324 C	345 T	365 C	372 T	12 A	31 C	81 C	93 A	102 C	126 T	153 T	169 G	210 C	213 T	216 G
20	4	41		A′	A′-1																
1								C													
		1																		C	
	10			A′	A′-2							T									
	1											T				G					
			2	A′	A′-3					C								T			
			1							C				G				T			
	3			B′			T		T				T		T		C				A
	3			C′			T				G				T		C		T		A

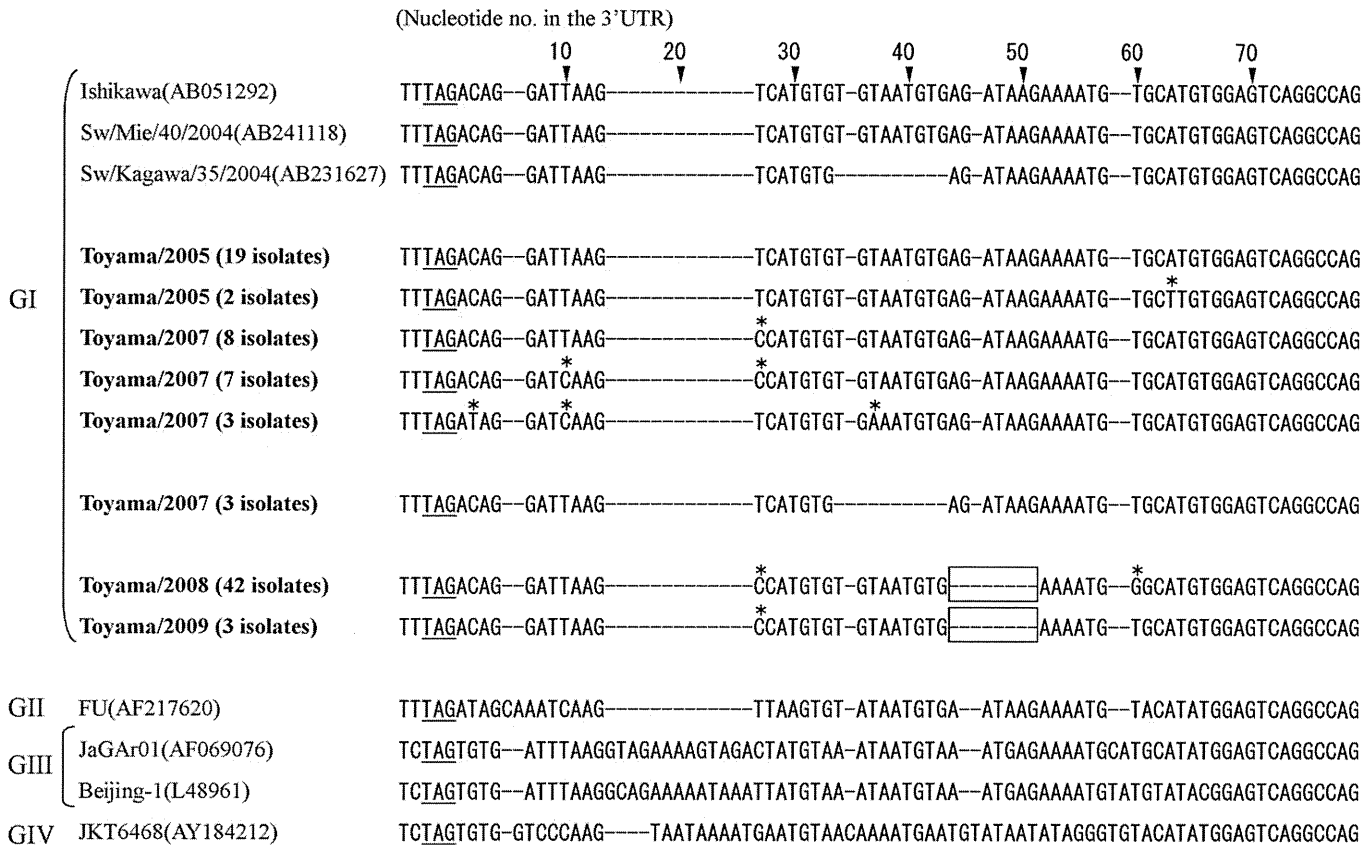

FIGURE 5. Alignment of nucleotide sequences of the 3′-untranslated regions (UTRs) of Toyama isolates and the reference strains of Japanese encephalitis virus (JEV), Toyama Prefecture, Japan. Japanese encephalitis virus isolates in Toyama Prefecture are shown as Toyama/year (number of isolates) in bold letters. Reference strains are shown by strain name (accession no.). GI–GIV indicates JEV genotypes. Deletions are indicated by hyphens. The stop codons are underlined. Novel deletion sites are in boxes. Nucleotides of strains isolated in this study that were different from Toyama/2005 (19 isolates) are indicated by asterisks.

"B′/B/Ishikawa type" and "C′/C/Ishikawa + Kagawa type" appeared but disappeared in 2008. In 2008, strains of "A′-1 (C/prM)/A-2 (E)" still circulated although the 3′UTR of these strains had a novel deletion. Major strains were "A′-1/A-3/ Ishikawa + Novel type" in 2008. In 2009, strains were "A′-3/A-2/ Ishikawa + Novel type". The results show that predominant strains in cluster "A′/A" changed from year to year but certain subcluster strains "A′-1/A-2" remained circulating during 2007–2008. Minor strains "B′/B" and "C′/C" were present only in one year (2007) and disappeared in later years.

Virus replication characteristics in tissue culture of Vero and C6/36 cells were examined among these isolates. Culture supernatants were collected one, two, three, and six days after infection of Vero (multiplicities of infection [MOI] were 0.01 and 0.001) and C6/36 cells (MOI = 0.001 and 0.0001) and virus titers in culture fluids were determined. Virus titers peaked at 2–3 days in Vero cells and at six days in C6/36 cells. The range of the peak virus titers was approximately 5×10^7–5×10^8 focus-forming units (FFU)/mL (MOI = 0.01 in Vero cells), 10^8–10^9 FFU/mL (MOI = 0.001 in Vero cells), 10^9–10^{10} FFU/mL (MOI = 0.001 in C6/36 cells) and 10^8–10^9 FFU/mL (MOI = 0.0001 in C6/36 cells). Among several isolates belonging to different clusters and having different deletions in the 3′UTR, virus replication did not correlate with the different clusters or deletion status.

DISCUSSION

There has been much discussion concerning how JEV appears every summer in Japan. One possible explanation is that the virus is introduced from tropical or subtropical zones of other countries in Asia every year. Another explanation is that JEV overwinters in Japan and re-emerges in early summer.

This study was performed to investigate how JEV maintains genetic continuity or undergoes genetic change locally for several years. Japanese encephalitis virus isolation and genetic characterization were performed in Toyama Prefecture, Japan, during 2005 to 2009.

Overall, strain "A′/A" seems to have remained in Toyama Prefecture and changed gradually. This fact may indicate that this type of JEV is a predominant strain that is endemic locally. The novel deletion in the 3′UTR might be an additional change. Conversely, strains "B′/B" and "C′/C" might be sporadically introduced to Japan and did not become predominant strains. Overwintering might be one of the factors for maintenance of predominant strain. Japanese encephalitis virus in Japan is considered to be a mixture of the overwintering type and a type from overseas because one subcluster was isolated only in Japan, and another type of JEV was also isolated elsewhere, such as in China and Vietnam.[13] Japanese encephalitis

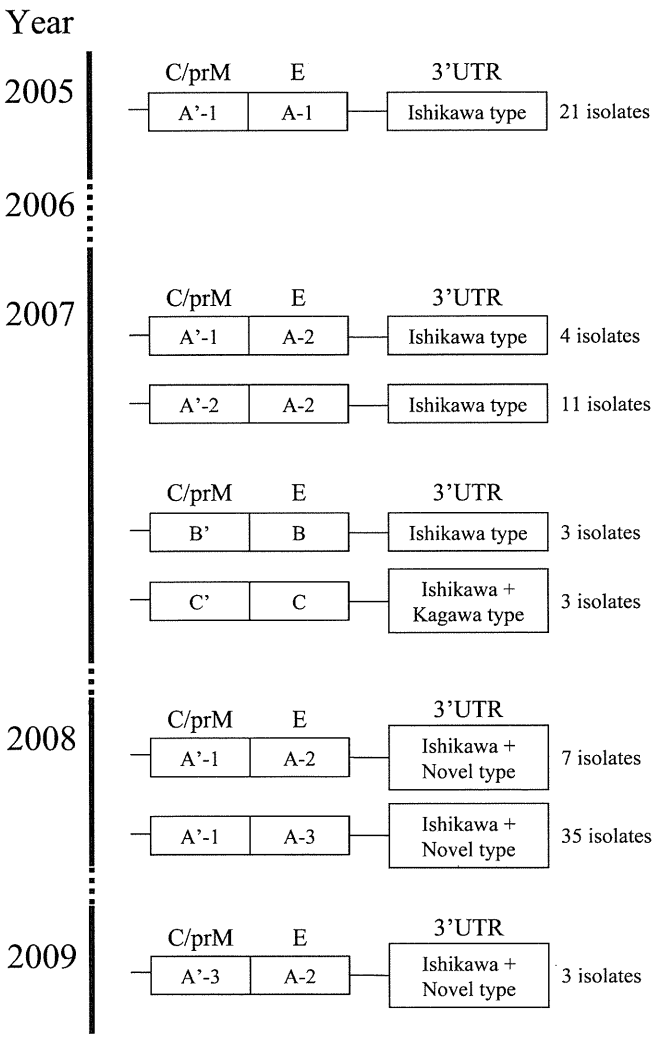

FIGURE 6. Changes in Japanese encephalitis virus (JEV) in Toyama Prefecture, Japan, during 2005–2009. The capsid/premembrane (C/prM) and envelope (E) genes are shown by cluster or subcluster names. The 3′ untranslated regions (UTRs) are shown by the deletion types indicated in Figure 5 Ishikawa type indicates the same deletion as Ishikawa (accession no. AB051292) and Sw/Mie/40/2004 (accession no. AB241118). Ishikawa + Kagawa type indicates the same deletion as Sw/Kagawa/35/2004 (accession no. AB231627). Ishikawa + Novel type indicates the novel deletion type observed in 2008 and 2009.

virus has not been isolated from overwintering mosquitoes in Japan, and JEV was shown to overwinter locally in Hokkaido, Japan because outbreaks of abortion in pigs caused by JEV were observed in early June, the interepidemic period of JEV.[20] In another report, JEV was maintained during winter in lizards experimentally, although it was not isolated from wild ones.[21] Therefore, the overwintering mechanism of JEV in Japan is still unclear. Conversely, JEV was isolated from overwintering mosquitoes in South Korea.[22] In Taiwan, one of the subtypes was shown to have been present for at least 11 years.[15] Because Japan, like Taiwan, consists of islands, some subtypes of JEV may also be maintained for several years in Japan. Although many isolates of JEV in Japan are considered to originate from southeast Asia, overseas migration of virus might be a rare event. Even if the viruses migrated from outside Japan, most of them might have been extinct. Our results support this hypothesis.

All JEV isolates in Toyama Prefecture were similar to strains in China and Vietnam, and these reference strains had already been isolated before strains in this study were isolated. This result also supports the theory that JEV was introduced from southeast Asia and continental eastern Asia to Japan.[13] Furthermore, strains of genotype III isolated before 1990 in Japan were similar to those in South Korea and Taiwan. All strains isolated in South Korea after 1991 belonged to genotype I, as did those in Japan. However, in Taiwan, strains of genotype III were isolated until 2002. These results indicate that the movement of JEV may be linked between Japan and South Korea. It is important to determine whether recent isolates in South Korea are similar to isolates in Toyama once nucleotide sequences of recent isolates in South Korea become available.

Strains isolated in 2005 and 2007 had the same deletions in the 3′UTR as the Ishikawa strain, and three strains in cluster C in 2007 had the same deletions as Sw/Kagawa-35/2004 strain. Japanese encephalitis virus strains isolated in 2008 and 2009 had an additional novel deletion in the 3′UTR, although they belonged to the same cluster as strains isolated in 2005 and 2007 on the basis of the E and C/prM genes. This novel deletion might have occurred in the JEV maintained in Toyama Prefecture or originated in another area and spread to Toyama Prefecture. Because JEV strains with this novel deletion have so far only been found in Toyama Prefecture, the former hypothesis seems to be likely. However, further analysis of isolates in other areas may clarify this issue.

Conversely, strains of "B′/B/Ishikawa type" and "C′/C/Ishikawa + Kagawa type" most likely migrated from other regions and then became extinct. They were detected in 2007 but were not isolated in 2008 and 2009. They might have disappeared in 2008 because they did not acclimate to local environmental conditions or they competed with other types of strains, or were not detected because of their low prevalence compared with those of prevalent strains.

