block viral replication at the assembly and/or release step still exists.

In contrast, our results using Huh-7 cells show that although FUT2 expression is a prerequisite for NV attachment and significantly enhances binding of wild-type virus as well as VLPs, it is not sufficient to lead to a productive infection and other factors are required. Although previous work had suggested that the block in viral replication in cell culture occurs at postbinding steps (20), our data rule out the presence of blocks at the level of RNA expression, replication, viral assembly, and progeny release.

Several possibilities can be hypothesized to explain why NV cannot spread to new cells: either cells lack some factor(s) required for viral entry and/or uncoating, or cellular antiviral responses inhibit viral replication. Based on data obtained using the NV replicon developed in Huh-7 cells, Chang et al. (7) suggested that innate immunity may have a role in the control of human norovirus replication, as reported for other animal members of the *Caliciviridae* family such as the murine norovirus (44) or porcine enteric calicivirus (6), since the NV replicon did not block the ability of Huh-7 cells to produce IFN in response to Sendai virus infection. In addition, expression of the NV replicon RNA was significantly reduced in the presence of exogenous IFN-α (7). Thus, it is possible that replication of wild-type NV RNA transfected into cells could activate the innate immune response, making other cells in the culture resistant to infection. However, whether a cellular innate immune response is activated in cells transfected with wild-type NV RNA or cells harboring the NV replicon remains to be determined.

A recent report describing a reverse genetics system for murine noroviruses using a recombinant fowlpox virus expressing T7 RNA polymerase (9) showed no differences in virus recovery when using Huh-7.5.1 cells or Vero cells, which are defective for IFN production, suggesting that the interferon system does not play a role in the restriction of virus recovery.

Our results show that NV replication is not enhanced in Huh-7.5.1 cells, which contain an inactivating mutation in RIG-I, suggesting that RIG-I pathway activation is not suppressing NV replication in Huh-7 cells. These results are not unexpected, since RIG-I mediates antiviral responses by recognition of single-stranded RNA bearing 5′ phosphates (18, 37), and it is believed that NV genomic and subgenomic RNAs are covalently linked to VPg. RIG-I and MDA-5 (melanoma differentiation-associated gene 5) are similar helicase proteins that induce type I IFN responses through the same signaling pathway, but RNA viruses are differentially recognized by RIG-I and MDA-5. While RIG-I is essential for the production of IFN in response to paramyxoviruses, influenza virus, and Japanese encephalitis virus, MDA-5 is critical for picornavirus detection (13, 26). Whether or not activation of cellular innate immune responses in NV RNA-transfected Huh-7 cells takes place through the MDA-5 signaling pathway will be a focus of our future studies.

If cellular antiviral responses do not account for the complete block of NV replication in cell culture, it is reasonable to think that cells may lack a cellular factor required for viral entry and/or uncoating. Besides interaction with H antigens, NV infection could require an interaction with a coreceptor that triggers internalization signals. Although our results cannot rule out the possibility that overexpression of FUT2 results in an expression level of carbohydrates sufficient to enhance binding of NV but not at the proper location on the cell surface or at the optimal density to allow successful viral entry, our observations lead us to predict that studies to identify other factors such as a coreceptor for internalization or a missing maturation/activation step that elicits uncoating are worthwhile. Understanding the molecular basis of the block(s) of NV infection in vitro should ultimately lead to the development of new cell culture systems to study NV replication and pathogenesis.

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REFERENCES

1. **Amano, J., and M. Oshima.** 1999. Expression of the H type 1 blood group antigen during enterocytic differentiation of Caco-2 cells. J. Biol. Chem. **274:**21209–21216.
2. **Asanaka, M., R. L. Atmar, V. Ruvolo, S. E. Crawford, F. H. Neill, and M. K. Estes.** 2005. Replication and packaging of Norwalk virus RNA in cultured mammalian cells. Proc. Natl. Acad. Sci. USA **102:**10327–10332.
3. **Bertolotti-Ciarlet, A., L. J. White, R. Chen, B. V. Venkataram Prasad, and M. K. Estes.** 2002. Structural requirements for the assembly of Norwalk virus-like particles. J. Virol. **76:**4044–4055.
4. **Blight, K. J., J. A. McKeating, and C. M. Rice.** 2002. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J. Virol. **76:**13001–13014.
5. **Burroughs, J. N., and F. Brown.** 1978. Presence of a covalently linked protein on calicivirus RNA. J. Gen. Virol. **41:**443–446.
6. **Chang, K. O., S. V. Sosnovtsev, G. Belliot, Y. Kim, L. J. Saif, and K. Y. Green.** 2004. Bile acids are essential for porcine enteric calicivirus replication in association with down-regulation of signal transducer and activator of transcription 1. Proc. Natl. Acad. Sci. USA **101:**8733–8738.
7. **Chang, K. O., S. V. Sosnovtsev, G. Belliot, A. D. King, and K. Y. Green.** 2006. Stable expression of a Norwalk virus RNA replicon in a human hepatoma cell line. Virology **353:**463–473.
8. **Chaudhry, Y., A. Nayak, M. E. Bordeleau, J. Tanaka, J. Pelletier, G. J. Belsham, I. O. Roberts, and I. G. Goodfellow.** 2006. Caliciviruses differ in their functional requirements for eIF4F components. J. Biol. Chem. **281:**25315–25325.
9. **Chaudhry, Y., M. A. Skinner, and I. G. Goodfellow.** 2007. Recovery of genetically defined murine norovirus in tissue culture by using a fowlpox virus expressing T7 RNA polymerase. J. Gen. Virol. **88:**2091–2100.
10. **Cheetham, S., M. Souza, T. Meulia, S. Grimes, M. G. Han, and L. J. Saif.** 2006. Pathogenesis of a genogroup II human norovirus in gnotobiotic pigs. J. Virol. **80:**10372–10381.
11. **Freymuth, F., A. Vabret, F. Rozenberg, J. Dina, J. Petitjean, S. Gouarin, L. Legrand, S. Corbet, J. Brouard, and P. Lebon.** 2005. Replication of respiratory viruses, particularly influenza virus, rhinovirus, and coronavirus in HuH7 hepatocarcinoma cell line. J. Med. Virol. **77:**295–301.
12. **Fuerst, T. R., and B. Moss.** 1989. Structure and stability of mRNA synthesized by vaccinia virus-encoded bacteriophage T7 RNA polymerase in mammalian cells: Importance of the 5′ untranslated leader. J. Mol. Biol. **2:**333–348.
13. **Gitlin, L., W. Barchet, S. Gilfillan, M. Cella, B. Beutler, R. A. Flavell, M. S. Diamond, and M. Colonna.** 2006. Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. Proc. Natl. Acad. Sci. USA **103:**8459–8464.
14. **Glass, P. J., L. J. White, J. M. Ball, I. LePare-Goffart, M. E. Hardy, and M. K. Estes.** 2000. Norwalk virus open reading frame 3 encodes a minor structural protein. J. Virol. **74:**6581–6591.
15. **Goodfellow, I., Y. Chaudhry, I. Gioldasi, A. Gerondopoulos, A. Natoni, L. Labrie, J. F. Laliberte, and L. Roberts.** 2005. Calicivirus translation initiation requires an interaction between VPg and eIF4E. EMBO Rep. **6:**968–972.
16. **Hardy, M. E., T. N. Tanaka, N. Kitamoto, L. J. White, J. M. Ball, X. Jiang, and M. K. Estes.** 1996. Antigenic mapping of the recombinant Norwalk virus capsid protein using monoclonal antibodies. Virology **217:**252–261.

17. **Herbert, T. P., I. Brierley, and T. D. Brown.** 1997. Identification of a protein linked to the genomic and subgenomic mRNAs of feline calicivirus and its role in translation. J. Gen. Virol. **78:**1033–1040.
18. **Hornung, V., J. Ellegast, S. Kim, K. Brzozka, A. Jung, H. Kato, H. Poeck, S. Akira, K. K. Conzelmann, M. Schlee, S. Endres, and G. Hartmann.** 2006. 5′-triphosphate RNA is the ligand for RIG-I. Science **314:**994–997.
19. **Hutson, A. M., F. Airaud, J. LePendu, M. K. Estes, and R. L. Atmar.** 2005. Norwalk virus infection associates with secretor status genotyped from sera. J. Med. Virol. **77:**116–120.
20. **Hutson, A. M., R. L. Atmar, and M. K. Estes.** 2004. Norovirus disease: changing epidemiology and host susceptibility factors. Trends Microbiol. **12:**279–287.
21. **Hutson, A. M., R. L. Atmar, D. M. Marcus, and M. K. Estes.** 2003. Norwalk virus-like particle hemagglutination by binding to H histo-blood group antigens. J. Virol. **77:**405–415.
22. **Jiang, X., D. Y. Graham, K. Wang, and M. K. Estes.** 1990. Norwalk virus genome cloning and characterization. Science **250:**1580–1583.
23. **Jiang, X., M. Wang, D. Y. Graham, and M. K. Estes.** 1992. Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein. J. Virol. **66:**6527–6532.
24. **Jiang, X., M. Wang, K. Wang, and M. K. Estes.** 1993. Sequence and genomic organization of Norwalk virus. Virology **195:**51–61.
25. **Katayama, K., G. S. Hansman, T. Oka, S. Ogawa, and N. Takeda.** 2006. Investigation of norovirus replication in a human cell line. Arch. Virol. **151:**1291–1308.
26. **Kato, H., O. Takeuchi, S. Sato, M. Yoneyama, M. Yamamoto, K. Matsui, S. Uematsu, A. Jung, T. Kawai, K. J. Ishii, O. Yamaguchi, K. Otsu, T. Tsujimura, C. S. Koh, C. Reis e Sousa, Y. Matsuura, T. Fujita, and S. Akira.** 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature **441:**101–105.
27. **Kelly, R. J., S. Rouquier, D. Giorgi, G. G. Lennon, and J. B. Lowe.** 1995. Sequence and expression of a candidate for the human secretor blood group α(1,2)fucosyltransferase gene (FUT2). Homozygosity for an enzyme-inactivating nonsense mutation commonly correlates with the non-secretor phenotype. J. Biol. Chem. **270:**4640–4649.
28. **Konduru, K., and G. G. Kaplan.** 2007. Stable growth of wild-type hepatitis A virus in cell culture. J. Virol. **80:**1352–1360.
29. **Lindenbach, B. D., M. J. Evans, A. J. Syder, B. Wolk, T. L. Tellinghuisen, C. C. Liu, T. Maruyama, R. O. Hynes, D. R. Burton, J. A. McKeating, and C. M. Rice.** 2005. Complete replication of hepatitis C virus in cell culture. Science **309:**623–626.
30. **Lindesmith, L., C. Moe, S. Marionneau, N. Ruvoen, X. Jiang, L. Lindblad, P. Stewart, J. LePendu, and R. Baric.** 2003. Human susceptibility and resistance to Norwalk virus infection. Nat. Med. **9:**548–553.
31. **Marionneau, S., F. Airaud, N. V. Bovin, J. LePendu, and N. Ruvoen-Clouet.** 2005. Influence of the combined ABO, FUT2, and FUT3 polymorphism on susceptibility to Norwalk virus attachment. J. Infect. Dis. **192:**1071–1077.
32. **Marionneau, S., N. Ruvoen, B. Le Moullac-Vaidye, M. Clement, A. Cailleau-Thomas, G. Ruiz-Palacios, P. Huang, X. Jiang, and J. LePendu.** 2002. Norwalk virus binds to histo-blood group antigens present on gastroduodenal epithelial cells of secretor individuals. Gastroenterology **122:**1967–1977.
33. **Monroe, S. S., S. E. Stine, L. Gorelkin, J. E. Herrmann, N. R. Blacklow, and R. I. Glass.** 1991. Temporal synthesis of proteins and RNAs during human astrovirus infection of cultured cells. J. Virol. **65:**641–648.
34. **Nakabayashi, H., K. Taketa, K. Miyano, T. Yamane, and J. Sato.** 1982. Growth of human hepatoma cell lines with differentiated functions in chemically defined medium. Cancer Res. **42:**3858–3863.
35. **Parker, T. D., N. Kitamoto, T. Tanaka, A. M. Hutson, and M. K. Estes.** 2005. Identification of genogroup I and genogroup II broadly reactive epitopes on the norovirus capsid. J. Virol. **79:**7402–7409.
36. **Parrino, T. A., D. S. Schreiber, J. S. Trier, A. Z. Kapikian, and N. R. Blacklow.** 1977. Clinical immunity in acute gastroenteritis caused by Norwalk agent. N. Engl. J. Med. **297:**86–89.
37. **Pichlmair, A., O. Schulz, C. P. Tan, T. I. Naslund, P. Liljestrom, F. Weber, and C. Reis e Sousa.** 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. Science **314:**997–1001.
38. **Schlesinger, S., and M. J. Schlesinger.** 2001. *Togaviridae*: the viruses and their replication, p. 895–916. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology, 4th ed. Lippincott, Williams and Wilkins, Philadelphia, PA.
39. **Sosnovtsev, S., and K. Y. Green.** 1995. RNA transcripts derived from a cloned full-length copy of the feline calicivirus genome do not require VpG for infectivity. Virology **210:**383–390.
40. **Straub, T. M., K. H. zu Bentrup, P. Orosz-Coghlan, A. Dohnalkova, B. K. Mayer, R. A. Bartholomew, C. O. Valdez, C. J. Bruckner-Lea, C. P. Gerba, M. Abbaszadegan, and C. A. Nickerson.** 2007. In vitro cell culture infectivity assay for human noroviruses. Emerg. Infect. Dis. **13:**396–403.
41. **Sumpter, R., Jr., Y.-M. Loo, E. Foy, K. Li, M. Yoneyama, T. Fujita, S. M. Lemon, and M. Gale, Jr.** 2005. Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. J. Virol. **79:**2689–2699.
42. **Wakita, T., T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H. G. Krausslich, M. Mizokami, R. Bartenschlager, and T. J. Liang.** 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. **11:**791–796.
43. **White, L. J., J. M. Ball, M. E. Hardy, T. N. Tanaka, N. Kitamoto, and M. K. Estes.** 1996. Attachment and entry of recombinant Norwalk virus capsids to cultured human and animal cell lines. J. Virol. **70:**6589–6597.
44. **Wobus, C. E., S. M. Karst, L. B. Thackray, K. O. Chang, S. V. Sosnovtsev, G. Belliot, A. Krug, J. M. Mackenzie, K. Y. Green, and H. W. Virgin.** 2004. Replication of Norovirus in cell culture reveals a tropism for dendritic cells and macrophages. PLoS Biol. **2:**e432.
45. **Yi, M., R. A. Villanueva, D. L. Thomas, T. Wakita, and S. M. Lemon.** 2006. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. Proc. Natl. Acad. Sci. USA **103:**2310–2315.
46. **Zhong, J., P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D. R. Burton, S. F. Wieland, S. L. Uprichard, T. Wakita, and F. V. Chisari.** 2005. Robust hepatitis C virus infection in vitro. Proc. Natl. Acad. Sci. USA **102:**9294–9299.

