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Short Communication

Region at amino acids 164 to 303 of the rabies virus glycoprotein plays an important role in pathogenicity for adult mice

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The authors have previously reported that the glycoprotein of the pathogenic Nishigahara strain of rabies virus is required to lethality for adult mice. A cluster region of amino acid substitutions exists at the positions 164 to 303 on the glycoprotein between avirulent and virulent strains. In this study, the authors generated a chimeric strain having the region at the positions 164 to 303 of the glycoprotein derived from the pathogenic Nishigahara strain in the genetic background of the avirulent RC-HL strain. The chimeric R(G 164–303) strain restores the lethality for adult mice. This result clearly shows that the region at the position 164 to 303 of glycoprotein plays an important role in the lethality for adult mice. Moreover, the authors observed that the lethality for adult mice correlated well with the viral growth in a brain but not with the pH-dependent fusion activity *in vitro*. *Journal of NeuroVirology* (2004) **10,** 131–135.

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Rabies virus belongs to the genus *Lyssavirus* of the family Rhabdoviridae and has an unsegmented negative-sense RNA as a genome. Reading from the 3′ to 5′ ends, the genome encodes the genes for a nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and polymerase (L) (Tordo *et al*, 1986). The G protein is composed of 505 amino acids and a signal peptide. The G protein is anchored to a lipid-bilayer envelope and participates in cell attachment, low pH-dependent membrane fusion, viral virulence, and induction of neutralizing antibodies.

Several previous studies have demonstrated that an arginine or lysine residue at position 333 of the G protein is necessary for lethality in adult mice. This phenomenon is common among representative laboratory strains of rabies virus, such as HEP-flury, CVS, and SAD B19 strains (Diallo, 1986; Dietzschold *et al*, 1983; Mebatsion, 2001; Morimoto *et al*, 2001; Seif *et al*, 1985; Tuffereau *et al*, 1989). However, the apathogenic RC-HL strain used as an inactivated vaccine for animals in Japan has same arginine residue at this position as that of the pathogenic parental Nishigahara strain, which is thought to have originated from the Pasteur strain of rabies virus (Goto *et al*, 1994; Ito *et al*, 1994). The RC-HL strain was derived from the Nishigahara strain after 330 passages in chicken embryos and cell cultures. The Nishigahara strain kills adult mice following intracerebral inoculation, whereas the RC-HL strain causes body weight reduction and piloerection but no lethal infection in adult mice.

Recently, we have rescued a recombinant RC-HL (rRC-HL) strain from cDNA of the RC-HL genome and have demonstrated that the G protein of the Nishigahara strain is associated with virulence of rabies virus in adult mice, by using the R(G) strain with the open reading frame of the G gene from the pathogenic Nishigahara strain in the background of the RC-HL genome (Ito *et al*, 2001b). This finding indicates that mutations at amino acid positions other than position 333 on the G protein are correlated with the difference between the pathogenicity of the RC-HL strain and that of the Nishigahara strain. It has been reported that amino acid substitutions in the region corresponding to antigenic site II (34 to 42, 198 to 200) also resulted in a reduction in pathogenicity for adult mice that had been intramuscularly

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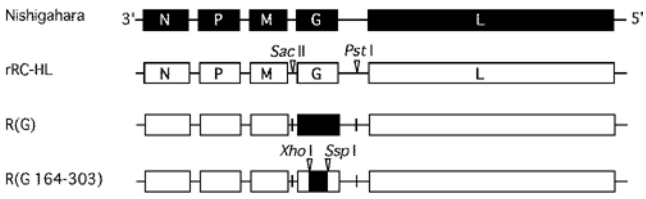

Figure 1 Schematic diagram of Nishigahara, rRC-HL, R(G), and R(G 164–303) strain genomes.

inoculated with rabies virus (Montano-Hirose *et al*, 1993; Prehaud *et al*, 1988). However, there is no report of other regions on the G protein being involved in the lethality of rabies virus.