All JEVs isolated from female *Cx. tritaeniorhynchus* mosquitoes were obtained on farms and belonged to genotype I. This result confirms that *Cx. tritaeniorhynchus* mosquitoes are the major vector of genotype I of JEV in Toyama Prefecture. Japanese encephalitis viruses were not only isolated from pigpens but also from a cattle shed. Because JEV is not known to cause viremia in cattle,[23,24] mosquitoes harboring JEV might have flown from other places, such as a pigpen 2 km away, to this shed. The fact that antibody nor JEV was not detected in seven cattle less than one year of age in 2009 supports the above hypothesis. In a previous report, cattle acquired antibody after experimental infection with JEV.[24] Because *Cx. tritaeniorhynchus* mosquitoes were few in number in 2009 (Figure 3A), there might have been little opportunity for the infection of cattle.

Although JEVs were isolated during August–October in 2005–2009 in this study, they were mainly isolated from the end of July through early September in Toyama Prefecture in 1966–1972 (Figure 3B),[18] during July–August in Nagasaki Prefecture in 1964–1973,[25,26] and during July–early September in Osaka Prefecture in 1968–1997.[27] Conversely, the number of *Cx. tritaeniorhynchus* mosquitoes at the end of July in the 1970s, and from the end of August to the beginning of September after the 1990s in Toyama Prefecture.[10] Thus, the late isolation of JEV in this study seems to correlate with the late increase in the number of *Cx. tritaeniorhynchus* mosquitoes. Although the reason for the late peak in the number of mosquitoes is not clear, it might be caused by the way that

insecticides are applied and/or the method of water control of rice fields.

From the late 1960s to the 1970s, rice fields were filled with water in May and *Cx. tritaeniorhynchus* mosquitoes developed during June–July. The growth of *Cx. tritaeniorhynchus* mosquitoes was suppressed by the first application of insecticide from a helicopter at the end of July, the second application in early August, and the drying of rice fields at the end of August before harvest time.[10,11] The application of insecticide by using a helicopter was stopped in 1995 because it disturbed ecologic systems around rice fields. Recently, because rice fields had been filled with water relatively late in the season, insecticide had been applied onto rice seedlings, and rice fields had been dried in June, *Cx. tritaeniorhynchus* mosquitoes did not develop in June. However, insecticides are not used frequently, except on seedlings. After the refilling of rice fields with water, *Cx. tritaeniorhynchus* mosquitoes may develop and peak during August–September. These factors may also affect the late prevalence of Japanese encephalitis, which has recently occurred mainly in September,[9] despite occurring in August in the past.[8]

Japanese encephalitis virus was isolated from mosquitoes in a pigpen after the peak in the number of female *Cx. tritaeniorhynchus* mosquitoes. This finding is in contrast to a previous report showing that JEV infections of mosquitoes occurred before or at the peak in the number of mosquitoes (Figure 3B).[18] At temperatures higher than 24°C, JEV reproduction in *Cx. tritaeniorhynchus* mosquitoes was found to be faster and virus titer was higher and peaked earlier after infection than at lower temperatures.[28] In the 1960s and 1970s, the number of *Cx. tritaeniorhynchus* mosquitoes peaked in July when the temperature was high. Japanese encephalitis virus might effectively reproduce in mosquitoes and peak in number at the time of the peak in the number of mosquitoes. Recently, the number of *Cx. tritaeniorhynchus* mosquitoes peaked in August and September, a period with a lower temperature. As a result, JEV might not have effectively reproduced in mosquitoes. Thus, the peak in the MIR of JEV followed the peak in the number of *Cx. tritaeniorhynchus* mosquitoes. This finding also means that recently the MIR for JEV in mosquitoes has not been high during August–September when the number of mosquitoes peaks. Therefore, the risk of infection in humans that are bitten by mosquitoes may now be lower in late summer and autumn than in summer. Furthermore, mosquitoes have more difficulty biting persons in autumn than in summer because people wear long sleeves. These factors might be related to the recent decrease in the prevalence of Japanese encephalitis in Japan, in addition to the effects of human vaccinations.

In conclusion, JEV still circulates between mosquitoes and pigs in Toyama Prefecture and is correlated with the prevalence of mosquitoes. However, the peak level of JEV circulation occurs later in the year than in the past. On the basis of the nucleotide sequence information derived from the E and C/prM genes, all isolates belong to genotype I. The major type of JEV might have remained in Toyama Prefecture and gradually changed over five years, and two types of JEV might have migrated from other countries and then become extinct. Japanese encephalitis virus isolates in 2008 and 2009 had a novel deletion in the 3′UTR.

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REFERENCES

1. Gubler DJ, Kuno G, Markoff L, 2007. Flaviviruses. Knipe DM, Howley PM, eds. *Fields Virology*. Fifth edition. Philadelphia, PA: Lippincott Williams and Wilkins, 1153–1252.
2. Hashimoto H, Nomoto A, Watanabe K, Mori T, Takezawa T, Aizawa C, Takegami T, Hiramatsu K, 1988. Molecular cloning and complete nucleotide sequence of the genome of Japanese encephalitis virus Beijing-1 strain. *Virus Genes 1:* 305–317.
3. Sumiyoshi H, Mori C, Fuke I, Morita K, Kuhara S, Kondou J, Kikuchi Y, Nagamatu H, Igarashi A, 1987. Complete nucleotide sequence of the Japanese encephalitis virus genome RNA. *Virology 161:* 497–510.
4. Uchil PD, Satchidanandam V, 2001. Phylogenetic analysis of Japanese encephalitis virus: envelope gene based analysis reveals a fifth genotype, geographic clustering, and multiple introductions of the virus into the Indian subcontinent. *Am J Trop Med Hyg 65:* 242–251.
5. Solomon T, Ni H, Beasley DW, Ekkelenkamp M, Cardosa MJ, Barrett AD, 2003. Origin and evolution of Japanese encephalitis virus in southeast Asia. *J Virol 77:* 3091–3098.
6. Burke DS, Leake CJ, 1988. Japanese encephalitis. Monath TP, ed. *The Arboviruses: Epidemiology and Ecology*. Volume III. Boca Raton, FL: CRC Press, 63–92.
7. Igarashi A, 1992. Japanese encephalitis virus, infection, and control. Kurstak E, ed. *Control of Virus Diseases*. New York: Marcel Dekker, Inc., 309–342.
8. Ogata T, 1985. Epidemiology of Japanese encephalitis in Japan [in Japanese]. *Rinsho To Uirusu 13:* 150–155.
9. Infectious Diseases Surveillance Center, 2008. Japanese encephalitis. *Annual Report 2006 National Epidemiological Surveillance of Vaccine-Preventable Disease* [in Japanese]. Tokyo: National Institute of Infectious Disease, 74–96.
10. Watanabe M, Hasegawa S, Obara M, Ando S, Yamauchi T, Takizawa T, 2011. *Long-term analyses of the population dynamics of Culex tritaeniorhynchus and Anopheles sinensis, and serological survey of Japanese encephalitis virus among swine in Toyama Prefecture, Japan, from 1969 to 2003.* Toyama, Japan: Skarafactory Ltd.

11. Kamimura K, 1998. Studies on the population dynamics of the principal vector mosquito of Japanese encephalitis [in Japanese]. *Med Entomol Zool 49:* 181–185.
12. Nerome R, Tajima S, Takasaki T, Yoshida T, Kotaki A, Lim CK, Ito M, Sugiyama A, Yamauchi A, Yano T, Kameyama T, Morishita I, Kuwayama M, Ogawa T, Sahara K, Ikegaya A, Kanda M, Hosoya Y, Itokazu K, Onishi H, Chiya S, Yoshida Y, Tabei Y, Katsuki K, Tabata K, Harada S, Kurane I, 2007. Molecular epidemiological analyses of Japanese encephalitis virus isolates from swine in Japan from 2002 to 2004. *J Gen Virol 88:* 2762–2768.
13. Nabeshima T, Loan HT, Inoue S, Sumiyoshi M, Haruta Y, Nga PT, Huoung VT, Del Carmen Parquet M, Hasebe F, Morita K, 2009. Evidence of frequent introductions of Japanese encephalitis virus from south-east Asia and continental east Asia to Japan. *J Gen Virol 90:* 827–832.
14. Ma SP, Yoshida Y, Makino Y, Tadano M, Ono T, Ogawa M, 2003. Short report: a major genotype of Japanese encephalitis virus currently circulating in Japan. *Am J Trop Med Hyg 69:* 151–154.
15. Jan LR, Yueh YY, Wu YC, Horng CB, Wang GR, 2000. Genetic variation of Japanese encephalitis virus in Taiwan. *Am J Trop Med Hyg 62:* 446–452.
16. Kumar S, Tamura K, Nei M, 2004. MEGA3: Integrated Software for Molecular Evolutionary Analysis and Sequence Alignment. *Brief Bioinform 5:* 150–163.
17. Kimura M, 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol 16:* 111–120.
18. Katori K, Matsuura K, Nakayama T, Imai S, Kamimura K, 1975. Epidemiological surveillance of Japanese encephalitis in Toyama prefecture for 10 years (2nd report) [in Japanese]. *Toyama-ken Noson Igaku Kenkyukai 6:* 55–61.
19. Takegami T, Ishak H, Miyamoto C, Shirai Y, Kamimura K, 2000. Isolation and molecular comparison of Japanese encephalitis virus in Ishikawa, Japan. *Jpn J Infect Dis 53:* 178–179.
20. Takashima I, Watanabe T, Ouchi N, Hashimoto N, 1988. Ecological studies of Japanese encephalitis virus in Hokkaido: interepidemic outbreaks of swine abortion and evidence for the virus to overwinter locally. *Am J Trop Med Hyg 38:* 420–427.
21. Doi R, Oya A, Shirasaka A, Yabe S, Sasa M, 1983. Studies on Japanese encephalitis virus infection of reptiles. II. Role of lizards on hibernation of Japanese encephalitis virus. *Jpn J Exp Med 53:* 125–134.
22. Rosen L, 1986. The natural history of Japanese encephalitis virus. *Annu Rev Microbiol 40:* 395–414.
23. Horimoto M, Sakai T, Goto H, 1987. Changes in antibody titers in cattle with Japanese encephalitis virus infection. *Indian J Med Res 86:* 695–701.
24. Ilkal MA, Dhanda V, Rao BU, George S, Mishra AC, Prasanna Y, Gopalkrishna S, Pavri KM, 1988. Absence of viraemia in cattle after experimental infection with Japanese encephalitis virus. *Trans R Soc Trop Med Hyg 82:* 628–631.
25. Hayashi K, Shichijo A, Mifune K, Matsuo S, Wada Y, Mogi M, Itoh T, 1973. Ecological studies on Japanese encephalitis virus: results of investigations in Nagasaki area, Japan, in 1969, 1970 and 1971. *Tropical Medicine 15:* 214–224.
26. Fukumi H, Hayashi K, Mifune K, Shichijo A, Matsuo S, Omori N, Wada Y, Oda T, Mogi M, Mori A, 1976. Ecology of Japanese encephalitis virus in Japan: I. Mosquito and pig infection with the virus in relation to human incidences. *Tropical Medicine 17:* 97–110.
27. Nakamura H, Yoshida M, Kimura A, Yumisashi T, Kimura T, Ueba N, Kunita N, 2002. Ecological studies on Japanese encephalitis (JE) in Osaka Prefecture 6. Surveillance of JE virus activity and the vector mosquito abundance during the years 1968–1997. *Med Entomol Zool 53:* 29–42.
28. Shichijo A, Mifune K, Hayashi K, Wada Y, Oda T, Omori N, 1972. Experimental infection of *Culex tritaeniorhynchus summorosus* mosquitoes reared in biotron with Japanese encephalitis virus [in Japanese]. *Tropical Medicine 14:* 218–229.

Reston Ebolavirus Antibodies in Bats, the Philippines

To the Editor: Filoviruses cause highly lethal hemorrhagic fever in humans and nonhuman primates, except for Reston Ebolavirus (REBOV), which causes severe hemorrhagic fever in macaques (*1*,*2*). REBOV epizootics among cynomolgus macaques occurred in 1989, 1990, 1992, and 1996 (*2*) and among swine in 2008 (*3*). African fruit bats have been suggested to be natural reservoirs for Zaire Ebolavirus and Marburg virus (*4*–*6*). However, the natural reservoir of REBOV in the Philippines is unknown. Thus, we determined the prevalence of REBOV antibody–positive bats in the Philippines.

Permission for this study was obtained from the Department of Environment and Natural Resources, the Philippines, before collecting bat specimens. Serum specimens from 141 wild-caught bats were collected at several locations during 2008–2009. The bat species tested are summarized in the Table. Captured bats were humanely killed and various tissues were obtained. Carcasses were then provided to the Department of Environment and Natural Resources for issuance of a transport permit.