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A pilot study on intradermal vaccination of Japanese rabies vaccine for pre-exposure immunization

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ABSTRACT

Japanese rabies vaccine, a purified chick embryo cell vaccine manufactured by Kaketsuken (PCEC-K), is normally given subcutaneously; however, this requires a large amount of vaccine, and the pre-exposure vaccination regimen requires 6 months to complete. These factors often hamper appropriate vaccination. Therefore, we examined whether this vaccine could induce adequate level of viral neutralizing antibody (VNA) when vaccinated according to the World Health Organization (WHO) intradermal regimen. Our pilot study showed that this regimen resulted in all subjects developing adequate VNA levels. Intradermal route was effective not only for pre-exposure but also for booster vaccination. The intradermal PCEC-K regimen was found to be safe and effective in inducing adequate VNA levels with the use of a smaller quantity of vaccine and within a shorter period of time.

1. Introduction

Rabies is a fatal viral encephalitis caused by the rabies virus. The virus is transmitted by the saliva of infected domestic or wild animals. Dogs are the most important reservoir of rabies in endemic countries [1]. Although the incubation period of rabies is usually 1–3 months, it may be long and variable. Since there is no effective treatment for rabies, the mortality rate is 100% after the appearance of clinical symptoms. Post-exposure vaccination is the only way to prevent disease. Although pre-exposure vaccination is available, its use is generally limited to laboratory personnel working with rabies virus, veterinarians, and travellers visiting rabies endemic areas [3].

With regards to pre-exposure vaccination, the World Health Organization (WHO) recommends that the vaccine be administered as either 1.0 ml intramuscularly or 0.1 ml intradermally on days 0, 7, and 28 [4]. However, according to the officially approved recommendation in Japan, Japanese rabies vaccine, a purified chick embryo cell culture rabies vaccine manufactured by Kaketsuken (PCEC-K), is usually administered subcutaneously on days 0, 28, and 180. This schedule includes a long interval between each dose, which hinders proper vaccination for travellers visiting rabies endemic areas. However, decreasing the number of doses is not an option, since Arai et al. [5] reported that vaccination given on days 0 and 28 did not adequately increase the viral neutralizing antibody (VNA) titre in 24% of subjects. Only the PCEC-K vaccine is approved and available in Japan. Thus, there is a limited supply of the vaccine available. In 2006, after two imported cases of rabies were reported in Japan, there was a lack of rabies vaccine even for veterinarians, rabies researchers, and laboratory personnel [6]. Vaccine shortages could be averted if significant neutralizing antibody levels could be induced by administrating a smaller amount of Japanese rabies vaccine and by decreasing the time interval between each inoculation. Therefore, in the present study, the immunogenicity of intradermal vaccination of PCEC-K vaccine for pre-exposure immunization was investigated in this pilot study.

2. Materials and methods

2.1. Subjects

A total of 55 subjects were enrolled; of these, 20 were prospectively vaccinated PCEC-K vaccine intradermally, and the remaining 35 subjects were formerly vaccinated PCEC-K subcutaneously. Serum samples were collected from each subject. The number of doses received and the date of last immunization were recorded for each subject. During the study period, none of the subjects took chloroquine; none of the subjects were pregnant, had any drug hypersensitivities, or were immunosuppressed.

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Table 1
Schedule of pre-immunization

Group			Total dose (ml)	n	M/F	Age	Blood collection
A	Pre-exposure ID	0.1 ml (2 sites) on days 0, 7, 28	0.6	20	8/20	24.2 ± 3.7	days 0, 14, 28, 42, 56, 82 and 208
B	Pre-exposure SC	1.0 ml on days 0, 28, 180	3.0	35	20/15	35.1 ± 12.4	1–120 months (19.9 ± 28.3)

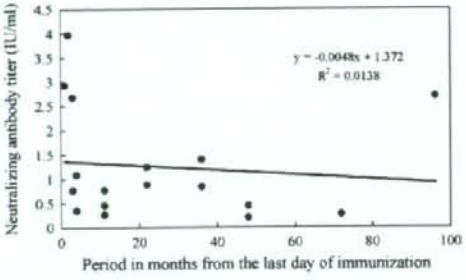

Fig. 1. Anti-rabies neutralizing antibody levels in serum samples obtained from 17 subjects who completed the recommended schedule and dose of PCEC-K vaccine. The y axis indicates the neutralizing antibody titre as measured using RFFIT, and the *x*-axis indicates the time period, in months, from the last day of immunization. There was no significant correlation between the neutralizing antibody level and the time period from the last day of immunization.

The effect of intradermal PCEC-K vaccine was assessed in 20 subjects (8 males, 12 females; age, 24.2 ± 3.7 years, range 21–33 years) (Group A). Each subject was vaccinated intradermally at two sites in the deltoid region using 0.1 ml of PCEC-K vaccine on days 0, 7 and 28, as per the schedule recommended by WHO (Table 1). To determine the effects of vaccination, serum samples were collected on days 0, 14, 28, 42, 56, 82, and 208.

The neutralizing antibody levels were also determined in 35 subjects (20 males, 15 females; age 35.1 ± 12.4 years old, range 20–72 years old) who were formerly vaccinated subcutaneously using 1.0 ml of PCEC-K vaccine (Group B). To determine the effects of vaccination, serum samples were collected from these subjects 19.9 ± 28.3 months (range, 1–120 months) from the last day of vaccination (Table 1). Among them, 10 subjects did not have adequate VNA levels, therefore, they were given one booster injection, either subcutaneously or intradermally. Five subjects (2 males, 3 females; age, 27.4 ± 7.6 years, range 22–40 years) received the booster subcutaneously; retrospective review showed that they received 1–3 doses of vaccine. Five females (age, 23.8 ± 2.5 years, range 22–28 years) received the booster injection intradermally at two sites. Retrospective review showed that they received only one dose of vaccine. To determine the effects of the booster, two serum samples were collected on days 14 and 28 after the booster vaccination.

Written informed consent was obtained from all subjects, and the study was approved by the Ethics Committee of Oita University.

2.2. Vaccine

PCEC-K vaccines manufactured by Kaketsuken (The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) were used in this study. These vaccines were inspected by the National Institute of Infectious Diseases (NIID), Japan. Lot No. RB02 was used for intradermal and booster vaccination. This lot's potency was measured by NIID and was found to be more than 2.5 international units (IU)/ml, which is the recommended accepted level by the WHO [5].

2.3. Neutralizing test

The VNA titre was determined using the rapid fluorescent focus inhibition test (RFFIT) [7]. All serum samples were inactivated by heating for 30 min at 56 °C. Each sample was two-fold diluted with Eagle medium containing 2% FCS in a 96-well plate. Challenge virus standard (CVS-11) was then added to each well and incubated in a 5% CO_2 incubator at 37 °C for 90 min. Subsequently, BHK-21 cells were added to each well, and the plates were incubated for a further 24 h. The specimens were fixed with acetone 90% and stained with FITC-labelled anti-rabies conjugate (FujiRebio, TFB) at 37 °C for 30 min. They were then examined using a fluorescence microscope. Each sample was measured in duplicate. The VNA titre was calculated by comparison with international standards [8]. A neutralizing antibody titre of more than 0.5 IU/ml was defined as adequate [9].

2.4. Statistical analysis

The Epi Info package for Windows was used for the statistical analysis. As far as the analysis of the effect of intradermal pre-exposure vaccine, it was assumed that neutralizing antibody acquired by intradermal vaccination would be 0% in non-vaccinated subjects and 80% in vaccinated subjects [10,11]. The sample size was calculated based on 0.8 power to detect a significant difference ($P < 0.05$, single-sided). Student's *t*-test for unpaired data was used to compare means. Associations between variables were examined using Pearson's product moment correlation coefficient. A P value of <0.05 was considered as significant.

3. Results

Firstly, the number of subjects, the total number of subcutaneous vaccinations, and the subjects' neutralizing antibody levels in Group B are shown in Table 2. The number of vaccinations varied;

Table 2
Serum neutralizing antibody levels after subcutaneous administration of PCEC-K vaccine

Inoculation number	1	2	3	4
Number of subjects	9	4	17	5
Neutralizing antibody				
GMTs (IU/ml)	0.19	0.27	0.93	1.90
Range (IU/ml)	0.16–0.24	0.18–0.44	0.27–3.93	0.29–9.00
$n \geq 0.5$ IU/ml	0/9	0/4	11/17	4/5
Seroconversion (%)	0	0	65	80

GMTs: geometric mean titre, range: lowest–highest vale. Subjects were considered as seroconverted when antibody titre was higher than 0.5 IU/ml.

Table 3
Serum neutralizing antibody levels after intradermal administration of PCEC-K vaccine

Day	0	14	28	42	56	84	208
Number of subjects	20	11	20	20	19	16	10
Neutralizing antibody							
GMTs (IU/ml)	0.13	2.34	2.21	4.46	2.49	1.71	0.60
Range	0.07–0.21	1.14–5.89	0.40–8.70	0.59–27.30	0.50–19.02	0.60–21.65	0.19–7.03
$n \geq 0.5$ IU/ml	0/20	11/11	18/20	20/20	19/19	16/16	5/10
Seroconversion (%)	0	100	90	100	100	100	50

GMTs: geometric mean titre, range: lowest–highest value subjects were considered seroconverted when the antibody titre was higher than 0.5 IU/ml.

most subjects received three times, and a few received four times. Subjects who received one or two vaccine doses did not develop adequate neutralizing antibody levels. Among the 17 subjects who received three vaccine doses, 11 (65.0%) had significant neutralizing antibody levels, but 6 (35.0%) did not. 4 (80.0%) of the 5 subjects who were given 4 doses of vaccine had significant neutralizing antibody levels.

To determine whether the time interval from the last dose of vaccination affected the neutralizing antibody titre, the correlation between the time from last immunization and the serum neutralizing antibody level was determined (Fig. 1); however, there was no significant correlation (correlation coefficient −0.11, $P = 0.65$). In 3 subjects, the neutralizing antibody level was not high despite a short-time interval from the last immunization.

The effects of intradermal PCEC-K vaccination in Group A are shown in Table 3. The neutralizing antibody level was increased significantly on day 14 (GMT 2.34 IU/ml). On day 28, only 2 (10%) of the 20 subjects had approximately 0.40 IU/ml of neutralizing antibody, which is less than the accepted level of neutralizing antibody (0.5 IU/ml). After the third vaccination, all subjects had adequate neutralizing antibody levels (GMT 4.46 IU/ml) in the serum obtained on day 42. On day 56, although the neutralizing antibody levels tended to decrease to GMT 2.49 IU/ml, all of the subjects still had adequate neutralizing antibody levels (GMT 1.71 IU/ml), even on day 84. On day 208, the neutralizing antibody level was below 0.5 IU/ml in half of the subjects.

With respect to the adverse effects of vaccination, 8 subjects developed slight skin pigmentation at the inoculation site, which gradually disappeared without incident, and 2 subjects developed mild and transient pain or itching at the inoculation site.

In addition to primary vaccination, we have also evaluated the effect of intradermal booster vaccination. In all intradermal booster vaccine recipients, the neutralizing antibody level increased significantly both after 2 weeks (GMT 7.79 IU/ml), and, after 4 weeks (GMT 6.40 IU/ml). Of note, the neutralizing antibody level tended to be higher in intradermal booster vaccine recipients than in subcutaneous booster vaccine recipients (Table 4).

4. Discussion

In November 2006, 2 imported cases of rabies were reported in Japan. Previously rabies had been prevalent in Japan, but it was gradually eradicated by the early 20th century after the introduction of compulsory registration and vaccination of dogs [2]. Therefore, the prevention of rabies re-emergence is a concern in rabies-free countries such as Japan, Australia, and New Zealand [12]. The number of Japanese travelling abroad is increasing each year. It is of concern that less than half of travellers receive post-exposure vaccination after a dog bite abroad [13]. These factors could contribute to the possible re-emergence of rabies in Japan if the virus were accidentally introduced to dogs.