A comparison of putative amino acid sequences in the G protein of RC-HL and Nishigahara strains showed that a cluster of amino acid substitutions exists in the region between amino acids 164 and 303 (Ito *et al*, 2001a). In order to determine whether this region is involved in the pathogenicity of rabies virus in adult mice, we generated a chimeric R(G 164–303) strain with the region between amino acids 164 and 303 of the G protein derived from the Nishigahara strain in the genomic background of the RC-HL strain.

Full-length plasmid pR(G 164–303) was constructed as follows (Figure 1). The *Xho*I-*Ssp*I cDNA fragment (639 bp) of the Nishigahara G gene was inserted into the plasmid pUC18-RGL*pst*I, which contained the G gene and G-L noncoding region of the RC-HL strain possessing a *Pst*I site as a genetic marker. A *Sac*II-*Pst*I fragment of this plasmid was inserted into the same site of pRC-HL(+), which encoded full-length genome sequence of the RC-HL strain (Ito *et al*, 2001b), and named pR(G 164–303).

The chimeric R(G 164–303) strain was rescued by the same method as that described previously (Ito *et al*, 2001b). We checked the virus antigen in cells inoculated with supernatant of transfected cells by an indirect immunofluorescence assay (IFA) using an anti-N protein monoclonal antibody (MAb) 8-1 (Minamoto *et al*, 1994). We confirmed that the nucleotide sequence of the G gene of rescued virus was the same as the expected sequence. We also checked the elimination of vaccinia virus, vTF7-3, from the stock of the R(G 164–303) strain by polymerase chain reaction (PCR) method as described previously (Ito *et al*, 2001b). Virus yield titrated by a focus assay in mouse neuroblastoma (NA) cells using MAb 8-1.

We confirmed the propagation of the R(G 164–303) strain in NA cells. Cell monolayers were inoculated with the R(G 164–303) strain at a multiplicity of infection (MOI) of 0.01 and incubated at 37°C. The supernatant of the cell cultures was collected at 1, 3, and 5 days post inoculation (p.i.) and virus titers were determined by IFAs. Virus growth curve of the R(G 164–303) strain was compared with those of rRC-HL, Nishigahara, and R(G) strains. Virus growth curves of these four strains in NA cells showed no significant differences.

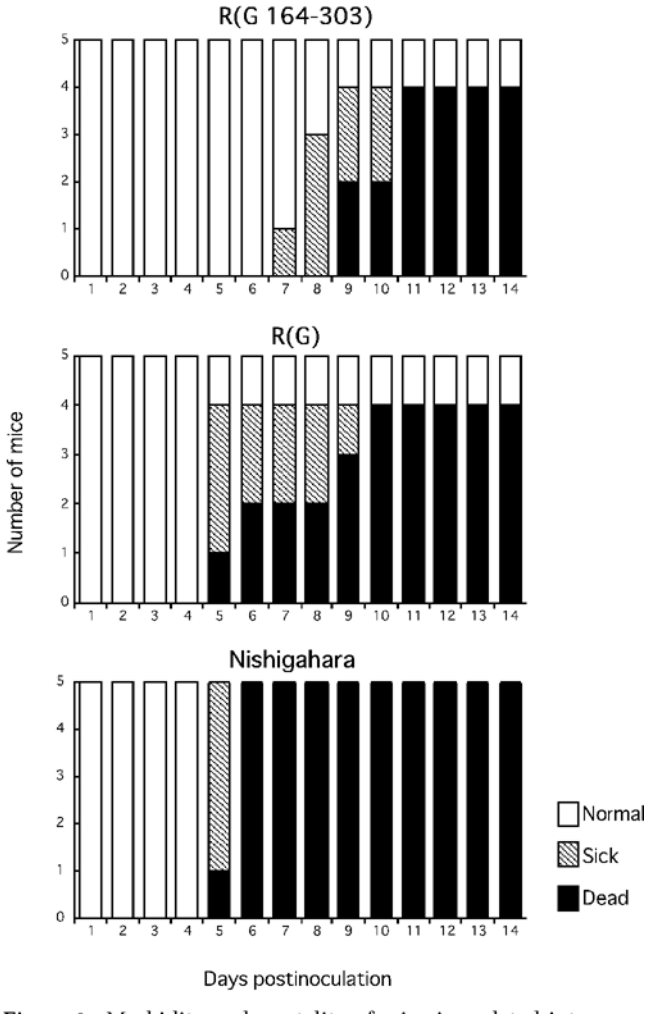

Figure 2 Morbidity and mortality of mice inoculated intracerebrally with R(G 164–303), R(G), and Nishigahara strains. Five mice per group were inoculated with 10 ffu of each virus. The morbidity and mortality of mice were observed for 2 weeks.