We used immunoglobulin (Ig) G ELISAs with recombinant nucleoprotein (NP) and glycoprotein (GP) of REBOV (*7*) to determine REBOV antibody prevalence. REBOV NP and GP were expressed and purified from Tn5 cells infected with recombinant baculoviruses AcResNP and AcResGPDTM, which express NP and the ectodomain of GP with the histidine tag at its C-terminus. We also used histidine-tagged recombinant Crimean-Congo hemorrhagic fever virus NP as a negative control antigen in the IgG ELISA to confirm specificity of reactivity.

In IgG ELISAs for bat specimens, positive results were detected by using rabbit anti-bat IgG and horseradish peroxidase–conjugated anti-rabbit IgG. Anti-bat (*Rousettus aegyptiacus*) rabbit IgG strongly cross-reacts with IgGs of other bat species, including insectivorous bats (*8*). Bat serum samples were 4-fold serially diluted (1:100–1:6,400) and tested by using IgG ELISAs. Results of IgG ELISAs were the sum of optical densities at serum dilutions of 1:100, 1:400, 1:1,600, and 1:6,400. Cutoff values (0.82 for both IgG ELISAs) were determined by using serum specimens from REBOV antibody–negative bats.

Among 16 serum samples from *R. amplexicaudatus* bats, 5 (31%) captured at either the forest of Diliman (14°38′N, 121°2′E) or the forest of Quezon (14°10′N, 121°50′E) had positive results in the IgG ELISA for REBOV NP, and 5 (31%) captured at the forest of Quezon had positive results in the IgG ELISA for REBOV GP. The REBOV NP antibody–positive bats serum samples were confirmed to be NP antibody positive in the IgG ELISA by using glutathione-S-transferase–tagged partial REBOV NP antigen (*9*). Three samples had positive results in both IgG ELISAs (Table). Serum samples from other bat species had negative results in IgG ELISAs.

All bat serum samples were also tested by indirect immunofluorescence assays (IFAs) that used HeLa cells expressing NP and GP (*10*). In the IFAs, 2 samples from *R. amplexicaudatus* bats captured at the forest of Diliman and the forest of Quezon had high titers (1,280 and 640, respectively) of NP-specific antibodies, and 1 sample from an *R. amplexicaudatus* bat captured at the forest of Quezon had a positive result in the GP-specific IFA (titer 20). All IFA-positive samples were also positive in the IgG ELISA (Table).

The forest of Diliman is ≈30 km from the monkey facility and the Bulacan farm where REBOV infections in monkeys and swine, respectively, were detected. The forest of Quezon is ≈60 km from the monkey facility. Samples from other bat species had negative results in IFAs. We also performed heminested reverse transcription PCR specific for the REBOV NP gene with spleen specimens from all 16 *R. amplexicaudatus* bats but failed to detect any REBOV-specific amplicons.

REBOV-specific antibodies were detected only in *R. amplexicaudatus* bats, a common species of fruit bat, in the Philippines. In Africa, *R. aegyptiacus* bats, which are genetically similar to *R. amplexicaudatus* bats, have been

Table. REBOV-specific IgG in *Rousettus amplexicaudatus* bats and other bats, the Philippines*

Bat ID	Collection site	ELISA optical density		IFA titer	
		REBOV NP	REBOV GP	REBOV NP	REBOV GP
1539	FD	**2.13**	–0.21	**1,280**	<20
1632	FQ1	**0.88**	0.2	<20	<20
1642	FQ1	0.36	**5.22**	<20	**20**
1643	FQ1	**1.26**	**0.92**	<20	<20
1651	FQ1	**1.61**	**1.02**	<20	<20
1657	FQ1	–0.45	**1.69**	<20	<20
1660	FQ1	**3.8**	**2.51**	**640**	<20

*Cutoff optical density of ELISA was 0.82 (sum of optical densities at serum dilutions of 1:100, 1:400, 1:1,600, and 1:6,400). Values in **boldface** are positive results. REBOV, Reston Ebolavirus; Ig, immunoglobulin; IFA, indirect immunofluorescence assay; ID, identification; NP, nucleoprotein; GP, glycoprotein; FD, forest of Diliman at the University of the Philippines Diliman campus; FQ1, forest at the Agricultural College in Province of Quezon, the Philippines. The other 9 *R. amplexicaudatus* bats collected at FQ1 had negative results for all assays. The following bat species also had negative results: 5 *Eonycteris spelaea*, 35 *Cynopterus brachyotis*, 38 *Ptenochirus jagoli*, 6 *Haplonycteris fischeri*, 2 *Macroglossus minimus*, 2 *Rhinolophus rufus*, 1 *Rhinolophus arcuatus*, 9 *Emballonura alecto*, 2 *Pipistrellus javanicus*, 5 *Scotophilus kuhlii*, 8 *Miniopterus australis*, 8 *M. schreibersi*, 1 *M. tristis tritis*, 1 *Hipposideros diadema*, 1 *Myotis macrotarsus*, and 1 bat of unknown species.

shown to be naturally infected with Zaire Ebolavirus and Marburg virus. Thus, *R. amplexicaudatus* bats are a possible natural reservoir of REBOV. However, only 16 specimens of *R. amplexicaudatus* bats were available in this study, and it will be necessary to investigate more specimens of this species to detect the REBOV genome or antigens to conclude the bat is a natural reservoir for REBOV.

We have shown that *R. amplexicaudatus* bats are putatively infected with REBOV or closely related viruses in the Philippines. Antibody-positive bats were captured at the sites near the study areas, where REBOV infections in cynomolgus monkeys and swine have been identified. Thus, bats are a possible natural reservoir of REBOV. Further analysis to demonstrate the REBOV genome in bats is necessary to conclude that the bat is a reservoir of REBOV.

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References

1. Miranda ME, Ksiazek TG, Retuya TJ, Khan AS, Sanchez A, Fulhorst CF, et al. Epidemiology of Ebola (subtype Reston) virus in the Philippines, 1996. J Infect Dis. 1999;179(Suppl 1):S115–9. doi:10.1086/514314
2. Morikawa S, Saijo M, Kurane I. Current knowledge on lower virulence of Reston Ebola virus [in French]. Comp Immunol Microbiol Infect Dis. 2007;30:391–8. doi:10.1016/j.cimid.2007.05.005
3. Barrette RW, Metwally SA, Rowland JM, Xu L, Zaki SR, Nichol ST, et al. Discovery of swine as a host for the Reston ebolavirus. Science. 2009;325:204–6. doi:10.1126/science.1172705
4. Leroy EM, Kumulungui B, Pourrut X, Rouquet P, Hassanin A, Yaba P, et al. Fruit bats as reservoirs of Ebola virus. Nature. 2005;438:575–6. doi:10.1038/438575a
5. Pourrut X, Souris M, Towner JS, Rollin PE, Nichol ST, Gonzalez JP, et al. Large serological survey showing cocirculation of Ebola and Marburg viruses in Gabonese bat populations, and a high seroprevalence of both viruses in *Rousettus aegyptiacus*. BMC Infect Dis. 2009;9:159. doi:10.1186/1471-2334-9-159
6. Towner JS, Amman BR, Sealy TK, Carroll SA, Comer JA, Kemp A, et al. Isolation of genetically diverse Marburg viruses from Egyptian fruit bats. PLoS Pathog. 2009;5:e1000536. doi:10.1371/journal.ppat.1000536
7. Saijo M, Niikura M, Ikegami T, Kurane I, Kurata T, Morikawa S. Laboratory diagnostic systems for Ebola and Marburg hemorrhagic fevers developed with recombinant proteins. Clin Vaccine Immunol. 2006;13:444–51. doi:10.1128/CVI.13.4.444-451.2006
8. Omatsu T, Ishii Y, Kyuwa S, Milanda EG, Terao K, Yoshikawa Y. Molecular evolution inferred from immunological cross-reactivity of immunoglobulin G among Chiroptera and closely related species. Exp Anim. 2003;52:425–8. doi:10.1538/expanim.52.425
9. Ikegami T, Saijo M, Niikura M, Miranda ME, Calaor AB, Hernandez M, et al. Immunoglobulin G enzyme-linked immunosorbent assay using truncated nucleoproteins of Reston Ebola virus. Epidemiol Infect. 2003;130:533–9.
10. Ikegami T, Saijo M, Niikura M, Miranda ME, Calaor AB, Hernandez M, et al. Development of an immunofluorescence method for the detection of antibodies to Ebola virus subtype Reston by the use of recombinant nucleoprotein-expressing HeLa cells. Microbiol Immunol. 2002;46:633–8.

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Acute Hepatitis C Outbreak among HIV-infected Men, Madrid, Spain

To the Editor: In the past decade, hepatitis C virus (HCV) has emerged as a sexually transmitted infection (STI) among HIV-infected men who have sex with men (MSM). The epidemic was originally reported in several northern European countries (England, France, Germany, and the Netherlands) (*1*) and soon after in Australia (*2*) and the United States (*3*). Acute HCV acquisition was associated with group sex, unprotected receptive anal intercourse, and according to some studies, concomitant STI (*4*). Molecular phylogenetic studies suggested evidence of an international transmission network of MSM within northern Europe (*1*). However, expansion of the HCV epidemic among MSM to Spain (*5*) or to other

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Inhibition of rabies virus propagation in mouse neuroblastoma cells by an intrabody against the viral phosphoprotein

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ABSTRACT

Rabies virus (RABV) is highly neurotropic and causes acute infection of the central nervous system. Death can be averted by prompt post-exposure prophylaxis; however, after clinical symptoms appear, the mortality rate is almost 100% and no reliable treatment is available. In this study, we investigated whether intracellular immunization using single-chain variable fragments (scFvs) against RABV phosphoprotein (RABV-P) could inhibit RABV propagation in neuronal cells. Of four scFv clones derived from an scFv phage-displayed library, scFv-P19 showed extremely high transfection efficiency and stable expression in mouse neuroblastoma (MNA) cells. The intracellular affinity and inhibition of RABV propagation were investigated using RABV-infected MNA cells pretransfected with the scFv-P19 gene. The specific interaction between scFv and RABV-P was confirmed by an immunoprecipitation assay and an indirect immunofluorescence assay showing that these molecules colocalized in the cytoplasm. Measurements of the spread of RABV in a culture well and the virus titer in the supernatant showed that RABV inhibition peaked 3 days after infection, at 98.6% and 99.9% inhibition, respectively. Although the mechanism of RABV inhibition by scFv-P19 is not clear, this scFv-based intracellular immunization could be a candidate for future RABV therapeutic studies if combined with appropriate delivery and application systems.

1. Introduction

Rabies virus (RABV) is a highly neurotropic virus that causes an acute infection of the central nervous system (CNS). RABV inoculated into peripheral tissues is hypothesized to enter nerve terminals either after replication in the peripheral skeletal muscle or directly, without prior replication (reviewed by Schnell et al. (2010)). The virus then travels via retrograde fast axonal transport within axons in peripheral nerves, through the spinal cord and dorsal root ganglia, to brain neurons. Following the invasion of the CNS, the virus rapidly disseminates within the CNS and spreads centrifugally to peripheral sites, including salivary glands, along neuronal routes, which can result in death. Transmission to and excretion from the salivary gland is essential for transmission of RABV to its next host. Death can be averted by prompt post-exposure prophylaxis (PEP). However, if PEP is delayed and clinical symptoms develop, the mortality rate is almost 100%. So far, only a few people have been reported to have survived after the onset of clinical disease (reviewed by Nigg and Walker (2009)). In 2004, it was reported that a patient had survived rabies encephalitis without PEP, after treatment including induction of coma and administration of an antiviral drug (Willoughby et al., 2005). Several laboratories have repeated this treatment program (reviewed by Nigg and Walker (2009)); however, few successful cases have been reported (Hemachudha et al., 2006) and the efficacy of this method is still controversial. To establish more stable and successful therapy, novel neuroprotective approaches based on specific anti-RABV agents are required.

Intracellular immunization (Baltimore, 1988) is a promising therapeutic technique that uses various forms of gene transfer to provide specific cellular resistance to viral infection. One approach is the degradation of viral messenger RNA by gene silencing mediated by antisense oligonucleotides (Stein et al., 2010), ribozymes (Nawtaisong et al., 2009), or RNA interference (RNAi; Dykxhoorn et al., 2003). Another approach is the inhibition of viral protein functions or interactions by intracellularly expressed antibodies, known as intrabodies (Marasco, 1997). Each approach has advantages for therapeutic application (Cao and Heng, 2005). Intrabodies have much higher specificity against target proteins than RNAi and far longer half-lives can be attained; however, intrabodies are more time-consuming and labor-intensive to generate. The most-explored form of intrabody is the single chain variable fragment (scFv), which consists of the VH and VL regions of the variable antigen-binding site of an immunoglobulin, connected with a short linker sequence. The scFvs possess several advantages over immunoglobulins as an intrabody format: a simple and compact structure, higher stability, and increased solubility. Intrabodies

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based on scFvs have been developed against human immunodeficiency virus-1 (Goncalves et al., 2002), hepatitis B virus (Yamamoto et al., 1999), hepatitis C virus (Karthe et al., 2008), rotavirus (Vascotto et al., 2004), herpes virus (Corte-Real et al., 2005), and flavivirus (Jiang et al., 1995). The intrabody-based strategy could be applied to the development of future therapeutic agents against rabies, because RABVs transfer exclusively cell-to-cell and possess highly distinct neurotropism.