The PCEC-K vaccine is prepared from an attenuated rabies strain (HEP-Flury) grown in primary cultures of chick embryo cells. Since 1980, PCEC-K has been the only rabies vaccine available for human use in Japan. For pre-exposure vaccination, PCEC-K is administered on days 0, 28, and 180; for post-exposure vaccination, it is given on days 0, 3, 7, 14, 28, and 90. Since the supply of rabies immunoglobulin (RIG) is scarce, and importing rabies vaccine from abroad is not permitted, PCEC-K is the only available means to control human rabies in Japan. Arai et al. reported that 3 doses of subcutaneous pre-exposure PCEC-K vaccination achieved adequate neutralizing antibody levels 1 month after the last inoculation [5]. In the present study, adequate neutralizing antibody levels were not detected in approximately 35% of the individuals who received 3 vaccine doses subcutaneously. In contrast to Arai's study, serum samples were not collected from all individuals 1 month after the last inoculation in the present study. However, the difference in the time interval between the last day of vaccination and the day of serum collection could not explain why adequate neutralizing antibody levels were not detected in the present study. It has been reported that the neutralizing antibody level elevation obtained using Japanese rabies vaccine (potency 3.6 IU/ml) was lower than that obtained with HDCV when these vaccines were administered with RIG [8]. However it has also been reported that there is no relationship between vaccine potency and the neutralizing antibody level over 2.5 IU/ml [14].

Table 4
Serum neutralizing antibody levels after PCEC-K booster vaccination by route of administration

Day after booster vaccination	0	14	28
Subcutaneous route (age 27.4 ± 7.6 years)			
GMTs (IU/ml)	0.13	4.12	2.53
Range (IU/ml)	0.03–0.30	0.80–9.99	0.38–19.00
$n \geq 0.5$ IU/ml	0/5	5/5	4/5
Seroconversion (%)	0	100	80
Intradermal route (age 23.8 ± 2.5 years)			
GMTs (IU/ml)	0.09	7.79	6.40
Range (IU/ml)	0.09–0.11	4.87–11.80	4.27–11.51
$n \geq 0.5$ IU/ml	0/5	5/5	5/5
Seroconversion (%)	0	100	100

GMTs: geometric mean titre, range: lowest–highest vale, subjects were considered as seroconverted, when antibody titre was higher than 0.5 IU/ml.

Since rabies vaccine is considered to be expensive in developing countries, the WHO recommends inoculating 0.1 ml of vaccine intradermally at two sites on days 0, 7, and 28 in order to reduce the amount of vaccine needed for immunization. In Japan, the recommended PCEC-K vaccination schedule differs from the WHO recommendation. With an increasing number of travellers going abroad, the possibility of rabies re-emergence in Japan might not be insignificant. Should rabies re-emerge, large quantities of the vaccine will be required. Another drawback of the Japanese rabies vaccination schedule is that half a year is required to complete vaccination. Therefore, use of the vaccine schedule recommended by the WHO is desirable for the PCEC-K vaccine in order to raise neutralizing antibody levels within a shorter period of time.

In the present study, the effect of the PCEC-K vaccine administered intradermally following the WHO-recommended schedule was assessed. All of the study subjects developed significant neutralizing antibody levels. One month after the final vaccination, the neutralizing antibody level tended to decrease, although it remained above the level required for protection (0.5 IU/ml) in all subjects. However, 6 months after immunization, the neutralizing antibody level decreased to less than 0.5 IU/ml in approximately half of the subjects; this is not a concern, since rabies vaccine recipients are considered to achieve significant neutralizing antibody levels following booster inoculation [15]. However, due to the modest sample size of the present study further study is needed to confirm the findings. In future, a large number of subjects are required to validate the WHO-recommended schedule for intradermal inoculation of PCEC-K vaccine. This regimen might also be appropriate when vaccine is in short supply. Furthermore, only mild adverse effects were noted when the vaccine was inoculated according to the WHO schedule in the present study.

Regarding booster vaccination, intradermal booster inoculation was better than subcutaneous booster inoculation in subjects who were initially given subcutaneous vaccination. Given the results of the present study with respect to booster vaccination, a smaller vaccine dose appears to be sufficient to induce adequate antibody levels in subjects who cannot complete the recommended PCEC-K vaccine schedule. A larger number of subjects might be required to further substantiate this finding.

In conclusion, the Japanese rabies vaccine was found to be highly effective when inoculated intradermally according to the schedule recommended by the WHO. Acceptable anti-rabies neutralizing antibody levels can be induced using smaller amounts of vaccine, and the vaccination schedule can be completed within a short period of time.

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References

[1] Steffen R, Banon A, deBernardis C. Vaccination priorities. Int J Antimicrob Agents 2003;21:175–80.
[2] Takayama N. Rabies control in Japan. Jpn J Infect Dis 2000;53(3):93–7.
[3] WHO Expert Committee on Rabies. Technical Report 931. Geneva, Switzerland: WHO; 2005.
[4] World Health Organization. WHO Recommendations on Rabies Post-Exposure Treatment and the Correct Technique of Intradermal Immunization Against Rabies. Geneva, Switzerland: WHO; 1997.
[5] Arai YT, Kimura M, Sakaue Y, Hamada A, Yamada K, Nakayama M, et al. Antibody responses induced by immunization with a Japanese rabies vaccine determined by neutralization test and enzyme-linked immunosorbent assay. Vaccine 2002;20:2448–53.
[6] Tamashiro H, Matibag GC, Ditangco RA, Kanda K, Ohbayashi Y. Revisiting rabies in Japan: Is there cause for alarm? Travel Med Infect Dis 2007;5(5):263–75.
[7] Smith JS, Yager PA, Baer GM. A rapid fluorescent focus inhibition test (RFFIT) for determining rabies virus-neutralizing antibody. In: Meslin FX, Kaplan MM, Koprowski H, editors. Laboratory techniques in rabies. 4th ed. WHO: Geneva; 1996. p. 181–92.
[8] Benjavongkulchai M, Kositprapa C, Limsuwun K, Khawplod P, Thipkong P, Chomchey P, et al. An immunogenicity and efficacy study of purified chick embryo cell culture rabies vaccine manufactured in Japan. Vaccine 1997;15:1816–9.
[9] WHO Expert Committee on Rabies. Eighth Report. World Health Organ Tech Rep Ser 1992; series 824.
[10] Lau C, Sisson J. The effectiveness of intradermal pre-exposure rabies vaccination in an Australian travel medicine clinic. J Travel Med 2002;9(6):285–8.
[11] Shaw MM, Leggat PA, Williams ML. Intradermal pre-exposure rabies immunization in New Zealand. Travel Med Infect Dis 2006;4(1):29–33.
[12] Infectious Agents Surveillance Report. Rabies as of 2006, Japan. March 2007. 28:3:3. [cited 29 April 2007. Available at URL:/http://idsc.nih.go.jp/iasr/28/325/tpc325.html5.
[13] Takayama N. Study on the subjects received post-exposure rabies vaccination in our vaccine clinic. Kansenshogaku Zasshi 1995;69(1):73–8 [in Japanese].
[14] Sudarshan MK, Mahendra BJ, Madhusudana SN, Narayana DH, Sanjay TV, Gangaboraiah, et al. Assessing the relationship between antigenicity and immunogenicity of human rabies vaccines. Human Vaccine 2005;1(5):187–90.
[15] Khawplod P, Wilde H, Benjavongkulchai M, Sriaroon C, Chomchey P. Immunogenicity study of abbreviated rabies preexposure vaccination schedules. J Travel Med 2007;14:173–6.

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EDITOR COMMUNICATED PAPER

A simple and rapid immunochromatographic test kit for rabies diagnosis

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List of Abbreviations: BHK-21, Baby hamster kidney-21; CAV2, canine adenovirus type 2; CCV, canine coronavirus; cDNA, complementary deoxyribonucleic acid; CDV, canine distemper virus; CHV, canine hepatitis virus; CPIV, canine parainfluenza virus; CPV, canine parvovirus; CVS, challenge virus standard; D-MEM, Dulbecco's modified Eagle's medium; dNTP, deoxyribonucleotide; ERA, Evelyn Rokitniki Abelseth; FAT, fluorescent antibody test; FBS, fetal bovine serum; FFU, focus forming unit; G, glycoprotein; HEP-Flury strain, high egg passage-Flury strain; ICT, immunochromatographic test; LD50, 50% lethal dose; M, matrix; MAb, monoclonal antibody; MEM, minimum essential medium; MMLV, Moloney murine leukemia virus; N, nucleoprotein; NASBA, nucleic acid sequence based amplification; OIE, World Organization for animal health; P, nucleocapsid-associated phosphoprotein; PBS, phosphate buffer saline; PET, post exposure treatment; RABV, rabies virus; RNA, ribonucleic acid; RNP, ribonucleoprotein complex; RT-PCR, reverse transcription and polymerase chain reaction; $TCID_{50}$, 50% tissue infectious dose; VSV, vesicular stomatitis virus; WHO, World Health Organization.

Key words
immunochromatographic test, rabies, rapid diagnosis.

ABSTRACT

In rabies endemic countries, funds and infrastructure are often insufficient to employ the approved gold standard for the definitive diagnosis of rabies: the direct fluorescent test. In the present study, two types (type 1 and 2) of an ICT kit were evaluated for detection of rabies. These were developed using monoclonal antibodies which recognize epitope II and III of the nucleoprotein of rabies virus. Both kits specifically detected all rabies virus strains and there was no cross reactivity with *Lyssaviruses* (Lagos, Mokola and Duvenhage), *Rhabdovirus* (VSV and Oita 296/1972) and other common canine-pathogenic viruses. In type 1, a single type of monoclonal antibody was used. It was capable of detecting recombinant nucleoprotein and showed sensitivity of 95.5% (42/44) and specificity of 88.9% (32/36) using brain samples from rabid dogs. In contrast, type 2 which was made of two different monoclonal antibodies had a lower sensitivity of 93.2% (41/44) and higher specificity of 100% (36/36). These ICT kits provide a simple and rapid method for rabies detection. They need neither cold chain for transportation nor complicated training for personnel. This diagnostic test is suitable for rabies screening, particularly in areas with a high prevalence of rabies and where the fluorescent antibody test is not available.

Rabies is a fatal enzootic disease caused by the RABV, genus *Lyssavirus* of the *Rhabdoviridae* family. RABV has a single, negative-stranded RNA genome and consists of five structural proteins: a nucleoprotein (N protein), a nucleocapsid-associated phosphoprotein (P protein), a matrix protein (M protein), a glycoprotein (G protein) and a polymerase (L protein) (1). The N gene is a highly conserved among *Lyssavirus* genotype 1 (RABV) and comparison of this gene has been employed in several epidemiological analyses. The envelope glycoprotein encoded by the G gene is responsible for receptor binding and viral invasion into host cells (2, 3). The glycoprotein is the main target for immune response and vaccine development (4–6).

RABV infects a wide range of mammalian species and causes fatal encephalitis. In Asia, the principal rabies reservoirs and/or transmitters are dogs, rather than wild animals such as foxes, bats, and raccoons. A recent estimate is that every year in Asia and Africa 55,000 people die of rabies (7). This is a modest estimate and the actual incidence of human rabies may be 100 times greater than officially recorded (7). The public health impact of rabies has been underestimated (8). Since rabies is fatal in all victims, diagnosis is often based on clinical manifestations only. However, definitive diagnosis of rabies can be obtained only by laboratory investigations, and can lead to the proper recommendation of PET using vaccine and globulin. Post-mortem diagnosis requires a FAT for detection of RABV in brain tissue. Definitive diagnosis of rabies infection using the "gold standard" of FAT as approved by both WHO and OIE is expensive and requires infrastructure and well-trained technicians (9, 10). Since many rabies endemic countries lack the above-mentioned requirements for the diagnosis of rabies, the exact number of rabies patients remains obscure. RT-PCR (11) and NASBA (12) methods have been successfully developed to detect rabies virus in tissue. However both of these techniques are out of reach in the areas of Asia and Africa where the virus is endemic.

Recently, we have developed a novel diagnostic test for RABV using immunochromatographic techniques. Our system can achieve rapid and sensitive detection for RABV using MAb which recognize the N protein of RABV. In this study we have shown the uniqueness of our rapid diagnostic test kit and evaluated its efficacy by comparing it with standard diagnostic techniques. The rapid diagnosis test is simple, time- and cost-saving. It can be used anywhere in the world and needs no special reagents or equipment. We anticipate this kit will be widely used in rabies-endemic areas and countries.

MATERIALS AND METHODS

Study samples

The diagnostic laboratories of the Queen Saovabha Memorial Institute (Thai Red Cross Society), National Institute of Health, the National Institute of Animal Health Laboratory and Chulalongkorn University serve as the main facilities for examining suspected rabid animal specimens in the central region of Thailand. The origin and the clinical history of each animal were recorded. Diagnoses were done using FAT on impression smears from hippocampus and brain stem. FAT-positive samples were stored at −80°C for further study.

Viruses and cells culture

Neuroblastoma NA cells of A/J mouse origin were grown at 37°C in MEM supplemented with 10% heat-inactivated FBS. BHK-21 cells were grown at 37°C in D-MEM supplemented with 10% FBS.

For RABV we used CVS-11, ERA, HEP-Flury and Nishigahara strains as laboratory strains of RABV plus the 1088 strain of street RABV (13–15). *Lyssaviruses* were represented by Lagos B-19, Mokola, and Duvenhage viruses which had previously been passaged in suckling mouse brain and stored in our laboratory. Similarly, vesicular stomatitis virus (VSV) and Oita 296/1972 virus isolated from horseshoe bat in Oita, Japan (16) were used to represent *Rhabdovirus*. Other viruses used in this study were as follows; canine adenovirus type 2 (CAV2), infectious canine hepatitis virus (CHV), canine parainfluenza virus (CPIV), canine coronavirus (CCV), canine parvovirus (CPV) and canine distemper virus (CDV).