First, we examined the lethality of R(G 164–303) strain in adult mice by intracerebral inoculation and compared the morbidity and mortality of mice inoculated with the R(G 164–303) strain with those of rRC-HL, Nishigahara, and R(G) strains reported previously (Ito *et al*, 2001b). Five 4-week-old female inbred ddY mice (Japan SLC, Shizuoka, Japan) were inoculated with 10 focus-forming units (ffu) of each strain and observed for 14 days. Four of the five mice inoculated with the R(G 164–303) strain subsequently developed neurological signs such as hyperactivity, tremor, and paralysis and died within 11 days p.i. (Figure 2). Rabies virus antigen was detected in brains of the dead mice by IFA. In the inoculation with the R(G 164–303) strain, surviving mouse reveals no symptoms such as weight reduction and hyperactivity. All of the mice inoculated with the Nishigahara strain developed the same neurological signs as those seen in mice inoculated with the R(G 164–303) strain and died within 6 days p.i. Onset of clinical signs

and death in mice inoculated with the R(G 164–303) strain was delayed compared with those in mice inoculated with the Nishigahara strain, but the difference of these observed in the mice inoculated with R(G 164–303) and R(G) strain was modest. In contrast with those results, all of the mice inoculated with the rRC-HL strain lost weight but recovered without any neurological signs (data not shown). Accordingly, the R(G 164–303) strain restores lethality for adult mice, as did the R(G) strain.

Production of antirabies antibodies of survived mice inoculated with rRC-HL and R(G 164–303) strains was checked at 14 days p.i. using IFAs. After inoculation with the R(G 164–303) strain, surviving mouse has no antibodies ($\leq$10). In case of the mice recovered from infection of the rRC-HL strain, high titers of antibodies, 1:400 to 1:1600, were shown in the mice serum.

For determination of the 50% lethal dose (LD_{50}) of each virus, groups of five 4-week-old ddY mice were intracerebrally inoculated with 0.03 ml of serial 10-fold dilution of each virus. After a 14-day observation period, the LD_{50} of each virus was calculated by the method of Reed and Müench (1938). The LD_{50} of the R(G 164–303) strain in adult mice was 7.7 ffu, whereas that of the rRC-HL strain was more than 1,000,000 ffu. The LD_{50} of Nishigahara and R(G) strain were 0.063 and 1.0 ffu, respectively (Ito *et al*, 2001b). Because the LD_{50} of the R(G 164–303) strain was about 120- and 7-fold higher than those of Nishigahara and R(G) strains, respectively, it was suggested that another regions expect for sequence at positions at 164 to 303 may be also related to the virulence for mice. However, these differences were small as compared with the difference in the LD_{50} between R(G 164–303) and rRC-HL strains. These results indicate that the central region at the positions 164 to 303 on the G protein of the Nishigahara strain mainly participated in the pathogenicity of the G protein for adult mice.

In order to clarify how this region alters the pathogenicity of rabies virus in adult mice, we compared some properties of the R(G 164–303) strain in adult mice brains with those of rRC-HL, Nishigahara, and R(G) strains. A previous study showed that pathogenic strains spread more rapidly and widely in the mouse brain than did apathogenic strains (Dietzschold *et al*, 1985). Yan *et al* (2002) demonstrated that the distribution of recombinant rabies viruses in brain, in which the G genes were replaced with those of other strains, was similar to those of strains from which the G gene was derived.