In this study, we developed several scFv-based intrabodies against RABV phosphoprotein (RABV-P). RABV-P was chosen as a target for intracellular immunization because it is involved in multiple functions through interactions with various viral or cellular proteins, such as encapsidation of viral genome RNA (Chenik et al., 1994), acting as a cofactor for RABV large protein (RABV-L, a viral RNA polymerase; Chenik et al., 1998), and inhibition of the cellular antiviral system induced by interferons (Vidy et al., 2007). Using a phage-displayed scFv library, we obtained four genetically independent scFv clones. One of these scFvs, when expressed transiently in mouse neuronal cell lines before RABV infection, severely inhibited the propagation and secretion of RABV and the spread of infection. This approach could be a prospective candidate for the establishment of novel therapeutic agents against RABV infection.

2. Materials and methods

2.1. Virus and viral protein

The fixed RABV strain CVS-11 was used in this study. The recombinant RABV-P protein was prepared as described previously (Motoi et al., 2005).

2.2. Selection of scFvs against RABV-P

Human single-fold scFv libraries I + J (Tomlinson I + J; a kind gift from MRC Centre for Protein Engineering, Cambridge, United Kingdom) underwent selection for the ability to bind RABV-P protein. The libraries are based on a single human framework for VH (V3-23/DP-47 and J_H4b) and Vκ (O12/O2/DPK9 and Jκ1) with side chain diversity incorporated at positions in the antigen binding site. In the libraries, the scFv genes were linked to a His-tag followed by a myc-tag and cloned into pIT2 phagemid vectors. The selection or "panning" process was essentially as described by the manufacturer's protocol (http://www.geneservice.co.uk/products/proteomic/datasheets/tomlinsonIJ.pdf). Briefly, Nunc immunotubes (Thermo Fisher, Wiesbaden, Germany) were coated with 100 μg/mL of RABV-P in PBS. After washes with PBS and blocking with PBS containing 2% skim milk, tubes were loaded with 10^{12}–10^{13} phages and incubated for 2 h. After intensive washes, bound phages were eluted by adding 500 μL of trypsin–PBS (2 mg/mL trypsin in PBS). The eluted phages were propagated and amplified in *Escherichia coli* TG1. After three rounds of panning, individual clones were grown in a 96-well U-bottom plate. Phages were produced by adding KM13 helper phages, and the binding to RABV-P by the monoclonal phages was confirmed by enzyme-linked immunosorbent assay (ELISA). As a negative control, scFvs against an unrelated protein, immunoglobulin G (IgG) of the fruit bat *Rousettus aegyptiacus* (a kind gift from Dr. T. Omatsu, Tokyo University) were selected following the same procedure.

2.3. ELISA

The ELISA plate was coated with either native RABV-P (2 μg/well) or denatured RABV-P (2 μg/well); the latter was generated by boiling RABV-P in 2% SDS for 5 min. Nonspecific binding was blocked by PBS containing 5% skim milk. The phages (diluted 1:10) or soluble scFvs (in *E. coli* HB2151 supernatant, diluted 1:10) were added to a set of two wells (native RABV-P and denatured RABV-P antigens) and incubated for 1 h at room temperature. After the plates had been washed, the bound phages were visualized by the addition of horseradish peroxidase (HRP)-conjugated protein L (Pierce, Rockford, IL, USA) and SureBlue TMB 1-component Microwell Peroxidase Substrate (KPL, Gaithersburg, MD, USA). The optical density (OD) was read at 450 nm using a Model 680 Microplate Reader (Bio-Rad, Hercules, CA, USA).

2.4. Phagemid DNA sequencing

The phagemid vector pIT2 was isolated using a Qiagen Plasmid Midi Kit (Qiagen, Venlo, Netherlands). The phagemids were sequenced using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the 373 DNA Sequencer (Applied Biosystems). The primers used to sequence the scFv inserts in the pIT2 vector were LMB3 (5′-CAGGAAACAGCTATGAC-3′) and pHEN (5′-CTATGCGGCCCCATTCA-3′). Deduced amino acid sequences of each α-RABV-P scFv were aligned using GENETYX Ver. 9 (Genetyx, Tokyo, Japan).

2.5. Expression and purification of soluble scFvs

RABV-P-reactive and genetically independent phage clones were selected for induction of soluble scFv production. *E. coli* HB2151 was transformed with phages and inoculated in 2× TY (tryptone–yeast) medium containing 100 μg/mL ampicillin and 0.1% glucose, then grown at 37 °C until the OD_{600} reached approximately 0.5. To induce the expression of soluble scFv, 1 mM isopropyl-β-D-thiogalactapyranoside was added to the culture. After incubation for 4 h at 30 °C, the culture was centrifuged at 2600 *g* for 20 min, and the pellet was resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole) and incubated on ice for 30 min. After the lysate was divided into aliquots in 2.0-mL tubes, glass beads (Sigma, St. Louis, MO, USA) were added, and the cells were disrupted with a Fast Prep Cell Disrupter (Qbiogene, Carlsbad, CA, USA) at speed 4 for 20 s. Following incubation on ice for 10 min and centrifugation at 12,000*g* for 5 min, the supernatant containing scFv and other *E. coli*-derived proteins was collected. For the purification of scFv, Ni–NTA nickel-charged resin (Qiagen) was added to the supernatant, and the mixture was rotated at 4 °C for 1 h. After centrifugation at 1000 *g* for 10 min, the pelleted Ni–NTA was washed three times with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole). Finally, scFv was eluted using elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole).

2.6. Immunoprecipitation

To examine the interaction between scFv and RABV-P *in vitro*, immunoprecipitation of *E. coli*-expressed soluble scFv against RABV-P was performed using magnetic beads (Dynabeads His-Tag Isolation & Pulldown; Invitrogen, Carlsbad, CA, USA). Briefly, mouse neuroblastoma (MNA) cells were grown as a monolayer in 6-well plates, infected with RABV at a multiplicity of infection (m.o.i.) of 10, and subsequently incubated in a 5% CO_2 incubator. After 48 h, the cells were lysed with 0.5 mL of lysis buffer (50 mM sodium phosphate [pH 8.0], 300 mM NaCl, 0.01% [vol/vol] Tween-20, 1% [vol/vol] Triton-X 100) at 4 °C for 1 h. The cell lysates were centrifuged at 20,000*g* for 30 min at 4 °C. The supernatants were collected, and 2.5 μg of scFvs were added and reacted for 1 h at 4 °C. The samples were mixed with Dynabeads and incubated for 10 min, washed four times with wash buffer (50 mM sodium phosphate [pH 8.0], 300 mM NaCl, 0.01% [vol/vol] Tween-20), and then

resuspended in 20 μL loading buffer (containing 4× NuPAGE LDS Sample Buffer [Invitrogen, Carlsbad, CA, USA], 10× NuPAGE Reducing Agent [Invitrogen]). After heating the sample for 10 min at 70 °C, the samples were subjected to SDS–polyacrylamide gel electrophoresis (PAGE).

To confirm the *in vivo* interaction between the intrabody and RABV-P, immunoprecipitation was performed using a Dynabeads Protein G Immunoprecipitation Kit (Invitrogen) and lysates of scFv-transfected MNA cells with or without RABV infection. To make the cell lysates, scFv-P19-pCAG was first transfected into MNA cells as described below (see Section 2.10). After 24 h, the cells were infected with CVS-11 (m.o.i. = 10) or mock-infected. At 48 h after infection, the cells were lysed, centrifuged, and collected as described above. The manufacturer's immunoprecipitation protocol was followed, using the buffer set included in the Kit. Briefly, 1.5 mg of Dynabeads were mixed with 2.0 μg of an anti-myc tag mouse monoclonal antibody (MoAb; MBL, Nagoya, Japan) and reacted for 10 min at room temperature. After washes with Ab Binding & Washing Buffer, the cell lysates were added and reacted for 10 min. After washes with Washing Buffer, the samples were suspended in a mixture of 20 μL Elution Buffer and 10 μL loading buffer. The samples were eluted by heating for 10 min at 70 °C, and were subjected to SDS–PAGE.

2.7. SDS–PAGE and Western blotting

The precipitated proteins were suspended in loading buffer and separated by electrophoresis in NuPAGE Novex Bis–Tris Mini Gels (Invitrogen) using MES Buffer (Invitrogen). In the upper buffer chamber, NuPAGE Antioxidant (Invitrogen) was added at a concentration of 0.25%. After SDS–PAGE, the proteins were transferred onto PVDF membranes using a semidry transblotter (Bio-Rad). The membranes were blocked for 1 h at room temperature (or overnight at 4 °C) with 5% skim milk (wt/vol) in PBS.

To detect RABV-P, a rabbit anti-RABV-P polyclonal antibody, obtained by DNA immunization, was diluted 1:5000 with 1% skim milk in PBS containing 0.05% (vol/vol) Tween-20 (PBS-T) and incubated with the membranes for 1 h at room temperature. Following four washes with PBS-T, an HRP-conjugated anti-rabbit IgG, diluted 1:5000 with 1% skim milk in PBS-T, was added and incubated for 1 h at room temperature. After four washes, the membranes were developed with ECL Plus Western Blotting Detection Reagent substrate solution (GE Healthcare). The signals were detected using VersaDoc (Bio-Rad).

To detect scFvs, an anti-myc tag mouse MoAb; was added to the primary antibody solution (above) at 0.5 μg/100 μL, and HRP-conjugated goat anti-mouse IgG (H + L) (Pierce) was added to the secondary antibody solution (above) at 1:5000.

2.8. Characterization of scFv affinity

To characterize the affinity of each scFv, ELISA against immobilized RABV-P was performed using peroxidase-labeled scFvs. First, 200 μg of each soluble scFv was peroxidase-labeled using a Peroxidase Labeling Kit-NH_2 (Dojindo, Kumamoto, Japan) in accordance with the manufacturer's instructions. After the labeling, serially diluted scFvs were applied to an ELISA plate coated with native RABV-P and detected as described above (see Section 2.3). The OD data was graphed by using Prism 5 (GraphPad, San Diego, CA, USA).

2.9. scFv vectors for intrabody expression

Each scFv gene cDNA was recovered from its pIT2 phagemid vector by PCR (primer sequences will be supplied on request). For intrabody expression, each scFv gene cDNA was inserted into pCAGGS-derived pCAGJ12bsr (Kojima et al., 2003; a kind gift from Dr. Asato Kojima, National Institute of Infectious Diseases, Japan), after the removal of the original insert, J12. The generated vectors were named scFv-P19-pCAG, scFv-P38-pCAG, scFv-P80-pCAG, scFv-P115-pCAG, and scFv-bat-IgG-pCAG (negative control).

2.10. Transfection and confirmation of intrabody expression

Each intrabody expression vector was transfected into MNA cells using Fugene HD (Roche) in accordance with the manufacturer's protocol. At 1–4 days after transfection, intrabody expression was confirmed with an indirect immunofluorescence assay (IFA). Briefly, after cell fixation in 3.6% formaldehyde and 0.4% Triton-X for 30 min at room temperature, the intrabody was stained using the anti-myc tag mouse MoAb as the primary antibody and an FITC-goat anti-mouse IgG (H + L) (Invitrogen) as the secondary antibody, then viewed under a fluorescence microscope (Nikon, Tokyo, Japan). For counterstaining, 0.002% Evans blue was added to the secondary antibody fluid.

2.11. Confirmation of the interaction between the intrabody and RABV-P in vivo

scFv-P19-pCAG was transfected into MNA cells as described above. After 24 h, the cells were infected with CVS-11 (m.o.i. = 10). At 24 h after infection, the cells were fixed in 3.6% formaldehyde and 0.4% Triton-X. The intrabody was stained as described above, and RABV-P was stained with rabbit anti-RABV-P serum as the primary antibody and TRITC-conjugated anti-rabbit IgG (Sigma) as the secondary antibody. Cells were visualized and the images were digitally captured by a BZ-8000 "Bio-zero" fluorescence microscope (KEYENCE, Osaka, Japan). At 24 h after transfection, the cells were lysed and collected, and then immunoprecipitated as described above.