Viral RNA extraction and RT-PCR

Total RNA was extracted from 0.2–0.5 g of brain specimen homogenates or cell-culture suspension using the acid-guanidinium thiocyanate phenol chloroform method (TRIzol; Gibco BRL, Gaithersburg, MD, USA). The N gene was amplified using a sense primer, designated as NF2850 (nt. 28–50, 5′-ACAGACAGCGTCAATGGCAGAGC-3′) and an anti-sense primer, designated as N660 (nt. 660–676, 5′-GTTTGGTATAGTACTCC-3′) (17). Primer position is indicated according to the N gene of CVS strain with the GenBank accession number DQ286762. One microgram of total RNA was reverse transcribed for synthesis of first strand cDNA using primer NF2850 and MMLV reverse transcriptase (Gibco BRL) at 37°C for 2 hr. First PCR reaction was done by using the NF2850/N660 primer set under the following conditions: briefly, 50-μl reaction

mixtures containing 5 μl of sample cDNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 20 μM each of the two primers, 200 μM dNTP, and 2 U of *Taq* polymerase (Promega Corp, Madison, WI, USA). The reaction mixture was subjected to 35 cycles of denaturation at 95°C for 0.5 min, annealing at 50°C for 0.5 min and extension at 72°C for 1.5 min. One tenth of the first PCR product was applied to the semi-nested PCR step. For this reaction, 10 g (nt. 66–82, 5′-CTACAATGGATGCCGAC-3′) was used for sense primer. The second PCR reaction was performed by using the 10 g/N660 primer set under the same conditions as the first PCR reaction except for annealing at 56°C. The amplicon was subjected to electrophoresis in 1% agarose gel and stained with ethidium bromide.

ICT kit

The test strip was constructed on the principles of immunochromatography using colloidal-gold-labeled MAb. We used two clones of MAb generated against the N protein of RABV. MAb10-41-F2 (18) and 87-3E2 recognize epitope III and II of the N protein respectively. Epitope mapping of these MAb was performed by competitive inhibition assay using MAb (kindly provided by Professor Minamoto, Gifu University) recognizing antigenic sites of N protein (19). One microgram/ml of anti-RABV N MAb 10-41-F2 was used for type 1 and MAb 87-3E2 for type 2. MAb was immobilized onto a nitrocellulose membrane for the test line zone and goat anti-mouse immunoglobulin antibody was immobilized for the control line zone to capture unbound MAb. A reagent pad containing colloidal gold-labeled MAb 10-41-F2 was located in front of the sample hole (Fig. 1a).

Brain tissue specimens were vigorously homogenized in mortar and 20% suspensions of the homogenate made in PBS and stored at –20°C until use. Before the test this homogenate was treated with an equal volume of B buffer containing 0.1% Triton-X, and 50 mM Tris-HCl, pH 7.0. Eighty μl of the treated sample was added to the sample hole of the test strip. Purified recombinant N protein solution or culture supernatant of virus was applied directly without any treatment. The final results were available within 15 min as signified by the appearance of a red-colored band at the test zone as well as at the control zone. When the colloidal gold is prepared to a certain size, it is visible as a red color after formation of antigen-antibody complex on the test strip (Fig. 1b).

Evaluation of ICT and determination of specificity and sensitivity of the kit

For the evaluation of ICT, five strains of RABV, three strains of *Lyssavirus*, two strains of *Rhabdovirus*, and six other viruses were examined. The sensitivity and specificity of ICT was determined by using 80 brain samples collected at Queen Saovabha Memorial Institute (Thai Red Cross Society) and comparing them with FAT as the reference method. Experimental infections or manipulation of RABV and *Lyssaviruses* were performed and stored in a Biosafety Level 3 facility in the Faculty of Medicine, Oita University, Japan.

RESULTS

Ability of ICT to detect RABV

Figure 1b shows a representative positive result of ICT after the appearance of a positive band in the test zone following the application of sample to the sample hole. First, we measured the detection limit of both kits using CVS-11 culture supernatant possessing an infectious titer of 4.0×10^7 FFU/ml ($=10^{4.28}$ $TCID_{50}/50\ \mu l$). Both kits detected approximately 10^{-3} fold diluted CVS-11 (4.0×10^3 FFU $= 10^{1.28}$ $TCID_{50}/50\mu l$). For practical use, brain homogenate material will be used for test samples. Therefore, the utility of ICT was determined using rabid-brain samples. Ten percent brain homogenate obtained from suckling mouse brain infected with strain 1088 was used and the infectious titer of this material was 1.0×10^7 FFU/ml ($=10^{4.23}$ $TCID_{50}/50\ \mu l$). Type 1 and type 2 could detect up to maximal 3000 times dilution of the original material (approximately 3.3×10^2 FFU $= 10^{0.75}$ $TCID_{50}/50\ \mu l$). Therefore, ICT showed a higher ability to detect RABV in brain homogenate than in culture supernatant. The detection limit of RT-PCR was $10^{-0.77}$ $TCID_{50}/50\ \mu l$ which is approximately 30-fold more sensitive than the ICT kit.

Our preliminary study revealed that MAb used in the present kit recognizes an epitope on the N protein of RABV which is a well-conserved amino acid sequence among RABV. The intensity of the test line of the kit is reduced depending on the serial dilution of the supernatant containing the virus. ICT was capable of detecting not only N protein obtained from cell culture or brain sample but also recombinant protein purified by column from *Escherichia coli* DH-5α transformed with pQE9-rNP harboring full length CVS N gene (20).

As shown in Table 1, we evaluated whether both kits could specifically detect laboratory strains and isolates of RABV (Fig. 1b), *Lyssavirus*, *Rhabdovirus*, and other viruses frequently infecting dogs. We found that both kits could specifically detect RABV and there was no cross reactivity with *Lyssaviruses* or *Rhabdoviruses*. Dogs suffering from

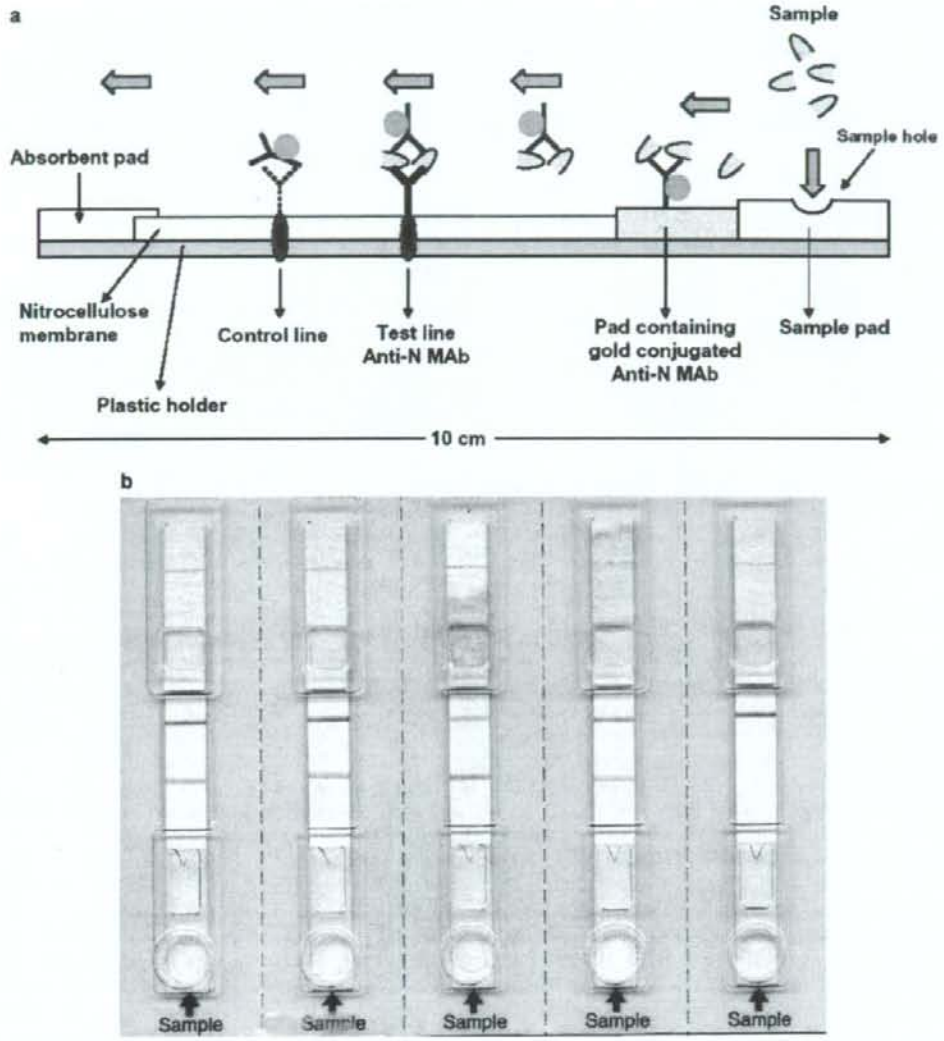

Fig. 1. (a) Diagram of rapid ICT strip for the detection of RABV. (b) ICT strip to detect RABV showing positive result. Positive bands were observed both in the test line and the control line. Lane 1: ERA, positive; lane 2: HEP-Flury, positive; lane 3: 1088, positive; lane 4: CVS-11, positive; lane 5: culture media, negative.

canine viral infections are often misdiagnosed as rabies. Our ICT showed negative reactions with CAV2, CHV, CPIV, CCV, CPV, and CDV.

During the preliminary analysis, both type 1 and type 2 showed false positive reactions with brain homogenate prepared only with PBS (data not shown). Sample treatment with B buffer reduced false positive reactions significantly in type 1 and eliminated them totally in type 2. Therefore, rabid dog brain samples treated by B buffer were used to evaluate the efficacy of ICT. The positive band on the test strip did not fade at room temperature for over a month.

Table 1 Ability of ICT to detect RABV, *Lyssavirus*, *Rhabdovirus* and other canine viruses

Virus		Origin	Result	
			Type 1	Type 2
RABV	CVS-11	culture sup	+	+
	ERA	culture sup	+	+
	HEP-Flury	culture sup	+	+
	Nishigahara	culture sup	+	+
	1088	SMB	+	+
Lyssavirus	Lagos B-19	SMB	–	–
	Mokola	SMB	–	–
	Duvenhage	SMB	–	–
Rhabdovirus	VSV	culture sup	–	–
	Oita-296/1972	SMB	–	–
Other viruses	CAV2	culture sup	–	–
	CHV	culture sup	–	–
	CPIV	culture sup	–	–
	CCV	culture sup	–	–
	CPV	culture sup	–	–
	CDV	culture sup	–	–

SMB: 10% suckling mouse brain

Efficacy of kits for clinical samples

To evaluate the efficacy of the kits for the diagnosis of RABV infections, eighty brain samples obtained from domestic dogs were tested. All tested samples were collected and stored for further analysis at the Queen Saovabha Memorial Institute (Thai Red Cross Society), National Institute of Health and the National Institute of Animal Health Laboratory of Thailand. Forty-four of the 80 samples were from rabid cases and the remaining 36 samples were from non-rabid cases as determined by FAT. Of the 44-rabies-positive samples, 42 cases tested positive and two negative using type 1. Of the 36 rabies-negative samples, four cases showed false positive results using type 1 (Table 2) in the form of very faint bands which could not easily be discriminated as negative results. Another two samples, which initially showed negative results using samples obtained from the hippocampus, showed positive results after repeated testing using samples from the brain stem. Furthermore, a few cases did not show positive results using 10% brain homogenate suspension but revealed positive results when 15–20% homogenate was used (data not shown). In terms of type 2, three cases of 44 rabies-positive samples tested negative but all rabies-negative samples showed negative results. Finally, with FAT as the reference method, results of tests on brain samples collected from the field indicated that the sensitivity and specificity of type 1 were 95.5% and 88.9% and that of type 2 were 93.2% and 100%, respectively.

Table 2 Concordance of ICT kit and FAT

	Type 1		Type 2	
	+	–	+	–
FAT +	42/44	2/44	41/44	3/44
FAT –	4*/36	32/36	0/36	36/36

*Very faint band, clearly identified as a negative

DISCUSSION

In the present study, we describe a simple and rapid diagnostic test for rabies infection based on the principle of immunochromatography on filter paper. The assay system was initially developed as a lateral-flow immunoassay for the detection of *Rotavirus* in fecal samples (21). Thereafter, this method has been widely accepted in various fields of laboratory diagnosis, including infectious diseases (22, 23).

Definitive *intra vitam* or postmortem diagnosis of rabies infection requires FAT detection of RABV in brain autopsy, skin biopsy, and corneal impression samples. FAT is approved by both WHO and OIE as the "gold standard" and available in several developing countries. FAT is a simple and reliable technique. Nevertheless, it requires expensive infrastructure and well-trained technicians, especially for postmortem diagnosis of rabid animals in endemic countries. More advanced techniques such as RT-PCR and NASBA have been successfully developed to detect RABV and approved for higher sensitivity. These are an alternative rapid way to confirm the diagnosis of rabies; however both methods need further skills and incur additional costs. More recently, a latex agglutination test (24) and direct rapid immunohistochemical test (25) have also been developed and evaluated using rabid samples. However these techniques are not yet widely accepted as an on-site diagnostic method in the areas of Asia and Africa where the virus is endemic. Since alternative detection systems for RABV such as RT-PCR and NASBA are based on the principal of gene amplification, they require several hours to obtain final results. Moreover, specific reagents and instruments are needed. PCR techniques are globally available for the diagnosis of various diseases; however socio-economical reasons limit the availability of these laboratory diagnostic methods in most rabies-endemic countries.