To determine the growth of each strain in the adult mouse brain, 4-week-old ddY mice were each intracerebrally inoculated with 100 ffu of each strain per 0.03 ml (Figure 3). Two mice for each strain were used and virus titers were expressed as log ffu per gram of brain. The virus titer of the R(G 164–303) strain was not be determined until 2 days p.i. but increased markedly on the 3 days p.i. and reached a peak of

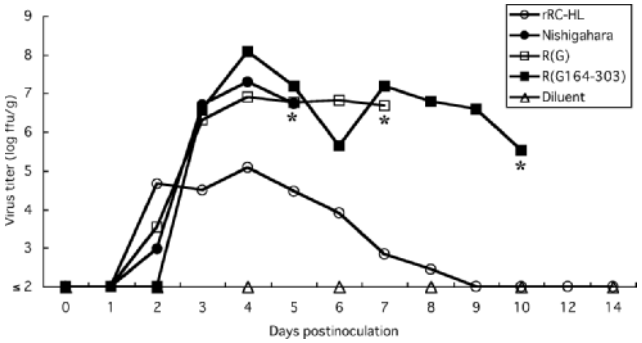

Figure 3 Virus growth of pathogenic and apathogenic strains in adult mice brains. Four-week-old ddY mice were inoculated intracerebrally with 100 ffu of R(G 164–303) (■), rRC-HL (○), Nishigahara (●), and R(G) (□) strains. As negative controls, mice were each inoculated with 0.03 ml of diluent (△). The asterisk indicates the time point at which all of the inoculated mice had died.

8.1 log ffu/g at 4 days p.i. The titer of the R(G 164–303) strain remained at 5.6 to 7.2 log ffu/g until death. The growth curve of the R(G 164–303) strain in the infected mouse brain was similar to those of pathogenic Nishigahara and R(G) strains. On the other hand, the virus titer of the apathogenic rRC-HL strain reached a peak of 5.0 log ffu/g at 4 days p.i. Afterward, the virus titer of the rRC-HL strain gradually declined and was not determined at 9 days p.i. It was revealed that the region at amino acids 164 to 303 in the G protein of the Nishigahara strain required for efficient viral propagation in adult mice brain.

The distribution of rabies virus antigen in the brain of 4-week-old BALB/c mice inoculated with each strain were analyzed by IFAs using an antirabies virus polyclonal antibody at 4 days p.i. Although the distribution pattern of the apathogenic rRC-HL strain was limited, the R(G 164–303) strain distributed widespread in the brain as well as those of pathogenic R(G) and Nishigahara strains (data not shown). This finding suggested that the region at amino acids 164 to 303 in the G protein of the Nishigahara strain was important for spread in the adult mouse brain. But further studies are needed to confirm accurately this finding.

It is likely that amino acids in this region are related to some functional changes in the G protein. Figure 4 shows a comparison of the deduced amino acid sequences of this region in RC-HL and Nishigahara strains. Although seven cysteine residues, which are thought to be associated with a disulphide bridge

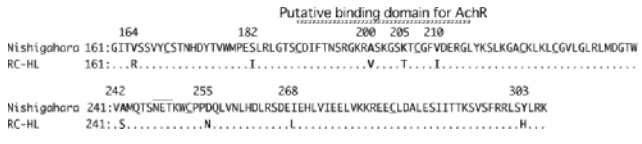

Figure 4 Comparison of the deduced amino acid sequences at amino acid positions 164 to 303 of the G protein in Nishigahara and RC-HL strains. The cysteine residues are underlined, and a predicted *N*-glycosylation site is marked with a line above the sequence.

and a potential *N*-glycosylation site, were completely conserved in this region, there are nine amino acid substitutions (positions 164, 182, 200, 205, 210, 242, 255, 268, and 303) in this region between RC-HL and Nishigahara strains.