2.12. Measurement of inhibition of RABV propagation by the intrabody

scFv-P19-pCAG was transfected into MNA cells in a 96-well plate as above. After 24 h, the cells were infected with CVS-11 (m.o.i. = 0.005). At 1 to 4 days after infection, inhibition of RABV propagation by the intrabody was measured by two methods: (i) measurement of the proportion of an area infected with RABV, and (ii) virus titration of the supernatant. To measure the infected area, the cells were stained with FITC Anti-Rabies Monoclonal Globulin (Fujirebio Diagnostics, Malvern, PA, USA), which targets RABV nucleoprotein (RABV-N). Images of RABV-infected (RABV-N-positive) cells were captured digitally as described above. In the digital image, the proportion of the fluorescence-emitting area relative to the whole well was calculated by using VH-H1A5 "VH analyzer" software (KEYENCE), then the data were graphed by using Prism 5. To measure the viral titer, the supernatant was collected, diluted 10-fold serially, and inoculated into MNA cells. After 48 h, the cells were stained by FITC Anti-Rabies Monoclonal Globulin and titrated by the Reed & Muench method (Reed and Muench, 1938).

3. Results

3.1. Selection of scFv clones against RABV-P

After three rounds of panning, a total of 192 phage clones were obtained. The binding ability of these phage clones was examined by ELISA against native RABV-P and denatured RABV-P, and 10 clones that showed equivalent reactivity for both native and denatured RABV-P were selected. DNA sequencing of scFv cDNA in each

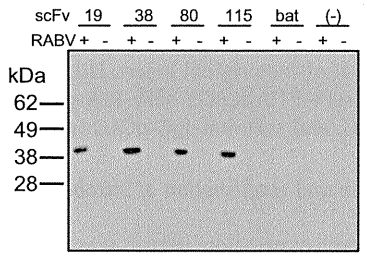

Fig. 1. *In vitro* interaction between scFvs and RABV-P confirmed by immunoprecipitation and Western blotting assays. Western blot analysis of lysates of RABV-infected and mock-infected MNA cells. The lysates were mixed with each scFv (scFv-P19, -38, -80, -115, -bat-IgG) or no scFv and immunoprecipitated with Dynabeads, blotted, and detected with a rabbit anti-RABV-P polyclonal antibody and HRP-conjugated anti-rabbit IgG.

of the 10 clones indicated that these clones converged to four genetically independent clones (Supplemental Fig. 1). *E. coli* HB2151 cells were infected with these phages to obtain soluble scFv for further characterization. After the purification of soluble scFvs using Ni–NTA resin, SDS–PAGE was performed to confirm the expression of scFvs. All four scFv clones showed a single protein band with a molecular weight of 29 kDa by SDS–PAGE (Supplemental Fig. 2). These scFvs were also confirmed to detect RABV-P by reducing Western blot analysis (data not shown), indicating that all four scFvs recognize linear epitopes.

We further performed an immunoprecipitation assay to examine the interaction *in vitro* between these scFvs and RABV-P. RABV-infected MNA cells were lysed 48 h after infection and incubated with each scFv. The protein mixture was precipitated by using magnetic beads to bind the histidine-tagged proteins. A distinct interaction between the scFvs and RABV-P was detected in a Western blot analysis with anti-RABV-P serum (Fig. 1) and the anti-myc MoAb (data not shown). To characterize the affinity of each scFv, we performed ELISA against immobilized RABV-P using peroxidase-labeled scFvs. According to the reaction curves (Supplemental Fig. 3), the affinity against RABV-P was highest in scFv-P19, followed by -115, -38, and -80.

3.2. Confirmation of intrabody expression

The scFv cDNAs of all four clones were inserted into pCAGGS-derived vectors to achieve intracellular expression as intrabodies. The generated intrabody expression vectors, scFv-P19-pCAG, scFv-P38-pCAG, scFv-P80-pCAG, and scFv-P115-pCAG, were transfected into MNA cells for transient expression, and their expression levels were monitored by IFA using an anti-myc tag MoAb. The transfection efficiency of the clones varied: scFv-P19 showed much higher transfection efficiency than the others, reaching more than 80% (estimated by eye; Fig. 2, top). Interestingly, all the scFv clones, but especially scFv-P19, showed highly stable expression without substantial diminution from 24 h until 96 h after transfection (Fig. 2). Considering its high transfection efficiency and stable expression, we selected scFv-P19 for further studies to examine *in vivo* binding with RABV-P and the ability to abrogate viral propagation.

3.3. Confirmation of the interaction between intrabodies and RABV-P in vivo

To examine the interaction between the scFv-P19 intrabody and RABV-P *in vivo*, MNA cells were transfected with scFv-P19-pCAG for transient expression, and 24 h later, the same cells were infected with CVS-11 (m.o.i. = 10). At 48 h after infection, intracellular localization of scFvs and RABV-P was observed by IFA using the anti-myc tag MoAb and anti-RABV-P polyclonal serum and compared with the localization in mock-infected MNA cells expressing scFv-P19. In mock-infected MNA cells, scFvs were located diffusely throughout the cytoplasm (Fig. 3A) without any particular condensation of fluorescence. In contrast, in CVS-11-infected MNA cells, some cells showed punctate fluorescent patterns against the background of diffuse fluorescence in the cytoplasm (Fig. 3B). We also observed this punctate fluorescence in CVS-11-infected cells stained with anti-RABV-P polyclonal serum (Fig. 3C), which is typically seen in cells infected with fixed RABV strains in culture and reportedly involves the intracellular accumulation of ribonucleoprotein complex to form Negri bodies (Lahaye et al., 2009). These punctate fluorescent patterns of scFv-P19 and RABV-P overlapped

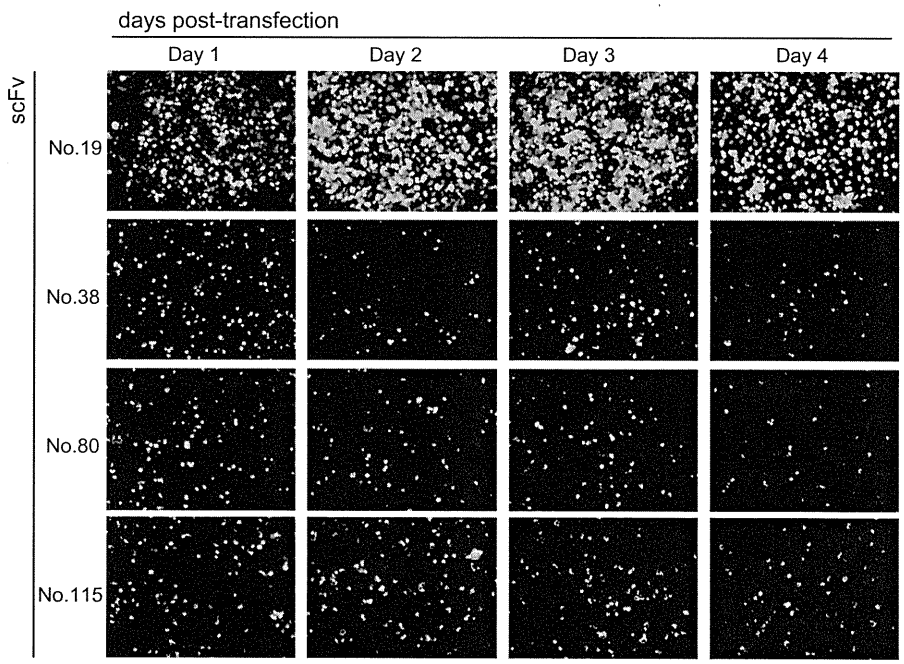

Fig. 2. Time course of intracellular expression of scFvs. In MNA cells transfected with the indicated scFv vectors, scFv was detected by a mouse anti-myc tag MoAb and FITC-conjugated anti-mouse IgG on days 1–4 after transfection. For counterstaining, 0.002% Evans blue was added to the secondary antibody fluid.

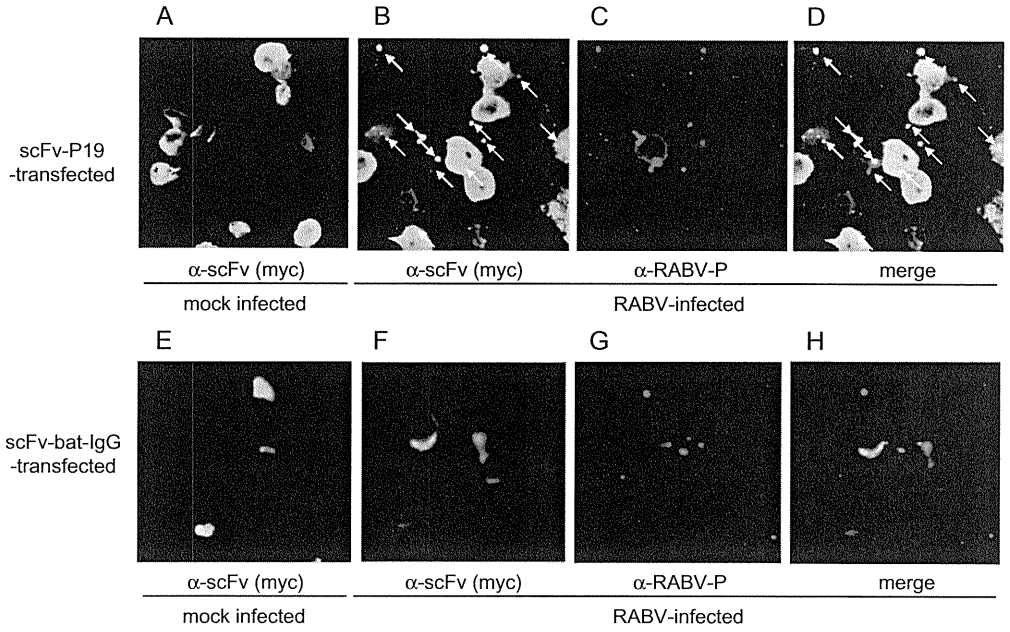

Fig. 3. Intracellular colocalization of scFv-P19 and RABV-P. MNA cells were pre-transfected with scFv-P19-pCAG (A–D) or scFv-bat-IgG-pCAG (E–H). In mock-infected MNA cells pre-transfected with scFv-expressing plasmids (A and E), scFv was detected with an anti-myc tag MoAb and FITC-conjugated anti-mouse IgG. In RABV-infected MNA cells pre-transfected with scFv-expressing plasmids, scFv was detected as described above (B and F) and RABV-P was detected with a rabbit anti-RABV-P polyclonal antibody and TRITC-conjugated anti-rabbit IgG (C and G). (D and H) Merged images of (B) and (C), or (F) and (G). Arrows in (B) and (D) indicate the punctate fluorescence of scFvs.

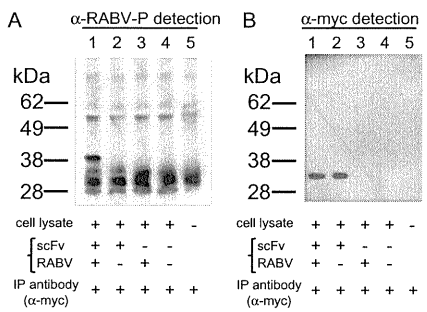

Fig. 4. *In vivo* interaction between scFv-P19 and RABV-P confirmed by immunoprecipitation and Western blotting assays. Western blot analysis of lysates of RABV-infected and mock-infected MNA cells transiently expressing scFv-P19. For detecting RABV-P (A), the lysates were immunoprecipitated (IP) with an anti-myc-tag MoAb, and the immunoblot was probed with a rabbit anti-RABV-P polyclonal antibody and detected by HRP-conjugated anti-rabbit IgG. For detecting scFv-P19 (B), the lysates were IP as above, and the immunoblot was probed with a mouse anti-myc tag MoAb and detected by HRP-conjugated anti-mouse IgG. In both images, cell lysates were immunoprecipitated (+, lanes 1–4) or only lysis buffer was used (–, lane 5). To prepare cell lysates, scFv-P19 was pre-transfected (+, lanes 1 and 2) or not transfected (–, lanes 3 and 4). After 24 h, the cells were inoculated with RABV (+, lanes 1 and 3) or mock-infected (–, lanes 2 and 4). In (A), the bands smaller than 30 kDa appear to be non-specific, because lane 5 (containing only the IP antibody) also has these bands. These non-specific bands might be due to the cross-reactivity of the detection antibody (anti-RABV-P) or the secondary antibody (anti-rabbit IgG) with the IP antibody.

in a merged image (Fig. 3D), indicating that these two molecules colocalized in the cytoplasm. In contrast, in MNA cells pre-transfected with scFv-bat-IgG-pCAG, the change in the fluorescence pattern of scFv was not observed after RABV infection even though the transfection efficiency was much lower than that of scFv-P19-pCAG, and the fluorescence of scFv and RABV-P did not overlap (Fig. 3E–H).

Subsequently, to obtain direct evidence of intracellular binding between scFv-P19 and RABV-P, we immunoprecipitated a lysate of CVS-11-infected MNA cells expressing scFv-P19 with an anti-myc MoAb 48 h after infection. A Western blot analysis using anti-RABV-P polyclonal serum (Fig. 4A) and the anti-myc MoAb (Fig. 4B) showed a 38-kDa RABV-P band (Fig. 4A, lane 1) and a 29-kDa scFv band (Fig. 4B, lane 1), whereas only the scFv band was present in mock-infected cells (Fig. 4B, lane 2). This indicates that these two molecules, scFv-P19 and RABV-P, can associate with each other when co-expressed in the cytoplasm.