Surveillance data is the most important information for rabies evaluation, successful animal control and rabies elimination from endemic areas. The cost and need for well trained personnel are obstacles to the availability of FAT laboratories in such areas. Therefore a rapid, accurate and low-cost means of laboratory diagnosis is

both necessary and desirable in order to make an on-site diagnosis of suspected cases, especially for *intra vitam* diagnosis of rabid animals. The rabies infection status of suspected animals is crucial for a rapid decision on PET, which should be started as soon as possible in any rabies exposed cases, but preferably only on the basis of a definite diagnosis, as the supply of rabies tissue culture vaccine is limited.

The ICT kit can detect RABV by recognizing the N protein, which is the most abundant in the virion or in RABV-infected tissue. Interestingly, the test was capable of detecting N protein without any treatments, although N protein is an internal protein of the virion. Why is the internally located N protein detected by the test? One possibility is that the MAb used in the ICT might recognize N protein which has leaked from the destroyed virion or infected tissue. Electron microscopic observation revealed that RABV particles contain several damaged virions and/or virions with protruding N protein (data not shown). Leakage of N protein from damaged RABV-infected brain tissue might be detected by the ICT. Therefore, detergent in B buffer possibly exposes N protein in the infected tissue in addition to reducing the incidence of non-specific reactions.

Regarding the diagnosis of rabies for PET, false negatives have more serious consequences than false positives, because misdiagnosis might have fatal consequences in rabies-exposed individuals. False negative results could occur due to the sequence of viral infection and distribution in the early stages of infection. For detection of rabies, selection of the brain region from which to obtain samples is important. The brain stem and hippocampus provide best results. Similar to FAT, any doubtful or negative results need to be confirmed by animal history or other laboratory techniques, such as RT-PCR, NASBA, cell culture or mouse inoculation tests depending on the facilities available in the local laboratory.

Evaluation of the ICT kit for the diagnosis of rabies using saliva or cerebrospinal fluid from living animals requires further study. The possibility of using this kit as an *intra vitam* diagnostic test might help to avoid the unnecessary killing of dogs in endemic countries.

In conclusion, our diagnostic test can provide results within 15 min, and the interpretation of the results does not require expertise and expensive equipment. This test does not need cold chain for transportation. Thus the kit is suitable for screening and surveillance of a large number of rabies-suspected animals in laboratories with proper facilities for biohazard in endemic areas because of its simple, rapid, reliable, and cost-saving properties. It will provide helpful information for decisions on the need for PET.

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REFERENCES

1. Wunner W.H., Larson J.K., Dietzschold B., Smith C.L. (1988) The molecular biology of rabies viruses. *Rev Infect Dis* **10**(Suppl. 4): S771–84.
2. Thoulouze M.I., Lafage M., Schachner M., Hartman U., Cremer H., Lafon M. (1998) The neural cell adhesion molecule is a receptor for rabies virus. *J Virol* **72:** 7181–90.
3. Tuffereau C., Benejean J., Blondel D., Kieffer G., Flamand A. (1998) Low-affinity nerve-growth factor receptor (P75NTR) can serve as a receptor for rabies virus. *EMBO J* **17:** 7250–529.
4. Cox J.H., Dietzschold B., Schneider L.G. (1977) Rabies virus glycoprotein. II. Biological and serological characterization. *Infect Immun* **16:** 754–9.
5. Macfarlan R.I., Dietzschold B., Wiktor T.J., Keil M., Houghten R., Lerner R.A., Sutcliffe J.G., and Koprowski H. (1984) T cell responses to cleaved rabies virus glycoprotein and to synthetic peptides. *J. Immunol.* **133:** 2448–752.
6. Wiktor T.J., Gyorgy E., Schlumberger D., Sokol G.A., and Koprowski H. (1973) Antigenic properties of rabies virus components. *J. Immunol.* **110:** 269–76.
7. Knobel D.L., Cleaveland S., Coleman P.G., Fevere E.M. (2005) Re-evaluating the burden of rabies in Africa and Asia. *Bull World Health Organ* **83:** 1–11.
8. Coleman P.G., Fevre E.M., Cleaveland S. (2004) Estimating the public health impact of rabies. *Emerg Infect Dis* **10**(1): 140–142.
9. Bourhy H., Rollin P.E., Vincent J., Sureau P. (1989) Comparative field evaluation of the fluorescent-antibody test, virus isolation from tissue culture, and enzyme immunodiagnosis for rapid laboratory diagnosis of rabies. *J Clin Microbiol* **27**(3): 519–523.
10. Meslin F.X., ed. (1996) *Laboratory techniques in rabies*. 4th edn. Geneva: WHO.
11. Crepin P., Audry L., Rotivel Y., Gacoin A., Caroff C., Bourhy H. (1998) Intravitam diagnosis of human rabies by PCR using saliva and cerebrospinal fluid. *J Clin Microbiol* **36**(4): 1117–21.
12. Wacharapluesadee S., Hemachudha T. (2001) Nucleic-acid sequence based amplification in the rapid diagnosis of rabies. *Lancet* **358:** 892–3.
13. Hirai H., Kawano H., Mifune K., Fujii H., Nishizono A., Shichijo A., Mannen K. (1992) Suppression of cell-mediated immunity by street rabies virus infection. *Microbiol Immunol* **36**(12): 1277–90.

14. Mifune K., Takeuchi E., Napiorkowski P.A., Yamada A., Sakamoto K. (1981) Essential role of T cell in the postexposure prophylaxis of rabies in mice. *Microbiol Immunol* **25**(9): 895–904.
15. Smith A.L., Tignor G.H., Mifune K., Motohashi T. (1977) Isolation and assay of rabies serogroup viruses in CER cells. *Intervirology* **8**: 92–9.
16. Iwasaki T., Inoue S., Tanaka K., Sato Y., Morikawa S., Hayasaki D., Moriyama M., Ono T., Kanai S., Yamada A., Kurata T. (2004) Characterization of Oita virus296/1972 of *Rhabdoviridae* isolated from a horseshoe bat bearing characteristics of both lyssavirus and vesiculovirus. *Arch. Virol.* **149**: 1139–54.
17. Nishizono A., Mannen K., Elio-Villa L.P., Tanaka S., Li K.S., Mifune K., Arca B.F., Cabanban A., Martinez B., Rodriguez A., Atienza V.C., Camba R., Resontoc N. (2002) Genetic analysis of rabies virus isolates in the Philippines. *Microbiol Immunol* **46**: 413–7.
18. Takita-Sonoda Y., Fujii H., Mifune K., Ito Y., Hiraga M., Nishizono A., Mannen K., Minamoto N. (1993) Resistance of mice vaccinated with rabies virus internal structural proteins to lethal infection. *Arch Virol* **132**: 51–65.
19. Minamoto N., Tanaka H., Hishida M., Goto H., Ito H., Naruse S., Yamamoto K., Sugiyama M., Kinjo T., Mannen K., Mifune K. (1994) Linear and conformation-dependent antigenic sites on the nucleoprotein of rabies virus. *Microbiol Immunol* **38**(6): 449–55.
20. Inoue S., Sato Y., Hasegawa H., Noguchi A., Yamada A., Kurata T., Iwasaki T. (2003) Cross-reactive antigenicity of nucleoproteins of lyssaviruses recognized by a monospecific antirabies virus nucleoprotein antiserum on paraffin sections of formalin-fixed tissues. *Pathol Int* **53**: 525–33.
21. Al-Yousif Y., Anderson J., Chard-Bergstom C., Kapil S. (2002) Development, evaluation, and application of lateral-flow immunoassay (Immunochromatography) for detection of rotavirus in bovine fecal samples. *Clin Diag Lab Immunol* **9**(3): 723–4.
22. Bai G.R., Sakoda Y., Mweene A.S., Kishida N., Yamada T., Minakawa H., Kida H. (2005) Evaluation of the ESPLINE INFLUENZA A&B-N kit for the diagnosis of avian and swine influenza. *Microbiol Immunol* **49**(12): 1063–7.
23. Kuroiwa Y., Nagai K., Okita L., Ukae S., Mori T., Hotsubo T., Tsutsumi H. (2004) Comparison of an immunochromatography test with multiplex reverse transcription-PCR for rapid diagnosis of respiratory syncytial virus infections. *J Clin Microbiol* **42**(10): 4812–4.
24. Kasempimolporn S., Saengseesom W., Lumlertdacha B., Sitprija V. (2000) Detection of rabies virus antigen in dog saliva using a latex agglutination test. *J Clin Microbiol* **38**(8): 3098–9.
25. Lembo T., Niezgoda M., Velasco-Villa A., Cleaveland S., Ernest E., Rupprecht C.E. (2006) Evaluation of a direct, rapid immunohistochemical test for rabies diagnosis. *Emerg Infect Dis* **12**(2): 310–3.

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Amino acid at position 95 of the matrix protein is a cytopathic determinant of rabies virus

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ABSTRACT

The molecular mechanism involved in cytopathogenicity of rabies virus has not been fully elucidated yet. A fixed rabies virus Nishigahara strain does not induce clear cytopathic effect (CPE) in mouse neuroblastoma (NA) cells, whereas Ni-CE strain, which was established after 100 passages of Nishigahara strain in chicken embryo fibroblast cells, induces CPE that is characterized by rounding, shrinkage and detachment of the cells. In this study, to identify which viral gene is associated with the CPE of Ni-CE strain, we analyzed chimeric viruses between Nishigahara and Ni-CE strains generated by reverse genetics systems of both strains. We showed that the matrix gene of Ni-CE strain is responsible for the CPE in NA cells. It was also demonstrated by infection of Nishigahara and Ni-CE mutants with a single amino acid substitution in the matrix protein (M) that an amino acid at position 95 of M is a cytopathic determinant of the virus. We also demonstrated that the CPE is, at least partly, due to apoptosis. This is the first report of identification of an amino acid residue in a rabies virus protein that is important for the cytopathogenicity of the virus.

1. Introduction

Rabies virus, which belongs to the genus *Lyssavirus* of the family *Rhabdoviridae*, causes severe neurological disease and death in almost all mammals, including humans. The genome encodes five structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and large protein (L) in that order from the 3′ end to 5′ end of the genome. Among the viral proteins, the M and G form the viral envelope together with a lipid membrane derived from host cells. The M participates in virion formation (Mebatsion et al., 1999) and regulation of the viral genome and mRNA syntheses (Finke et al., 2003). The G is specifically responsible for binding to receptors on host cells (Lentz et al., 1984; Thoulouze et al., 1998; Tuffereau et al., 1998) and is also known as the major pathogenic determinant for adult mice (Dietzschold et al., 1983; Ito et al., 2001b; Seif et al., 1985; Tuffereau et al., 1989).

Accumulating evidence indicates that cytopathogenicity of rabies virus inversely correlates with the viral pathogenicity for mice. It is well known that an attenuated strain induces apoptosis more strongly in infected cells both in vitro and in vivo than does a virulent strain (Jackson et al., 2006; Morimoto et al., 1999; Prehaud et al., 2003). Detailed observations in previous studies have revealed that the G plays an important role in the induction of apoptosis: it has been shown that expression level of the G in attenuated strain-infected cells is higher than that in virulent strain-infected cells and that the high level of expression appears to concomitantly induce apoptosis in the infected cells (Morimoto et al., 1999; Prehaud et al., 2003; Thoulouze et al., 1997). This finding is supported by results obtained by Faber et al. (2002) showing that overexpression of the G by infection of a recombinant rabies virus carrying two G genes in the genome results in enhancement of apoptosis. Prehaud et al. (2003) reported that expression of the G from an attenuated strain induces apoptosis more strongly than that from a virulent strain in the absence of other viral components.

Some studies have shown that rabies virus M is also important for cytopathogenicity. Nakahara et al. (1999) reported that M expressing cells displayed typical morphology of degenerated cells within a few days and that a cell line stably expressing the M could not be established because of cytotoxicity. We have also tried to establish an M expressing cell line in order to propagate an M gene-deficient rabies virus, RC-HLΔM strain that had been generated as a rabies virus vaccine candidate (Ito et al., 2005). Using an inducible expression system, we successfully established M expressing cells. However, only a very small amount of M was expressed in the cells

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due to the cytopathogenicity of the protein, thereby resulting in less efficient propagation of RC-HLΔM strain.

Kassis et al. (2004) demonstrated that expression of M from a field isolate (street virus) of rabies virus induces apoptosis in the absence of other viral components, although the apoptosis was induced at a remarkably retarded manner compared to that induced by M from Mokola and Lagos bat viruses, which are other members of the genus *Lyssavirus*. It was speculated that the difference in the induction time of apoptosis explains the high pathogenicity of rabies virus and less pathogenicities of Mokola and Lagos bat viruses. As mentioned above, previous studies have shown that the G and M of rabies virus are responsible for the cytopathogenicity via induction of apoptosis. However, the amino acid residue in these proteins that works as the cytopathic determinant has not been identified yet. This hinders elucidation of the molecular mechanism by which rabies virus shows cytopathogenicity and understanding the basis of the viral pathogenicity in vivo.