Among these, the amino acid substitution at position 164 is overlapped with the predicted low pH–induced fusion domain of rabies virus between amino acids 103 and 179 of the G protein (Durrer *et al*, 1995). Low pH–dependent fusion is thought to be associated with the ability of the virus to spread from cell to cell (Dietzschold *et al*, 1985), and it has been reported that the pH threshold for fusion could influence the pathogenicity of Rhabdoviridae (Desmezieres *et al*, 2003; Gaudin *et al*, 1999). We examined the low pH–dependent fusion activity of R(G 164–303), rRC-HL, Nishigahara, and R(G) strains. NA cells on 10-well Teflon-coated plates were infected with each strain at an MOI of 1 and incubated for 24 h at 32°C. Cells were exposed with the fusion medium consisted of Eagle's minimum essential medium (MEM) and a small amount of 2-morpholinoethanesulfonic acid monohydrate and *N*-2-hydroxyethylpiperazine *N′*-2-ethanesulfonic acid and adjusted to pH 5.4, 5.8, 6.1, and 7.1, respectively (Struck *et al*, 1981). After exposure to fusion medium adjusted to pH 5.4 and 5.8, over 75% of cells infected with each strain were fused. None of the infected cells exposed to pH 7.1 fusion medium were fused. In the case of pH 6.1 fusion medium, fusion was induced in cells infected with rRC-HL and R(G 164–303) strains, but not in cells infected with Nishigahara and R(G) strains. Accordingly, we found that the apathogenic rRC-HL strain was able to induce fusion at a higher pH (pH 6.1) than that at which pathogenic Nishigahara and R(G) strains induced fusion (pH 5.8). However, the pH threshold of fusion activity observed in R(G 164–303) strain-infected NA cells was identical with that of the rRC-HL strain. These results suggested that there was no correlation between the ability to cause cell fusion and lethality of the R(G 164–303) strain in adult mice, though the possibility that this is due to the difference in assay methods or materials, such as protein-expressed cells and virus-infected cells, cannot be excluded. Together with the data reported previously (Durrer *et al*, 1995), these data might allow to exclude the amino acids sequence at positions 164 to 179 from the putative low pH–dependent fusion domain at amino acids 103 to 179 of the G protein and to propose the sequence from 103 to 163 as the new putative low pH fusion domain.

Amino acid substitutions at positions 200, 205, and 210 in the G protein of the RC-HL strain exist in a putative binding domain for the nicotinic acetylcholine receptor (nAChR) (amino acids 198 to 214) (Lentz *et al*, 1982), which is thought to be one of the rabies virus receptors. It has been reported that nACh may related to binding of virus at the neuromuscular junction (Lewis *et al*, 2000) and susceptibility of different animal species to rabies virus (Baer *et al*, 1990; Jackson, 2002). Although, these substitutions may affect the binding ability of rabies virus to nAChR, we could not clarify this possibility because the route of inoculation employed in this study (intracerebral) would bypass the steps of traveling of rabies virus from peripheral site to central nervous system.

Accordingly, viral growth and distribution in mouse brain of the R(G 164–303) strain were consistent with those of pathogenic strains, but the viral growth in cultured cells and the low pH–dependent fusion activity were in accord with those of the apathogenic rRC-HL strain. These discrepancies suggest that host factors responding to the G protein may be responsible for the difference between the lethalities of Nishigahara and RC-HL strains for mice. Host factors that have been suggested to be involved in the pathogenicity of rabies virus include T and B lymphocytes (Galelli *et al*, 2000; Hooper *et al*, 1998; Lafon, 2002; Perry and Lodmell, 1991; Smith, 1981; Smith *et al*, 1982; Sugamata *et al*, 1992; Weiland *et al*, 1992), apoptosis of infected neurons (Morimoto *et al*, 1999; Theerasurakarn and Ubol, 1998), cytokines (Marcovistz *et al*, 1994), affinity to host-cell receptor (Dietzschold *et al*, 1985), and nitric oxide (Ubol *et al*, 2001). Hence, the difference between virus titers in the mouse brain of pathogenic strains and the apathogenic rRC-HL strain appeared in the early phase of infection (3 days p.i.), suggesting that innate immunity might be involved in the lethality of rabies virus for adult mice. Further studies are needed to determine the mechanisms underlying the pathogenicity of rabies virus.

In conclusion, we have clearly shown that the region of the G protein between amino acids 164 and 303 plays an important role in virus pathogenicity for adult mice. It is thought that nine amino acids at positions 164, 182, 200, 205, 210, 242, 255, 268, and 303 in this region contribute to the pathogenicity of rabies virus for adult mice. Further studies to determine the most important amino acid related to the lethality of the Nishigahara strain among the nine amino acids are now in progress.

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