Taken together, these results suggest that scFv-P19 is able to recognize and bind to the target protein, RABV-P, intracellularly and to function as an intrabody.

3.4. Inhibition of RABV propagation by the intrabody

Finally, we determined the inhibitory effect of intracellularly expressed scFv-P19 on RABV propagation. MNA cells in 96-well plates were transiently transfected with scFv-P19-pCAG, and 24 h later the cells were infected with CVS-11 at an m.o.i. of 0.005. In this assay, we aimed to observe how the intrabody could inhibit the spread of RABV infection from a limited initial area to simulate the early phase of real viral infection *in vivo*. In order to have the infected area as limited as possible on day 1, we used a low m.o.i. here. We used two separate methods to compare the inhibitory effect of scFv-P19 in scFv-expressing cells and nontransfected cells: measuring the inhibition of the spread of RABV-infected cells in a culture plate (Figs. 5 and 6A), and titration of viruses in the supernatant (Fig. 6B). The data were collected for 4 days after the infection, during which the intracellular expression of scFv-P19 was high and stable (Fig. 5, bottom panel). Cells expressing scFv-P19 substantially inhibited the spread of the RABV-infected area for 4 days (Fig. 5, middle panel), whereas in nontransfected control cells, RABV infection had spread throughout the well within that time (Fig. 5, top panel). The highest inhibitory effect (calculated from the ratio of the proportion of RABV-infected cells in scFv-expressing cells to that in nontransfected cells) was achieved at 3 days after infection ($[1-0.91/66.99] \times 100 = 98.6\%$ inhibition; Fig. 6A). In the experiment involving virus titration, the highest inhibitory effect (calculated from the ratio of the virus titer in

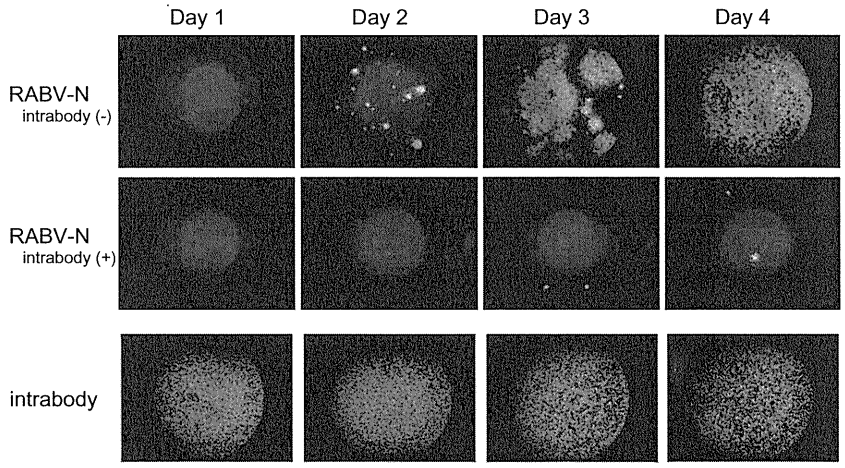

Fig. 5. Comparison of the spread of RABV infection between intrabody-positive and intrabody-negative MNA cells. CVS-11-infected MNA cells without pre-expression of scFv-P19 (top panel), or with pre-expression (middle and bottom panel). The infected cells were stained by FITC Anti-Rabies Monoclonal Globulin on days 1–4 after infection, and the areas that stained positive were compared. In the bottom panels, scFv expression was monitored by staining with a mouse anti-myc tag MoAb and FITC-conjugated anti-mouse IgG; FITC-derived images of scFvs were digitally converted to violet color to avoid confusion with the FITC-derived images of RABV-N in the top and middle panels.

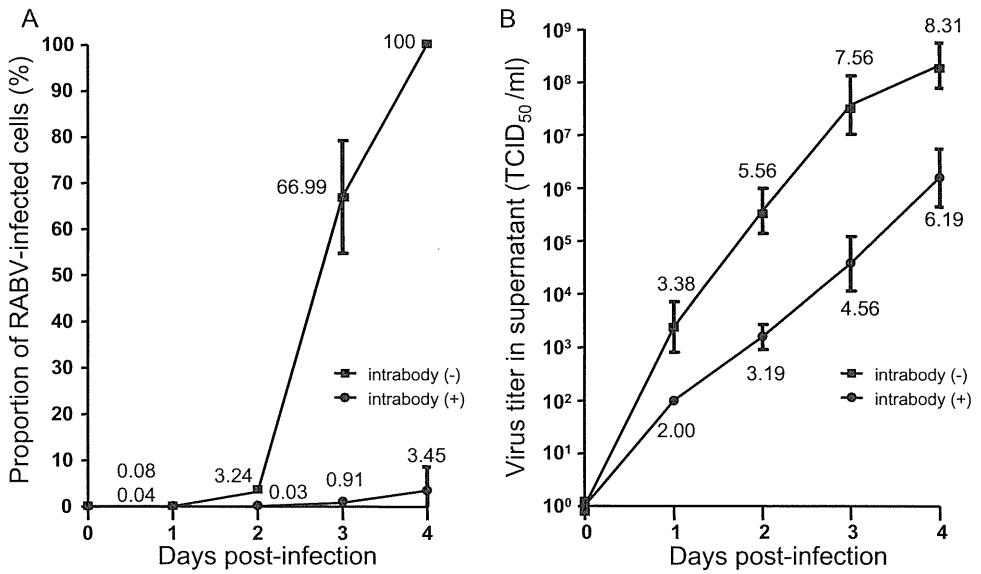

Fig. 6. Inhibition of RABV propagation in MNA cells by intrabody scFv-P19. (A) Time course of the proportion of RABV-infected area relative to the whole well, in a 96-well plate of CVS-11-infected MNA cells without pre-expression of scFv-P19 (■), or with pre-expression of scFv-P19 (●); data were derived from the digitally captured images shown in Fig. 5. Values are mean ± standard error of the mean (SE); $N = 4$. (B) Time course of the virus titer ($TCID_{50}$/ml) in the supernatant of CVS-11-infected MNA cells without pre-expression of scFv-P19 (■), or with pre-expression of scFv-P19 (●). Values are mean ± SE; $N = 4$. The numbers on the graph indicate the logarithm of the mean.

scFv-expressing cells to that in nontransfected cells) was also achieved at 3 days after infection ($[1-10^{4.56}/10^{7.56}] \times 100 = 99.9\%$ inhibition; Fig. 6B)

These results indicate that scFv-P19, when expressed intracellularly as an intrabody, is effective at inhibiting RABV propagation until at least 4 days after infection.

4. Discussion

To our knowledge, this is the first report of intrabody-mediated inhibition of RABV propagation and secretion in studies using mouse neuronal cell lines. A significant reduction in RABV propagation was achieved by pretransfection of an scFv expression vector expressing scFv-P19, which was directed against RABV-P. Another intracellular immunization approach against RABV infection, RNAi-based gene silencing, has also been reported to inhibit RABV propagation (Brandao et al., 2007) and to reduce the replication of the viral genome in mouse neuronal cell lines (Israsena et al., 2009). Also, the intracellular expression of peptides mimicking the N-terminus of RABV-P has been shown to have antiviral activity (Castel et al., 2009). However, no studies of antibody-based intracellular immunization aimed at future therapeutic application have been reported, although several research groups have been developing scFvs or Fabs against RABV proteins for diagnosis or prevention. Intracellular immunization based on scFvs is worthy of investigation because scFvs have several advantages, such as high specificity and long half-lives. Many studies have utilized hybridomas producing MoAbs as a source of scFv; however, phage-display libraries have also been used in recent studies be-

cause they provide benefits such as high diversity in the antigenic repertoire and the ability to rapidly select specific antibodies without animal immunization. In this study, we used human single-fold scFv libraries, Tomlinson I + J, to select scFvs against RABV-P.

One of the obstacles to using scFvs or other antigen-binding molecules as intrabodies is the need to preserve their affinity *in vivo*. Many scFvs have been reported to fold incorrectly *in vivo* and to lose their affinity due to the highly reducing conditions inside cells (Ramm et al., 1999; Wörn and Plückthun, 2001). To minimize this problem, our screening procedure was designed to focus on the selection of scFvs recognizing linear epitopes. Both native and denatured antigens were used as coating antigens in ELISA screening, and only clones showing equivalent reactivity against both were selected. Consequently, all four scFvs obtained in this study reacted with RABV-P in a reducing Western blot analysis, indicating that these clones recognize linear epitopes as expected. Considering the substantial inhibition that scFv-P19 showed against RABV propagation, the strategy of targeting scFv recognizing linear epitopes might be generally useful for future intrabody studies.

We chose RABV-P as the target protein because it contributes to diverse functions through interactions with several viral and cellular proteins. These functions occur at various stages of the RABV life cycle and include encapsidation of viral genome RNA (together with RABV-N and -L), acting as a cofactor for viral RNA polymerase (RABV-L), and inhibiting the interferon-induced cellular antiviral system by binding to signal transducer and activator of transcription 1 (STAT1) and STAT2 to retain them in the cytoplasm (reviewed by Schnell et al., 2010). These multiple functions of RABV-P may be achieved by its efficient modular organization (Gerard et al., 2009) and by the synthesis of four N-terminally truncated P products (P2–P5) showing different subcellular localizations (Blondel et al., 2002): full-length P and P2 are located in the cytoplasm, whereas P3, P4, and P5 are in the nucleus. The detailed three-dimensional structure of P has only been obtained for its C-terminal domain, and how it produces these multiple functions through interactions with various protein counterparts is still not fully understood. The intrabodies against RABV-P obtained in this study were expected to inhibit at least one of these functions, leading to the eradication of viral propagation. Because the epitopes recognized by scFv-P19 (and other clones) have not yet been determined, at the moment there is no evidence regarding which RABV-P function(s) these scFvs block. Our observation that scFv-P19 colocalized with RABV-P in Negri bodies, as well as being diffusely distributed in the cytoplasm, raises the possibility that scFv-P19 inhibits several RABV-P functions in different subcellular locations. Negri bodies, composed of RABV ribonucleoproteins (RABV-N, -P, -L proteins and the RABV genome) and several cellular proteins, are considered to be a marker of RABV infection and are key structures for the synthesis of descendant viral particles (Lahaye et al., 2009). To understand the mechanism through which the scFvs inhibit viral propagation, epitope mapping of the scFvs combined with detailed three-dimensional analysis of RABV-P will be required.

In this study, pretransfection of the scFv-P19 gene into MNA cells substantially inhibited the propagation of RABVs and reduced the virus titer in the supernatant to 1:1000 of that of nontransfected cells at 3 days after infection. This indicates that scFv-P19 could be a prospective candidate for an intrabody; however, this remarkable inhibition might have been achieved partially due to an unexpectedly higher transfection efficiency compared to other scFv clones (scFv-P38, -80, and -115). Although the cause of the difference in transfection efficiency is not clear, a more stable and reliable expression and delivery system should be explored in future studies. One plausible option would be to use a viral vector, which would enable both higher transfection efficiency and strict tropism. These features are especially advantageous for therapeutic application against RABV, which shows extremely high tropism for neuronal cells and propagates by cell-to-cell transmission. By using a viral vector, the other three clones we identified (scFv-P38, -80, and -115), which were not explored further in this study due to low transfection efficiency, might attain higher expression and an inhibition potency as powerful as scFv-P19.

Intrabody research generally consists of two dimensions: the determination of a candidate molecule as an intrabody, and the establishment of an appropriate delivery or application system. This study is the first to report the identification of a candidate molecule that reduces RABV propagation when pretransfected. However, considering the potential of intrabodies as therapeutic tools against RABV infection, post-exposure applications should also be investigated. In this study, we were not able to examine post-exposure application of intrabodies because the high transfection efficiency required for a post-exposure study could not be attained with the transient expression system used. A combination of the scFvs generated in this study and efficient delivery systems involving viral vectors could be powerful tools for future practical studies of post-exposure applications.

In conclusion, we demonstrated that scFv-P19, when pre-expressed as an intrabody in MNA cells, substantially inhibited the propagation of RABV CVS-11. This scFv-based intracellular immunization could be a candidate for a future therapeutic tool against RABV infection if combined with an appropriate delivery and application system, such as a viral vector.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2011.04.016.

References

Baltimore, D., 1988. Gene therapy. Intracellular immunization. Nature 335, 395–396.

Blondel, D., Regad, T., Poisson, N., Pavie, B., Harper, F., Pandolfi, P.P., De Thé, H., Chelbi-Alix, M.K., 2002. Rabies virus P and small P products interact directly with PML and reorganize PML nuclear bodies. Oncogene 21, 7957–7970.