We have previously established Ni-CE strain of rabies virus after 100 passages of a laboratory strain (fixed virus), Nishigahara strain, in chicken embryo fibroblast cells (unpublished data). In contrast to Nishigahara strain, which causes lethal infection in adult mice after intracerebral inoculation, the Ni-CE strain is highly attenuated, only causing non-lethal infection in mice (Shimizu et al., 2006). Another notable difference between the biological characters of the two strains is seen in their cytopathic effect (CPE)-inducing abilities in mouse neuloblastoma (NA) cells: Ni-CE strain induces a CPE in infected cells, characterized by rounding, shrinkage and detachment of the cells, whereas Nishigahara strain does not induce a clear CPE. In this study, to clarify the mechanism by which the two strains differ in CPE-inducing ability, we sought to identify which viral gene and amino acid in the gene product are associated with the CPE. For this purpose, by using a reverse genetics system of Nishigahara strain (Yamada et al., 2006), we generated a series of chimeric viruses by replacing each gene in the Nishigahara genome with the corresponding gene from Ni-CE strain. We inoculated these chimeric viruses into NA cells, together with Ni-CE-based chimeric viruses with each gene from Nishigahara strain generated previously (Shimizu et al., 2006). The infection experiments clearly indicated that the M gene of Ni-CE strain is responsible for the CPE in NA cells. We also demonstrated by infection experiments using Nishigahara and Ni-CE mutants with a single amino acid substitution in the M that an amino acid at position 95 of M is a cytopathic determinant of the virus. Furthermore, we showed that the CPE is, at least partly, due to induction of apoptosis.

2. Materials and methods

2.1. Cells and viruses

NA cells were maintained in Eagle's minimum essential medium supplemented with 10% fetal calf serum. Nishigahara and Ni-CE strains were previously generated by a reverse genetic system (Shimizu et al., 2006; Yamada et al., 2006). A series of chimeric viruses with each open reading frame (ORF) of the viral gene from Nishigahara strain in the Ni-CE genome, CE(NiN), CE(NiP), CE(NiM), CE(NiG) and CE(NiL) strains (Fig. 1), were recovered from the full-length genome plasmids previously (Shimizu et al., 2006). Stocks of all strains were prepared in NA cells.

2.2. Generation of chimeric and mutant rabies viruses

Full-length genome plasmids of chimeric viruses with each ORF from Ni-CE strain in the Nishigahara genome [Ni(CEN), Ni(CEP), Ni(CEM), Ni(CEG) and Ni(CEL) strains] (Fig. 1) were constructed by introducing cDNA fragments derived from Ni-CE strain into the genome plasmid of Nishigahara strain reported previously (Yamada et al., 2006) using conventional molecular cloning methods. We also constructed genome plasmids of Nishigahara and Ni-CE mutants carrying an amino acid substitution at position 29 or 95 in the M [Ni(M;29E,95V) and Ni(M;29D,95A), and CE(M;29D,95A) and CE(M;29E,95V)]. Detailed information of the construction of these plasmids is available from the authors on request. The chimeric viruses and the mutants were recovered from these genome plasmids using a reverse genetics system as reported previously (Ito et al., 2003). Briefly, three helper plasmids (pT7IRES-RN, -RP, and -RL) expressing rabies virus N, P and L under the control of T7 RNA polymerase were transfected to BHK/T7-9 cells, which stably express the polymerase, together with respective full-length genome plasmids using TransIT-LT1 reagent (Mirus Bio Corp., Madison, WI, USA). After incubation for 5–7 days, viruses in culture supernatants were collected. Stocks of recombinant viruses were prepared in NA cells. The authenticity of each gene of recovered viruses was confirmed by restriction endonuclease digestion and/or partial sequencing of RT-PCR fragments.

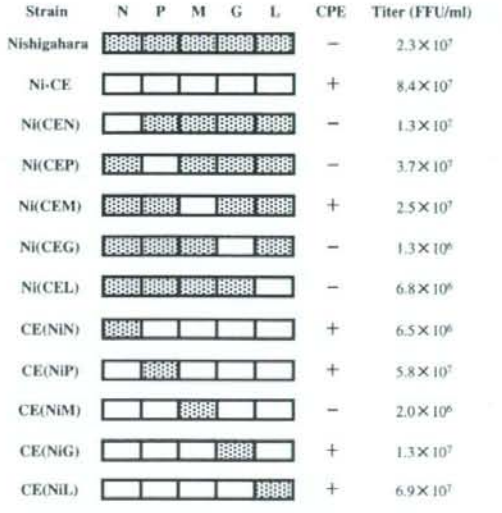

Fig. 1. CPE-inducing ability and growth of Nishigahara and Ni-CE strains and their chimeric strains in NA cells. Shaded and open boxes represent ORFs derived from Nishigahara and Ni-CE strains, respectively. The Nishigahara strain was recovered from cloned cDNA in our previous study (Yamada et al., 2006). The Ni-CE strain and Ni-CE-based chimeric viruses were also established previously (Shimizu et al., 2006). The CPE-inducing ability of each virus in NA cells at 3 days post-infection (dpi) is shown with a plus sign (+) or a minus sign (−). Virus titer of each virus in the supernatant of NA cells at 3 dpi is indicated on the right.

2.3. Observation of CPE caused by each virus

A monolayer culture of NA cells was infected with each virus at a multiplicity of infection (MOI) of 1. The cells were fixed at 4 days post-infection (dpi) with 2% paraformaldehyde for 60 min and 100% methanol for 2 min. Then the cells were stained by the IFA method with anti-N monoclonal antibody (MAb) 8-1 to identify the infected cells (Minamoto et al., 1994). The change in morphology of infected cells was examined with a Biozero fluorescent microscope (BZ-8000 series) (Keyence, Osaka, Japan).

2.4. Virus growth in cultured cell

A monolayer culture of NA cells in a 6-well plate was infected with each virus at an MOI of 0.01. Viruses in the culture supernatants were harvested at 3 dpi and titrated in NA cells by indirect fluorescent antibody (IFA) staining using the anti-N MAb stated above.

2.5. Observation of CPE caused by expression of M from plasmid

PCR-amplified cDNA fragments of the M gene from Nishigahara and Ni-CE strains and the mutants of each M gene were cloned into pcDNA3.1(+) (Invitrogen, Groningen, The Netherlands). Based on the amino acids at positions 29 and 95 in the M expressed from these plasmids, the resulting plasmids were designated as pcDNA-M(29D,95V), -M(29E,95A), -M(29D,95A) and -M(29E,95V). The M expressed from pcDNA-M(29D,95V) and -M(29E,95A) are identical to those of Nishigahara and Ni-CE strains, respectively, and pcDNA-M(29D,95A) and -M(29E,95V) express mutant M with a single amino acid substitution. These plasmids (0.5 μg/well) were transfected into NA cells grown in a 24-well tissue culture plate with TransIT-Neural reagent (Mirus Bio Corp., Madison, WI, USA). At 2 days post-transfection, the cells were fixed with 2% paraformaldehyde for 60 min and 100% methanol for 2 min and then stained by the IFA method with anti-M MAb 53-D-3-3 (kindly provided by Dr. K. Mifune). The change in morphology of the M expressing cells was examined with a Biozero fluorescent microscope.

2.6. Detection of apoptotic cells in NA cells infected with each virus

NA cells grown on an 8-well chamber slide were infected with each virus at an MOI of 1 and were fixed with 4% paraformaldehyde at 3 dpi. Apoptotic cells in the infected cells were detected by the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling (TUNEL) assay using an In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. The results of TUNEL assays were examined using a Biozero fluorescent microscope. We randomly chose four microscope fields and determined the ratio of the numbers of TUNEL-positive cells in total cell numbers in the four fields (more than 1200 cells). Significant difference tests were performed with the chi-square test for comparison of two proportions.

3. Results

3.1. Identification of the viral gene related to induction of CPE

To identify which viral gene is associated with the difference in CPE-inducing ability between Nishigahara and Ni-CE strains, we generated five Nishigahara-based chimeric viruses containing each ORF of the viral gene from Ni-CE strain in the Nishigahara genome (Fig. 1). Together with five Ni-CE-based chimeric viruses harboring one of the Nishigahara ORFs, which were previously established (Shimizu et al., 2006) (Fig. 1), we inoculated these viruses into NA cells at an MOI of 1 and checked whether a CPE is induced in the infected cells.

Ni-CE strain induces CPE in infected NA cells, which is characterized by rounding, shrinkage and detaching of the cells (Fig. 2B, left), whereas the parental Nishigahara strain does not induce a clear CPE (Fig. 2A, left). To determine infected cells, we also carried out the IFA staining with an anti-N MAb. Since fluorescent signals were not observed in mock-infected cells after IFA staining (Fig. 2G, right), it was confirmed that this method enables specific detection of rabies virus-infected cells (Fig. 2A–F, right). The CPE-inducing abilities of chimeric Ni(CEN) and CE(NiN) strains, which have N-ORF of Ni-CE strain in the Nishigahara genome and that of Nishigahara strain in the Ni-CE genome, respectively, were indistinguishable from the abilities of their parental strains (Fig. 2C and D). Similarly, replacement of the single ORF of P, G and L genes with the respective ORF of another strain did not affect CPE-inducing abilities of the parental strains (data not shown). In contrast, Ni(CEM) strain, which has M-ORF of Ni-CE strain in the Nishigahara genome, induced a CPE differently from the parental Nishigahara strain (Fig. 2E). On the other hand, CE(NiM) strain, which has M-ORF of Nishigahara strain in the Ni-CE genome, lost CPE-inducing ability (Fig. 2F). However, fluorescent signals of N of CE(NiM) strain in infected cells (Fig. 2F, right) were slightly weaker than those of other strains (Fig. 2A–E, right), raising the possibility that less efficient growth of CE(NiM) strain in NA cells, but not the intrinsic property of M of Nishigahara strain, resulted in loss of the CPE-inducing ability. To examine this possibility, we titrated infectivity of each virus in the culture supernatant from infected NA cells at 3 dpi (Fig. 1). The titer of CE(NiM) strain (2.0×10^6 focus-forming units [FFU]/ml) was slightly lower than those of most other strains, ranging from 6.5×10^6 to 8.4×10^7 FFU/ml. Notably, Ni(CEG) strain with G-ORF of Ni-CE strain in the Nishigahara genome also grew less efficiently, the titer being 1.3×10^6 FFU/ml. Although the influence of the less efficient growth of CE(NiM) strain needs to be considered further, it was apparent that Ni(CEM) strain acquired strong CPE-inducing activity. This suggested that the difference between CPE-inducing abilities of the two strains was determined by the M gene.

3.2. Identification of the amino acid in M that is related to induction of CPE

Next, we determined which amino acid in M is responsible for the CPE. Since only two amino acid differences were found in the M of the two strains at position 29 (Asp to Glu, indicated as a mutation from Nishigahara strain to Ni-CE strain) and position 95 (Val to Ala) (Shimizu et al., 2006) (Fig. 3A), we generated Nishigahara and Ni-CE mutants with a single amino acid substitution at both positions and checked their CPE-inducing abilities. CPE-inducing abilities of Nishigahara and Ni-CE mutants with a mutation at position 29 [Ni(M;29E,95V) and CE(M;29D,95A)] were not distinguishable from those of the respective parental viruses (Fig. 3B-1 and -3). In contrast, interestingly, replacement of the amino acid (Val to Ala or Ala to Val) at position 95 drastically affected the abilities of the parental strains: the Nishigahara mutant with Ala at position 95, Ni(M;29D,95A) strain, induced a clear CPE (Fig. 3B-2), whereas the Ni-CE mutant with Val at the same position, CE(M;29E,95V), did not induce a CPE (Fig. 3B-4). These findings suggested that Ala at position 95 is important for the CPE by Ni-CE strain. Notably, similarly to CE(NiM) strain (Fig. 2F, right), slightly weak fluorescent signals of N were observed in CE(M;29E,95V)-infected cells (Fig. 3B-4, right). Also, the titer of CE(M;29E,95V) strain (6.2×10^6 FFU/ml) was slightly lower than those of other mutants ($7.5–7.7 \times 10^7$ FFU/ml) at 3 dpi (Fig. 3).