Brandao, P.E., Castilho, J.G., Fahl, W., Carnieli, P., Oliveira Rde, N., Macedo, C.I., Carrieri, M.L., Kotait, I., 2007. Short-interfering RNAs as antivirals against rabies. Braz. J. Infect. Dis. 11, 224–225.

Cao, T., Heng, B.C., 2005. Intracellular antibodies (intrabodies) versus RNA interference for therapeutic applications. Ann. Clin. Lab. Sci. 35, 227–229.

Castel, G., Chtéoui, M., Caignard, G., Préhaud, C., Méhouas, S., Réal, E., Jallet, C., Jacob, Y., Ruigrok, R.W.H., Tordo, N., 2009. Peptides that mimic the amino-terminal end of the rabies virus phosphoprotein have antiviral activity. J. Virol. 83, 10808–10820.

Chenik, M., Chebli, K., Gaudin, Y., Blondel, D., 1994. In vivo interaction of rabies virus phosphoprotein (P) and nucleoprotein (N): existence of two N-binding sites on P protein. J. Gen. Virol. 75, 2889–2896.

Chenik, M., Schnell, M., Conzelmann, K.K., Blondel, D., 1998. Mapping the interacting domains between the rabies virus polymerase and phosphoprotein. J. Virol. 72, 1925–1930.

Corte-Real, S., Collins, C., Aires da Silva, F., Simas, J.P., Barbas, C.F., Chang, Y., Moore, P., Goncalves, J., 2005. Intrabodies targeting the Kaposi sarcoma-associated herpesvirus latency antigen inhibit viral persistence in lymphoma cells. Blood 106, 3797–3802.

Dykxhoorn, D.M., Novina, C.D., Sharp, P.A., 2003. Killing the messenger: short RNAs that silence gene expression. Nat. Rev. Mol. Cell Biol. 4, 457–467.

Gerard, F.C.A., Ribeiro, E.D.A., Leyrat, C., Ivanov, I., Blondel, D., Longhi, S., Ruigrok, R.W.H., Jamin, M., 2009. Modular organization of rabies virus phosphoprotein. J. Mol. Biol. 388, 978–996.

Goncalves, J., Silva, F., Freitas-Vieira, A., Santa-Marta, M., Malhó, R., Yang, X., Gabuzda, D., Barbas, C., 2002. Functional neutralization of HIV-1 Vif protein by intracellular immunization inhibits reverse transcription and viral replication. J. Biol. Chem. 277, 32036–32045.

Hemachudha, T., Sunsaneewitayakul, B., Desudchit, T., Suankratay, C., Sittipunt, C., Wacharapluesadee, S., Khawplod, P., Wilde, H., Jackson, A.C., 2006. Failure of therapeutic coma and ketamine for therapy of human rabies. J. Neurovirol. 12, 407–409.

Israsena, N., Supavonwong, P., Ratanasetyuth, N., Khawplod, P., Hemachudha, T., 2009. Inhibition of rabies virus replication by multiple artificial microRNAs. Antiviral Res. 84, 76–83.

Jiang, W., Venugopal, K., Gould, E.A., 1995. Intracellular interference of tick-borne flavivirus infection by using a single-chain antibody fragment delivered by recombinant Sindbis virus. J. Virol. 69, 1044–1049.

Karthe, J., Tessmann, K., Li, J., Machida, R., Daleman, M., Häussinger, D., Heintges, T., 2008. Specific targeting of hepatitis C virus core protein by an intracellular single-chain antibody of human origin. Hepatology 48, 702–712.

Kojima, A., Yasuda, A., Asanuma, H., Ishikawa, T., Takamizawa, A., Yasui, K., Kurata, T., 2003. Stable high-producer cell clone expressing virus-like particles of the Japanese encephalitis virus e protein for a second-generation subunit vaccine. J. Virol. 77, 8745–8755.

Lahaye, X., Vidy, A., Pomier, C., Obiang, L., Harper, F., Gaudin, Y., Blondel, D., 2009. Functional characterization of Negri bodies (NBs) in rabies virus-infected cells: evidence that NBs are sites of viral transcription and replication. J. Virol. 83, 7948–7958.

Marasco, W.A., 1997. Intrabodies: turning the humoral immune system outside in for intracellular immunization. Gene Ther. 4, 11–15.

Motoi, Y., Inoue, S., Hatta, H., Sato, K., Morimoto, K., Yamada, A., 2005. Detection of rabies-specific antigens by egg yolk antibody (IgY) to the recombinant rabies virus proteins produced in *Escherichia coli*. Jpn. J. Infect. Dis. 58, 115–118.

Nawtaisong, P., Keith, J., Fraser, T., Balaraman, V., Kolokoltsov, A., Davey, R.A., Higgs, S., Mohammed, A., Rongsriyam, Y., Komalamisra, N., Fraser, M.J., 2009. Effective suppression of Dengue fever virus in mosquito cell cultures using retroviral transduction of hammerhead ribozymes targeting the viral genome. Virol. J. 6, 73.

Nigg, A.J., Walker, P.L., 2009. Overview, prevention, and treatment of rabies. Pharmacotherapy 29, 1182–1195.

Ramm, K., Gehrig, P., Plückthun, A., 1999. Removal of the conserved disulfide bridges from the scFv fragment of an antibody: effects on folding kinetics and aggregation. J. Mol. Biol. 290, 535–546.

Reed, L., Muench, H., 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27, 493–497.

Schnell, M.J., McGettigan, J.P., Wirblich, C., Papaneri, A., 2010. The cell biology of rabies virus: using stealth to reach the brain. Nat. Rev. Microbiol. 8, 51–61.

Stein, C.A., Hansen, J.B., Lai, J., Wu, S., Voskresenskiy, A., Høg, A., Worm, J., Hedtjärn, M., Souleimanian, N., Miller, P., Soifer, H.S., Castanotto, D., Benimetskaya, L., Ørum, H., Koch, T., 2010. Efficient gene silencing by delivery of locked nucleic acid antisense oligonucleotides, unassisted by transfection reagents. Nucleic Acids Res. 38, e3.

Vascotto, F., Campagna, M., Visintin, M., Cattaneo, A., Burrone, O.R., 2004. Effects of intrabodies specific for rotavirus NSP5 during the virus replicative cycle. J. Gen. Virol. 85, 3285–3290.

Vidy, A., El Bougrini, J., Chelbi-Alix, M.K., Blondel, D., 2007. The nucleocytoplasmic rabies virus P protein counteracts interferon signaling by inhibiting both nuclear accumulation and DNA binding of STAT1. J. Virol. 81, 4255–4263.

Willoughby, R.E., Tieves, K.S., Hoffman, G.M., Ghanayem, N.S., Amlie-Lefond, C.M., Schwabe, M.J., Chusid, M.J., Rupprecht, C.E., 2005. Survival after treatment of rabies with induction of coma. N. Engl. J. Med. 352, 2508–2514.

Wörn, A., Plückthun, A., 2001. Stability engineering of antibody single-chain Fv fragments. J. Mol. Biol. 305, 989–1010.

Yamamoto, M., Hayashi, N., Takehara, T., Ueda, K., Mita, E., Tatsumi, T., Sasaki, Y., Kasahara, A., Hori, M., 1999. Intracellular single-chain antibody against hepatitis B virus core protein inhibits the replication of hepatitis B virus in cultured cells. Hepatology 30, 300–307.

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Original Article

Molecular Epidemiology of Rabies Virus in Vietnam (2006–2009)

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SUMMARY: This study was aimed at determining the molecular epidemiology of rabies virus (RABV) circulating in Vietnam. Intra vitam samples (saliva and cerebrospinal fluid) were collected from 31 patients who were believed to have rabies and were admitted to hospitals in northern provinces of Vietnam. Brain samples were collected from 176 sick or furious rabid dogs from all over the country. The human and canine samples were subjected to reverse transcription-polymerase chain reaction analysis. The findings showed that 23 patients tested positive for RABV. Interestingly, 5 rabies patients did not have any history of dog or cat bites, but they had an experience of butchering dogs or cats, or consuming their meat. RABV was also detected in 2 of the 100 sick dogs from slaughterhouses. Molecular epidemiological analysis of 27 RABV strains showed that these viruses could be classified into two groups. The RABVs classified into Group 1 were distributed throughout Vietnam and had sequence similarity with the strains from China, Thailand, Malaysia, and the Philippines. However, the RABVs classified into Group 2 were only found in the northern provinces of Vietnam and showed high sequence similarity with the strain from southern China. This finding suggested the recent influx of Group 2 RABVs between Vietnam and China across the border. Although the incidence of rabies due to circulating RABVs in slaughterhouses is less common than that due to dog bite, the national program for rabies control and prevention in Vietnam should include monitoring of the health of dogs meant for human consumption and vaccination for workers at dog slaughterhouses. Further, monitoring of and research on the circulating RABVs in dog markets may help to determine the cause of rabies and control the spread of rabies in slaughterhouses in Vietnam.

INTRODUCTION

Rabies is a fatal zoonotic disease caused by rabies virus (RABV), which belongs to the genus *Lyssavirus* of family *Rhabdoviridae*. Rabies exists in more than 150 countries and territories worldwide. A recent estimate indicates that more than 55,000 people die of rabies every year. However, the actual incidence of human rabies may be 100 times higher than the officially reported numbers, and most of the fatalities due to rabies occur in African and Asian countries (6). In recent years, the number of cases of human rabies in Vietnam, the Philippines, Laos, Indonesia, and China has been rapidly increasing (2,4,8,10,11,13,15). In Vietnam, 362 cases of human rabies have been reported from 2007 to 2010, and the rabies epidemic has occurred in 25–27 provinces (4). The main reservoirs and transmitters of rabies in Vietnam are dogs rather than wild animals such as foxes, bats, and raccoons. Furthermore, most dogs are unvaccinated, and domestic transport and import/export of animals, including dogs and cats, are not well regulated (8). Therefore, determining the genotype of the circulating RABV is very important to understand its evolutionary relationship with the local as well as regional strains and to elucidate the dynamics of the widespread transmission of this disease. The objective of this study was to determine the molecular epidemiology of the RABV circulating in Vietnam.

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MATERIALS AND METHODS

Study samples: We collected brain samples from 176 dogs, of which 100 were sick dogs found at slaughterhouses in the northern provinces. These 100 suspected rabies-infected dogs showed at least one of the following signs: refusal to eat or discontinuation of eating, excessive salivation, aggressiveness, and paralyses. The other 76, which were furious rabid dogs, were from the central, highland, and southern provinces in Vietnam. The diagnosis of rabies in those dogs was performed either using fluorescent antibody test (FAT) with the polyclonal antibody raised against the nucleoprotein (N) of RABV (Sanofi Diagnostics, Pasteur, France) or using reverse transcription-polymerase chain reaction (RT-PCR) with a OneStep RT-PCR Kit (Qiagen, Hilden, Germany). Intra vitam samples of saliva (SLV) and cerebrospinal fluid (CSF) were collected from 31 suspected rabies-infected humans who were admitted to national hospitals located in the northern provinces of Vietnam. RT-PCR analysis confirmed that these sam-

ples contained RABV.

Viral RNA extraction and RT-PCR analysis: Total RNA was extracted from 0.3–0.5 g of homogenized brain samples using the RNeasy Mini Kit (Qiagen) and from 0.5 ml of SLV and CSF using the QIAamp Viral RNA Mini Kit (Qiagen). The part of the N gene was then amplified using the QIAGEN OneStep RT-PCR Kit, using a sense primer N7 (nt. 15–34; 5′-ATG TAA CAC CTC TAC AAT GG-3′) and an anti-sense primer JW6E (nt. 601–619; 5′-CAG TTG GCA CAC ATC TTG TG-3′) in a 50-μl reaction mixture containing 400 μM of each dNTP, 10 μl of 5× buffer, 30 μM of each primer, 2 μl enzyme mix, 20 μl distilled water, and 10 μl of the template RNA. The reaction mixture was then subjected to reverse transcription at 50°C for 30 min, followed by heating at 95°C for 15 min, and then 35 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and elongation at 72°C for 1.5 min. The mixture was further incubated at 72°C for 15 min to complete elongation. The amplicon was then subjected to electrophoresis in 2% agarose gel and stained with ethidium bromide.

Nucleotide sequencing: The RT-PCR product was excised from the gel and purified using the QIA quick Gel Extraction Kit (Qiagen). Cycle sequencing was performed using N7 and JW6E primers with the ABI 3100 Genetic Analyzer. The nucleotide sequences of the N gene of RABV strains reported in this paper have been submitted to the DDBJ/EMBL/GenBank nucleotide sequence databases (accession numbers AB614372–AB614393 and AB628210–AB628214).

Phylogenetic analysis: The partial nucleotide sequence of N gene (nucleotide position, 85–473) was determined and compared with the representative sequences obtained from the GenBank (www.ncbi.nlm.nih.gov). The accession numbers (the name of strain, country) of the representative sequences are as follows: AB299032 (HCM1, Vietnam); AB299033 (HCM2, Vietnam); AB299034 (HCM5, Vietnam); AB299035 (HCM6, Vietnam); AB299036 (HCM7, Vietnam); AB299037 (HCM8, Vietnam); AB299038 (HCM9, Vietnam); AB299039 (HCM10, Vietnam); AB116579 (VN3, Vietnam); AB116580 (VN52, Vietnam); U22653 (8738THA, Thailand); U22916 (8677MAL, Malaysia); AB070759 (PHI123-01, Philippines); AB 070761 (PHI127-03, Philippines); AB116581 (PHI103, Philippines); AB116582 (PHI114, Philippines); AB070817 (Mdn183/45, Philippines); EF555102 (CQQJDN06, China); EF555106 (SDJNCN01, China); EF555112 (GDZQDN45, China); U22918 (94260NEP, Nepal); EF555098 (CQQJDN02, China); EF555099 (CQQJDN03, China); and EF555100 (CQQJDN04, China). Mokola virus, AY333111 (Eth-16, Ethiopia), was used as an out-group. Complete alignment of nucleotide sequences was performed using ClustalX, version 2.0 (9). MegAlign software version 7.0 (DNASTAR, Madison, Wis., USA) was used to analyze homologies of the nucleotide and deduced amino acid sequences. The neighbor-joining method in MEGA4 version 4.0 (12) was used for constructing the phylogenetic tree with 1,000 bootstrap replications. Epidemiology map was constructed using HealthMapper software, version 4.2, which was supported by the World Health Organization.

RESULTS

Prevalence of rabies in humans and animals: From 2007 to 2009, 31 residents of the northern provinces of Vietnam who were suspected of having rabies were admitted to the Bach Mai Hospital or the Institute for Tropical and Infectious Diseases. We used the CSF and/or SLV samples of these patients for the laboratory diagnosis of rabies. Direct RT-PCR analysis of the patients' SLV and/or CSF samples confirmed that 23 of the 31 (74%) rabies suspected patients were infected with RABV (Table 1). Out of these 23 patients, 12 (52%) had been bitten by dogs or cats and 5 (22%) were involved in the butchering of sick cats or dogs, whereas 6 (26%) did not have any history of dog/cat bites or the butchering of sick animals. In this study, brain samples were collected from 100 sick dogs from slaughterhouses in the northern provinces. The findings of FAT and RT-PCR analysis confirmed that, out of those 100 dogs, 2 (2.0%) were infected with RABV. We also found that 15 (16.4%) of the 76 dogs from the southern and highland provinces were infected with RABV (Table 1).

Phylogenetic analysis: Phylogenetic analysis was performed on 27 strains of RABVs of canine and human origin (Table 2). In the phylogenetic tree, the RABVs

Table 1. Diagnostic results of rabies suspected cases of human and animals (2006–2009)

Location		Human	Animal
Region	Province	Positive/ total (%)	Positive/ total (%)
North	Ha Tay	10/12 (83.3)	2/72 (2.8)
	Hanoi	—	0/4
	Phu Tho	4/4	—
	Bac Ninh	1/1	—
	Hoa Binh	3/3	0/10
	Yen Bai	1/1	—
	Son La	1/1	0/1
	Nghe An	1/1	—
	Lang Son	1/1	0/6
	Tuyen Quang	1/1	—
	Vinh Phuc	0/1	0/1
	Thai Binh	0/1	—
	Unknown	0/1	—
	Ninh Binh	—	0/6
Subtotal	14 provinces	23/28 (82.1)	2/100 (2)
South and Highland	Gia Lai	0/3	3/3
	Lam Dong	—	1/1
	Ho Chi Minh	—	5/53
	Long An	—	1/4
	Soc Trang	—	2/3
	An Giang	—	1/1
	Dong Nai	—	1/4
	Tay Ninh	—	1/1
	Binh Duong	—	0/3
	Tra Vinh	—	0/1
	Tien Giang	—	0/1
	My Tho	—	0/1
Subtotal	12 provinces	0/3 (0)	15/76 (16.4)
Total		23/31 (74.2)	17/176 (9.7)

Table 2. Rabies virus strains used for the phylogenetic analysis isolated from rabies human and dogs, 2006–2009

Case	Location	History	Strain	Year of isolation	Genbank accession no.
Human	Ha Tay	Dog bite	H010607	2007	AB614379
			H040707	2007	AB614380
			H111007	2007	AB614385
			H240808	2008	AB614392
		Dog butchering	H020607	2007	AB628214
			H200608	2008	AB614390
			H280509	2009	AB614393
		Unknown	H140208	2008	AB614387
	Hoa Binh	Dog bite	H230808	2008	—
		Unknown	H170408	2008	AB614389
	Phu Tho	Dog bite	H060907	2007	AB628212
			H071007	2007	AB614382
			H210608	2008	AB614391
		Dog butchering	H091007	2007	AB614384
	Yen Bai	Dog butchering	H050707	2007	AB614381
	Tuyen Quang	Unknown	H150308	2008	AB614388
	Lang Son	Unknown	H130108	2008	AB614386
	Son La	Unknown	H080807	2007	AB614383
Dog	Gia Lai	Aggressive and human attacked	D156	2009	AB628211
			D157	2009	AB614377
			D158	2009	AB614378
	Ho Chi Minh	Aggressive and human attacked	D153	2007	AB614376
			D155	2007	—
	Lam Dong	Aggressive and human attacked	D154	2007	AB628213
	Soc Trang	Aggressive and human attacked	D150	2006	AB614373
	An Giang	Aggressive and human attacked	D151	2006	AB614374
	Long An	Aggressive and human attacked	D152	2007	AB614375
	Ha Tay	Collected from slaughterhouses	D010807	2007	AB614372
			D060807	2007	AB628210

circulating in Vietnam were classified into two major groups: Group 1 consisting of RABVs distributed in the northern, southern, and highland provinces and Group 2 consisting of the strains isolated in the northern provinces (Fig. 1).

Group 1 consisted of RABVs from Thailand, Malaysia, the Philippines, and China, as well as the 11 viruses isolated from humans in the northern provinces and all RABVs from dogs in the highland and southern provinces of Vietnam. Group 1 was further divided into 2 subgroups as shown in Fig. 1. Subgroup 1A consisted of RABVs from Thailand and Malaysia, and all RABVs isolated from dogs located in the southern provinces of Vietnam, and the strains of Ho Chi Minh City, as reported by Yamagata et al. (14). All highland virus strains clustered together (virus strains from D1560609VN to D1580609VN). Subgroup 1B consisted of RABVs from the Philippines and China as well as the 11 RABVs of human origin that were isolated from residents of the northern provinces. Interestingly, the RABV strain H130108VN was clustered with the strain SDJNCN01 from a rabid cow from China. The virus strain H130108VN isolated from a human in Lang Son province located at the Vietnam-China border.

Group 2 consisted of the strains of both human and canine origin isolated in the northern provinces of Vietnam as well as the strains from southern China.

Geographical distribution: The distribution of RABV strains circulating in Vietnam is shown in Fig. 2. The RABV strains in Group 1 were found in 11 of northern, southern, and highland provinces/cities. In contrast, the RABV strains in Group 2 were isolated exclusively in the northern provinces of Vietnam. The circulating of RABV strains belonging to both Group 1 and Group 2 were found in Ha Tay and Phu Tho provinces that experienced a major rabies epidemic (Fig. 2).

DISCUSSION

In Vietnam, the Ministry of Health has devised a system for human rabies surveillance. Thus far, this system has reported the annual data on the number of human deaths due to rabies on the basis of clinical diagnosis alone and not laboratory confirmation. To the best of our knowledge, this is the first report of human rabies confirmed by laboratory diagnosis.

According to the annual reports of the human rabies surveillance program in Vietnam in recent years, human rabies cases have mainly occurred in the northern and highland provinces, particularly in Ha Tay and Phu Tho

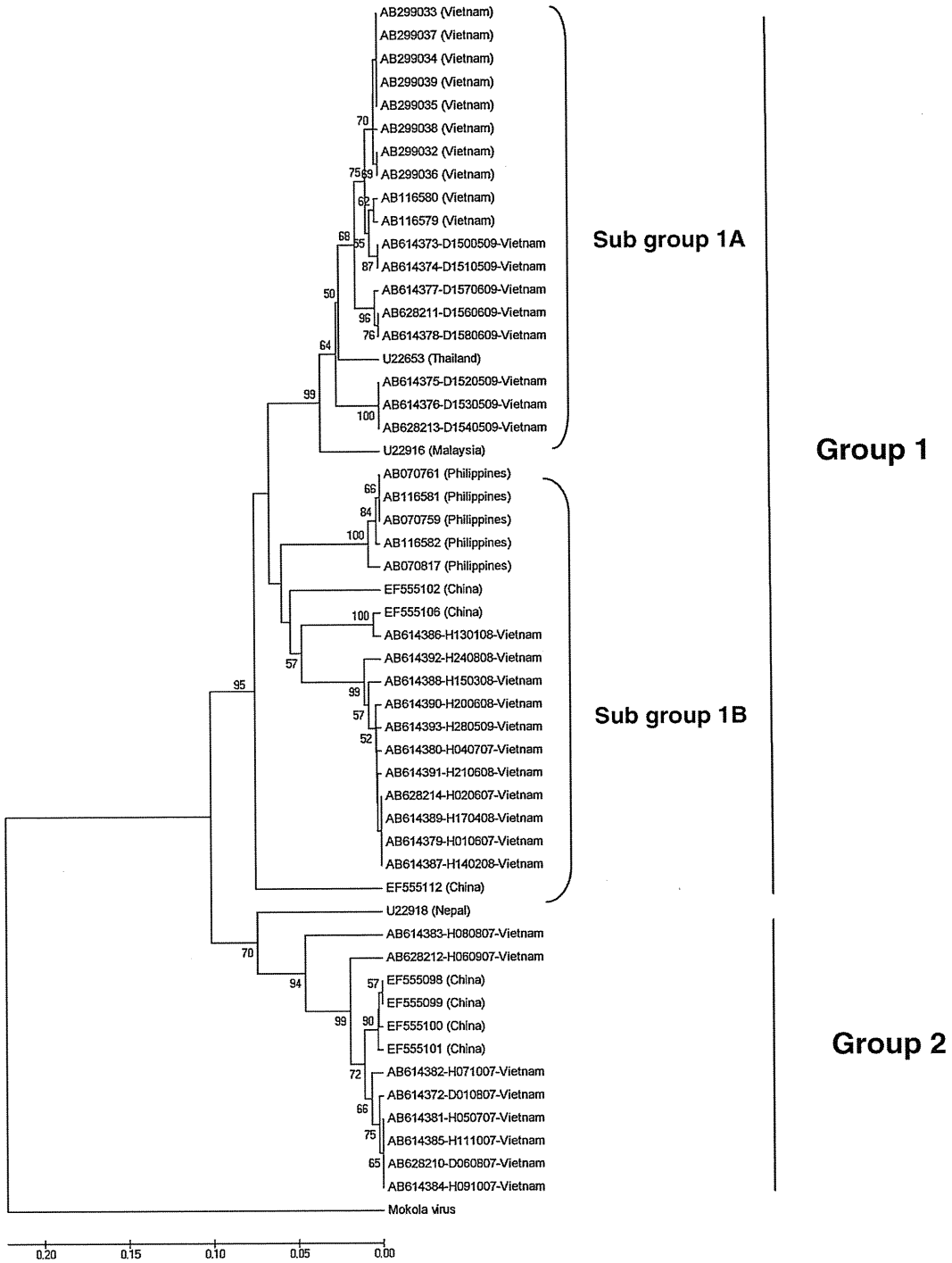

Fig. 1. Neighbor-joining phylogenetic tree based on partial N gene sequences (388 bp) of 27 RABV strains from humans and animals in Vietnam, 2006–2009. Partial nucleotide sequence of N gene (position from 85 to 473) was determined and compared with the representative sequences achieved in the GenBank (www.ncbi.nlm.nih.gov) described as the accession numbers (country). Mokola virus, AY333111 (Eth-16, Ethiopia), was used for an outgroup. Bootstrap values expressed as percentage of 1,000 replicates are shown at tree nodes.

provinces (4). From 2007 to 2009, RABV infection was confirmed in 23 of the 31 rabies suspected patients from 13 provinces. Out of these 23 patients with rabies, 10 were reported in Ha Tay province and 4 were reported in the Phu Tho province. The findings showed that 5 of the 23 rabies patients did not have any history of dog or cat bites, but they had an experience of butchering dogs or cats, or consuming their meat. RABV was detected in 2 of 72 (2.8%) dogs from the slaughterhouses in northern provinces.

Vietnamese communities commonly raise stray dogs, but the dogs are not vaccinated; vaccination coverage is only 10–20% (8). Furthermore, domestic and international transportation of animals is not well regulated in Vietnam. This lack of control has led to the spread of rabies from one region to others, and consequently, to the increase in the number of cases of human rabies. Therefore, strict measures for the control of rabies must