3.3. Induction of CPE by M expressed from plasmid

As mentioned above, there was a possibility that the less efficient growth of CE(NiM) and CE(M;29E,95V) strains in NA cells resulted in loss of their CPE-inducing abilities. To clarify this point, we constructed plasmids expressing M of Nishigahara and Ni-CE strains, pcDNA-M(29D,95V) and pcDNA-M(29E,95A), respectively. After transfection of these plasmids into NA cells, we checked

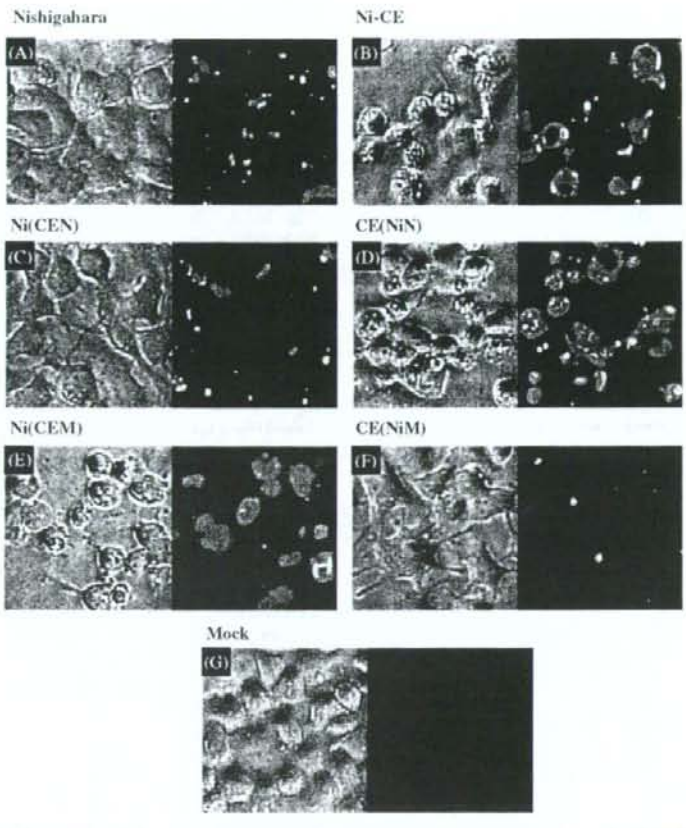

Fig. 2. CPE-inducing ability of Nishigahara strain, Ni-CE strain and their chimeric strains in NA cells. NA cells were infected with Nishigahara (A), Ni-CE (B), Ni(CEN) (C), CE(NiN) (D), Ni(CEN) (E), CE(NiM) (F) and mock (G) at an MOI of 1. At 4 dpi, NA cells were fixed and stained with anti-N MAb and were analyzed by phase-contrast (left) or fluorescence (right) microscopy with an (10×) objective. Representative phase-contrast and fluorescence images of the same field are shown in each strain.

whether the expressed M induced a CPE in the absence of other viral components. To identify the M expressing cells, IFA staining with an anti-M MAb was also performed at 2 days post-transfection. Since fluorescent signals were not observed in the empty vector-transfected cells after IFA staining (Fig. 4A, right), it was indicated that this method specifically stained M expressing cells (Fig. 4B–E, right). We found that expression of M of Nishigahara strain did not affect morphology of NA cells (Fig. 4B), whereas that of Ni-CE strain induced CPE characterized by rounding and shrinkage of cells (Fig. 4C). Notably, in contrast to NA cells expressing Nishigahara M (Fig. 4C, right), cells expressing Ni-CE M did not contain fluorescent signals of the protein in the dendritic processes (Fig. 4C, right), clearly showing morphological change of cells expressing Ni-CE M. This finding showed that viral proteins other than M are not required for CPE induction by Ni-CE strain. Next, we examined whether a single amino acid substitution at position 95 alters the CPE-inducing ability of M. When Ala at position 95 in Ni-CE M was replaced with Val, the mutant protein lost CPE-inducing ability (Fig. 4D). Replacement of Val at the same position in Nishigahara M with Ala conferred CPE-inducing ability to the protein (Fig. 4E). These results are consistent with results obtained from infection experiments using Nishigahara and Ni-CE strains (Fig. 2) and their mutants (Fig. 3). These findings clearly indicated that the loss of CPE-inducing ability of CE(NiM) and CE(M;29E,95V) strains was not due to their less efficient growth but due to the intrinsic property of M and that, consequently, the amino acid at position 95 in M of Ni-CE strain is important for the CPE.

3.4. Involvement of apoptosis in the induction of CPE

The cell rounding and shrinkage observed in the CPE-positive cells (Figs. 2, 3B and 4) are characteristic of apoptotic cells (Jackson and Rossiter, 1997; Kopecky and Lyles, 2003a). Also, the results of a previous study showing that rabies virus M expressed from a plasmid induces apoptosis (Kassis et al., 2004) are consistent with the results of the present study showing the involvement of the protein in the induction of a CPE. These facts prompted us to investigate whether the CPE induced by M from the Ni-CE strain is due to apoptosis or not. Therefore, we compared percentages of apoptotic cells in NA cells infected with Nishigahara and Ni-CE strains and the chimeric Ni(CEM) and CE(NiM) strains after applying the TUNEL assays (Fig. 5). While we detected 3.5% of TUNEL-positive cells in the mock-infected cells, 9.2% of the positive cells were observed in the cells infected with Nishigahara strain, indicating that even infection with Nishigahara strain results in some apoptosis in infected cells ($P < 0.01$). However, infection with Ni-CE strain more strongly induced apoptosis than did Nishigahara strain, as clearly shown by the higher percentage of TUNEL-positive cells (18.1%) in Ni-CE strain-infected cells ($P < 0.01$). Similar to the results from observation of the CPE (Fig. 2), interestingly, the percentage of apoptotic

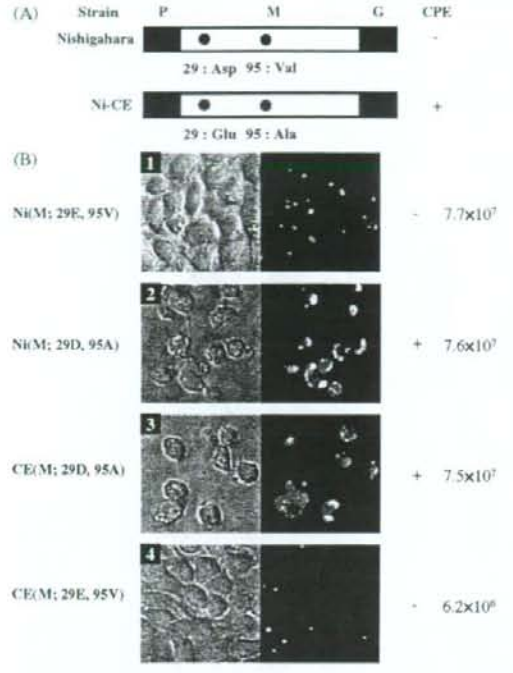

Fig. 3. CPE-inducing ability and productivity of the Nishigahara and Ni-CE mutants in NA cells. (A) Difference in amino acids at positions 29 and 95 in the M between the Nishigahara and Ni-CE strains. (B) Amino acids at positions 29 and 95 in M of these viruses are indicated in parentheses. NA cells were infected with the Nishigahara mutants, Ni(M;29E,95V) (1) and Ni(M;29D,95A) (2), and the Ni-CE mutants, CE(M;29D,95A) (3) and CE(M;29E,95V) (4), at an MOI of 1. At 4 dpi, NA cells were fixed and stained with an anti-N MAb and were analyzed by phase-contrast (left) or fluorescence (right) microscopy with an (10×) objective. Representative phase-contrast or fluorescence images were observed in the same fields in each strain. CPE-inducing ability of these mutants in NA cells at 3 dpi and virus titers in the supernatant of NA cells at 3 dpi are shown in the same manner as that in Fig. 3.

cells in the chimeric Ni(CEM) strain-infected cells (21.3%) was significantly higher than that in Nishigahara strain-infected cells. Also, the percentage in the CE(NiM) strain-infected cells (6.0%) was significantly lower than that in Ni-CE strain-infected cells. These data clearly indicated that the M gene determines not only the CPE-inducing ability but also the apoptosis-inducing ability of the virus and that the CPE is, at least partly, due to apoptosis. To further confirm this finding, we also compared percentages of chromatin concentration-positive cells in NA cells infected with these strains after nuclear staining with Hoechst 33342 and obtained very similar results (data not shown).

Next, we examined the contribution of the amino acid at position 95 in M to the induction of apoptosis. The percentage of TUNEL-positive cells in NA cells infected with Ni(M;29D,95A) strain (15.8%) was significantly higher than that in the cells infected with Nishigahara strain. Also, the percentage of TUNEL-positive cells in the cells infected with CE(M;29E,95V) strain (11.9%) was significantly lower than that in the cells infected with Ni-CE strain. On the other hand, there was no statistically significant difference between the percentages of TUNEL-positive cells in Nishigahara and Ni(M;29E,95V) strain-infected cells and also between those in Ni-CE and CE(M;29D,95A) strain-infected cells. Thus, it was indicated that the amino acid at position 95 in M more strongly contributes to determination of the apoptosis-inducing ability than does the amino acid at position 29.

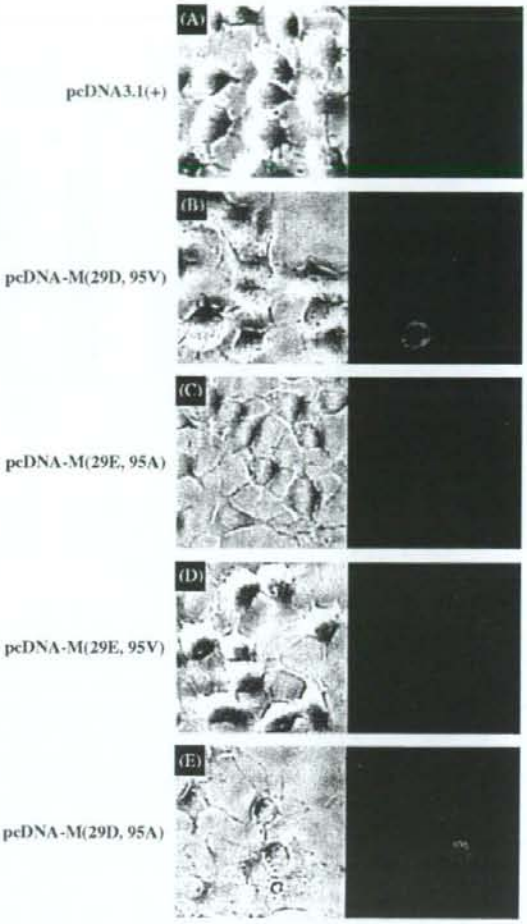

Fig. 4. CPE-inducing ability of M from Nishigahara, Ni-CE and their mutants in NA cells. NA cells were transfected with pcDNA3.1(+) (empty vector) (A), pcDNA-M(29D,95V) (B), -M(29E,95A) (C), -M(29E,95V) (D) and -M(29D,95A) (E). At 2 days post-transfection, NA cells were fixed and stained with an anti-M MAb and were analyzed by phase-contrast (left) or fluorescence (right) microscopy with an (10×) objective. Representative phase-contrast and fluorescence images of the same field are shown in each M.

4. Discussion

Previous studies have focused on apoptosis to explain cytopathogenicity of rabies virus and have demonstrated that the G and M play an important role in induction of apoptosis (Faber et al., 2002; Kassis et al., 2004; Morimoto et al., 1999; Prehaud et al., 2003). However, little is known about the molecular mechanism by which these viral proteins show cytopathogenicity. In this study, we clearly showed that an amino acid residue at position 95 in the M of rabies virus is important for the CPE and induction of apoptosis in NA cells. We determined for the first time the amino acid residue in the viral protein that is responsible for cytopathogenicity of rabies virus.

Kassis et al. (2004) reported that lyssavirus (street rabies virus, Mokola virus and Lagos bat virus) infection and sole expression of these M induced apoptosis in cultured cells. It was also shown that the apoptosis caused by expression of street rabies virus M was

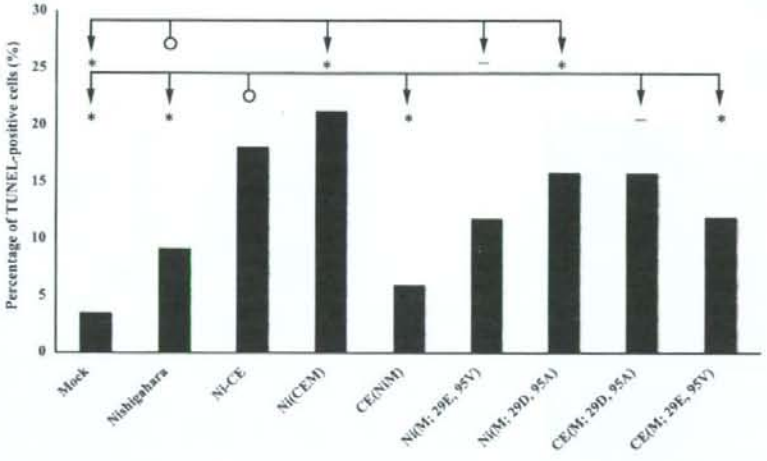

Fig. 5. Apoptosis-inducing ability of each virus in NA cells. NA cells were infected with each virus at an MOI of 1. Apoptotic cells in the infected cells were detected by TUNEL staining at 3 dpi. A total of more than 1200 TUNEL-positive and -negative cells were counted in four microscope fields randomly chosen, and the positive rates were determined. Significant difference tests (chi-square test) were performed between the rate of apoptotic cells of Nishigahara strain or Ni-CE strain (shown with open circles) and the rates of other strains (indicated with arrows). *: $P < 0.01$; –: no significant difference.

retarded compared to that caused by expression of M of Mokola and Lagos bat viruses. Based on this finding, it was speculated that this time difference accounts for the high pathogenicity of street rabies virus and less pathogenicities of the other viruses. However, Kassis et al. (2004) did not obtain direct evidence indicating a relationship between the apoptosis and the pathogenicity of the virus. Meanwhile, in the present study, CE(NiM) strain was found to have lost the ability to induce clear apoptosis in infected cells, in contrast to the parental Ni-CE strain, which causes cell death in infected cells (Fig. 5). Interestingly, we previously demonstrated that this CE(NiM) strain killed adult mice after intracerebral inoculation, whereas the Ni-CE strain caused only non-lethal infection in mice (Shimizu et al., 2006). These findings suggested that induction of apoptosis by expression of M, similarly to that of G (Faber et al., 2002; Morimoto et al., 1999; Prehaud et al., 2003), inversely correlates with the viral pathogenicity for mice.

As far as is known, the Ni-CE strain is the only strain that has an Ala residue at position 95 in the M. On the other hand, the parental Nishigahara strain contains a Val residue at the position (Ito et al., 2001a) as do other known rabies viruses (Conzelmann et al., 1990; Hiramatsu et al., 1993; Rayssiguier et al., 1986). Since the Ala and Val residues are classified as hydrophobic amino acids, the change is a conservative substitution, which is generally thought not to drastically affect protein structure. Nevertheless, the substitution affects the ability of M to induce a CPE and apoptosis in infected cells. Elucidation of the crystal structure of rabies virus M will be necessary to determine the reason. Interestingly, residues 89–107 of the M are known as a hydrophobic domain that is presumed to interact with a membrane lipid of host cells (Capone and Ghosh, 1984; Tordo et al., 1986). Although, the Ala and Val residues are the same hydrophobic amino acids, the hydrophobicity of the Val residue was stronger than that of the Ala residue. Therefore, the substitution might affect the hydrophobicity of the domain, thereby changing the biological character of M.

It was shown that both of the chimeric CE(NiM) and Ni(CEG) strains grew less efficiently in NA cells (Fig. 1), suggesting an incompatibility between M from Nishigahara strain and G from Ni-CE strain. Similarly, growth of an Ni-CE mutant, CE(M;29E,95V) strain, in the cells was impaired, implying that the amino acid residue at position 95 in M is responsible for the interaction with G as well as the cytopathogenicity (Fig. 3). We recently reported that there are only two amino acid substitutions in G between Nishigahara and Ni-CE strains: Tyr to Arg at position 50 and Ser to Ile at position 182 (Shimizu et al., 2006). Either or both of the substitutions in G are thought to participate in the interaction with M protein. How these residues in M and G determine the interaction between the two proteins needs to be elucidated in the future. Since little is known about the molecular mechanism of interaction between M and G, it would be interesting to further analyze these chimeric and mutant viruses to obtain new findings about the interaction.

Following the study by Kassis et al. (2004), this is only the second report on involvement of rabies virus M in cytopathogenicity. Thus, the cytopathic function of the M is still not understood well. In contrast, many studies have shown that M of vesicular stomatitis virus (VSV), which belongs the same family, *Rhabdoviridae*, as rabies virus, participates in the cytopathogenicity of the virus: expression of VSV M also results in induction of apoptosis (Gaddy and Lyles, 2005; Kopecky and Lyles, 2003b; Kopecky et al., 2001). On the other hand, the M is also known to contribute to depolymerization of cytoskeletal elements such as microtubles and/or intermediate filaments and the resulting cell rounding (Jayakar and Whitt, 2002; Kopecky and Lyles, 2003a; Lyles and McKenzie, 1997). Therefore, the CPE induced by rabies virus may not only be due to apoptosis but also disruption of cytoskeletal elements, if rabies virus M of Ni-CE strain has the same function.

Rabies virus M is also known to play a critical role in assembly and budding of the progeny virus (Mebatsion et al., 1999). Previously, we generated an M gene-deficient rabies virus, RC-HLΔM strain, as a rabies vaccine candidate with high levels of immunogenicity and safety, which infects cells and synthesizes viral proteins other than M but does not produce progeny virus in the infected cells (Ito et al., 2005). We established an M expressing cell line and found that the cells did not effectively support the growth of the RC-HLΔM strain, because the cytotoxicity of the M limited its own expression level in the cells (Ito et al., 2005). In the present study, we clearly showed that M of Nishigahara strain has low cytopathicity. Therefore, it is worth establishing a cell line expressing low cytopathic Nishigahara M for more efficient propagation of RC-HLΔM.

In this study, we showed that an Ala residue at position 95 in rabies virus M is the cytopathic determinant of the virus and that apoptosis is involved in the cytopathogenicity. To elucidate the molecular mechanism, the intracellular signaling pathway involved in the apoptosis needs to be clarified in the future. Previous studies have indicated that rabies virus induces apoptosis via both caspase-dependent and -independent pathways (Sarmento et al., 2006; Thoulouze et al., 2003). Thus, it would be interesting to examine whether apoptosis induced by Ni-CE strain and the chimeric and mutant viruses used in this study also results from activation of both pathways or not. Furthermore, identification of the host cell factor that interacts with M with Ala at position 95 is also necessary to elucidate the mechanism. We believe that elucidation of the mechanism for cytopathogenicity of rabies virus would be useful for a better understanding of the pathogenicity of the virus in vivo.

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References

Capone, J., Ghosh, H.P., 1984. Association of the nucleocapsid protein N of vesicular stomatitis virus with phospholipid vesicles containing the matrix protein M. Can. J. Biochem. Cell Biol. 62 (11), 1174–1180.

Conzelmann, K.K., Cox, J.H., Schneider, L.G., Thiel, H.J., 1990. Molecular cloning and complete nucleotide sequence of the attenuated rabies virus SAD B19. Virology 175 (2), 485–499.

Dietzschold, B., Wunner, W.H., Wiktor, T.J., Lopes, A.D., Lafon, M., Smith, C.L., Koprowski, H., 1983. Characterization of an antigenic determinant of the glycoprotein that correlates with pathogenicity of rabies virus. Proc. Natl. Acad. Sci. U.S.A. 80 (1), 70–74.

Faber, M., Pulmanausahakul, R., Hodawadekar, S.S., Spitsin, S., McGettigan, J.P., Schnell, M.J., Dietzschold, B., 2002. Overexpression of the rabies virus glycoprotein results in enhancement of apoptosis and antiviral immune response. J. Virol. 76 (7), 3374–3381.

Finke, S., Mueller-Waldeck, R., Conzelmann, K.K., 2003. Rabies virus matrix protein regulates the balance of virus transcription and replication. J. Gen. Virol. 84 (Pt 6), 1613–1621.

Gaddy, D.F., Lyles, D.S., 2005. Vesicular stomatitis viruses expressing wild-type or mutant M proteins activate apoptosis through distinct pathways. J. Virol. 79 (7), 4170–4179.

Hiramatsu, K., Mannen, K., Mifune, K., Nishizono, A., Takita-Sonoda, Y., 1993. Comparative sequence analysis of the M gene among rabies virus strains and its expression by recombinant vaccinia virus. Virus Genes 7 (1), 83–88.

Ito, N., Kakemizu, M., Ito, K.A., Yamamoto, A., Yoshida, Y., Sugiyama, M., Minamoto, N., 2001a. A comparison of complete genome sequences of the attenuated RC-HL strain of rabies virus used for production of animal vaccine in Japan, and the parental Nishigahara strain. Microbiol. Immunol. 45 (1), 51–58.

Ito, N., Takayama, M., Yamada, K., Sugiyama, M., Minamoto, N., 2001b. Rescue of rabies virus from cloned cDNA and identification of the pathogenicity-related gene: glycoprotein gene is associated with virulence for adult mice. J. Virol. 75 (19), 9121–9128.

Ito, N., Takayama-Ito, M., Yamada, K., Hosokawa, J., Sugiyama, M., Minamoto, N., 2003. Improved recovery of rabies virus from cloned cDNA using a vaccinia virus-free reverse genetics system. Microbiol. Immunol. 47 (8), 613–617.

Ito, N., Sugiyama, M., Yamada, K., Shimizu, K., Takayama-Ito, M., Hosokawa, J., Minamoto, N., 2005. Characterization of M gene-deficient rabies virus with advantages of effective immunization and safety as a vaccine strain. Microbiol. Immunol. 49 (11), 971–979.

Jackson, A.C., Rossiter, J.P., 1997. Apoptosis plays an important role in experimental rabies virus infection. J. Virol. 71 (7), 5603–5607.

Jackson, A.C., Rasalingam, P., Weli, S.C., 2006. Comparative pathogenesis of recombinant rabies vaccine strain SAD-L16 and SAD-D29 with replacement of Arg333 in the glycoprotein after peripheral inoculation of neonatal mice: less neurovirulent strain is a stronger inducer of neuronal apoptosis. Acta Neuropathol. (Berl) 111 (4), 372–378.

Jayakar, H.R., Whitt, M.A., 2002. Identification of two additional translation products from the matrix (M) gene that contribute to vesicular stomatitis virus cytopathology. J. Virol. 76 (16), 8011–8018.

Kassis, R., Larrous, F., Estaquier, J., Bourhy, H., 2004. Lyssavirus matrix protein induces apoptosis by a TRAIL-dependent mechanism involving caspase-8 activation. J. Virol. 78 (12), 6543–6555.

Kopecky, S.A., Willingham, M.C., Lyles, D.S., 2001. Matrix protein and another viral component contribute to induction of apoptosis in cells infected with vesicular stomatitis virus. J. Virol. 75 (24), 12169–12181.

Kopecky, S.A., Lyles, D.S., 2003a. The cell-rounding activity of the vesicular stomatitis virus matrix protein is due to the induction of cell death. J. Virol. 77 (9), 5524–5528.

Kopecky, S.A., Lyles, D.S., 2003b. Contrasting effects of matrix protein on apoptosis in HeLa and BHK cells infected with vesicular stomatitis virus are due to inhibition of host gene expression. J. Virol. 77 (8), 4658–4669.

Lentz, T.L., Wilson, P.T., Hawrot, E., Speicher, D.W., 1984. Amino acid sequence similarity between rabies virus glycoprotein and snake venom curaremimetic neurotoxins. Science 226 (4676), 847–848.

Lyles, D.S., McKenzie, M.O., 1997. Activity of vesicular stomatitis virus M protein mutants in cell rounding is correlated with the ability to inhibit host gene expression and is not correlated with virus assembly function. Virology 229 (1), 77–89.

Mebatsion, T., Weiland, F., Conzelmann, K.K., 1999. Matrix protein of rabies virus is responsible for the assembly and budding of bullet-shaped particles and interacts with the transmembrane spike glycoprotein G. J. Virol. 73 (1), 242–250.

Minamoto, N., Tanaka, H., Hishida, M., Goto, H., Ito, H., Naruse, S., Yamamoto, K., Sugiyama, M., Kinjo, T., Mannen, K., et al., 1994. Linear and conformation-dependent antigenic sites on the nucleoprotein of rabies virus. Microbiol. Immunol. 38 (6), 449–455.

Morimoto, K., Hooper, D.C., Spitsin, S., Koprowski, H., Dietzschold, B., 1999. Pathogenicity of different rabies virus variants inversely correlates with apoptosis and rabies virus glycoprotein expression in infected primary neuron cultures. J. Virol. 73 (1), 510–518.

Nakahara, K., Ohnuma, H., Sugita, S., Yasuoka, K., Nakahara, T., Tochikura, T.S., Kawai, A., 1999. Intracellular behavior of rabies virus matrix protein (M) is determined by the viral glycoprotein (G). Microbiol. Immunol. 43 (3), 259–270.

Prehaud, C., Lay, S., Dietzschold, B., Lafon, M., 2003. Glycoprotein of nonpathogenic rabies viruses is a key determinant of human cell apoptosis. J. Virol. 77 (19), 10537–10547.

Rayssiguier, C., Cioe, L., Withers, E., Wunner, W.H., Curtis, P.J., 1986. Cloning of rabies virus matrix protein mRNA and determination of its amino acid sequence. Virus Res. 5 (2–3), 177–190.

Sarmento, L., Tseggai, T., Dhingra, V., Fu, Z.F., 2006. Rabies virus-induced apoptosis involves caspase-dependent and caspase-independent pathways. Virus Res. 121 (2), 144–151.

Seif, I., Coulon, P., Rollin, P.E., Flamand, A., 1985. Rabies virulence: effect on pathogenicity and sequence characterization of rabies virus mutations affecting antigenic site III of the glycoprotein. J. Virol. 53 (3), 926–934.

Shimizu, K., Ito, N., Mita, T., Yamada, K., Hosokawa-Muto, J., Sugiyama, M., Minamoto, N., 2006. Involvement of nucleoprotein, phosphoprotein, and matrix protein genes of rabies virus in virulence for adult mice. Virus Res. 123 (2), 154–160.

Thoulouze, M.I., Lafage, M., Montano-Hirose, J.A., Lafon, M., 1997. Rabies virus infects mouse and human lymphocytes and induces apoptosis. J. Virol. 71 (10), 7372–7380.

Thoulouze, M.I., Lafage, M., Schachner, M., Hartmann, U., Cremer, H., Lafon, M., 1998. The neural cell adhesion molecule is a receptor for rabies virus. J. Virol. 72 (9), 7181–7190.

Thoulouze, M.I., Lafage, M., Yuste, V.J., Baloul, L., Edelman, L., Kroemer, G., Israel, N., Susin, S.A., Lafon, M., 2003. High level of Bcl-2 counteracts apoptosis mediated by a live rabies virus vaccine strain and induces long-term infection. Virology 314 (2), 549–561.

Tordo, N., Poch, O., Ermine, A., Keith, G., Rougeon, F., 1986. Walking along the rabies genome: is the large G-L intergenic region a remnant gene? Proc. Natl. Acad. Sci. U.S.A. 83 (11), 3914–3918.

Tuffereau, C., Leblois, H., Benejean, J., Coulon, P., Lafay, F., Flamand, A., 1989. Arginine or lysine in position 333 of ERA and CVS glycoprotein is necessary for rabies virulence in adult mice. Virology 172 (1), 206–212.

Tuffereau, C., Benejean, J., Blondel, D., Kieffer, B., Flamand, A., 1998. Low-affinity nerve-growth factor receptor (P75NTR) can serve as a receptor for rabies virus. EMBO J. 17 (24), 7250–7259.

Yamada, K., Ito, N., Takayama-Ito, M., Sugiyama, M., Minamoto, N., 2006. Multigenic relation to the attenuation of rabies virus. Microbiol. Immunol. 50 (1), 25–32.