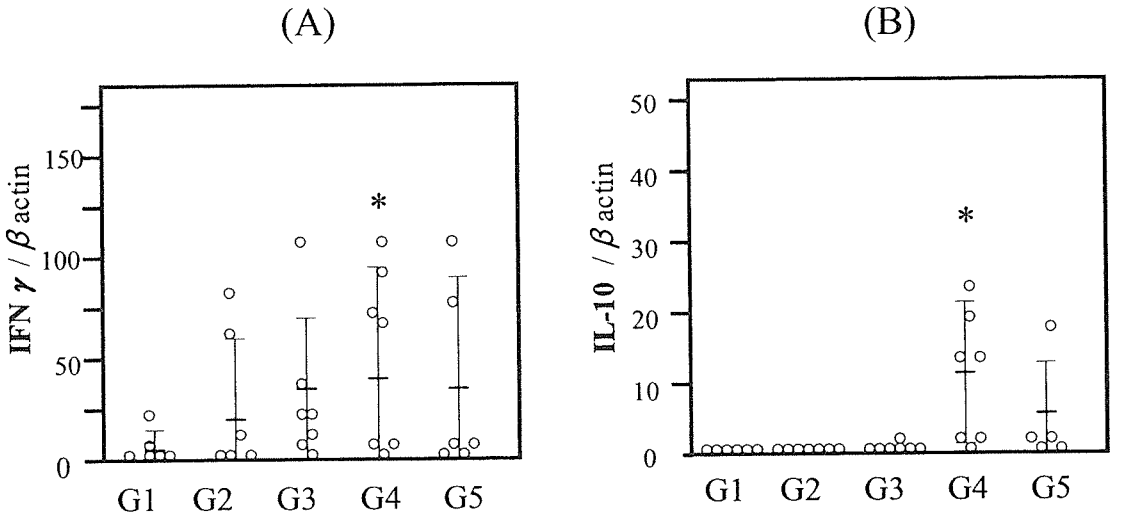

FIG. 7. Expression of IFN-γ (A) and IL-10 (B) in the gastric tissue of mice treated with the LB-pulsed (G2), the FKB-pulsed (G3), the WCS-pulsed (G4), and the unpulsed (G5) Jaws II cells was determined by real-time PCR (see Materials and Methods), and cytokine amounts were compared to those in mice treated with PBS alone (G1). Standard curves were generated using external standards for each cytokine DNA molecule with a known copy number and were used to estimate mRNA copy numbers in each sample. A significant increase in IFN-γ expression was observed in G4 mice. A significant increase in IL-10 expression was also seen in G4 mice. The asterisks indicate statistical significance at P of <0.05. The results are expressed as the geometric means (bold lines) ± standard deviations of results for each mouse.

pulsed (G3), the WCS-pulsed (G4), and the unpulsed (G5) Jaws II cells and compared them to those in tissues of mice treated with PBS alone (G1). A significant increase in IFN-γ expression was observed only in G4 mice (Fig. 7A). A significant increase in IL-10 expression was seen only in G4 mice (Fig. 7).

DISCUSSION

DCs are categorized into two subtypes, the myeloid type and the plasmacytoid type (2, 33). The plasmacytoid-type DC is able to convert into the myeloid-type DC, responding to surrounding conditions (9, 10, 39). When cell surface markers of Jaws II cells were analyzed by FACS, CD11c and CD11b were detected whereas CD8α was not (Fig. 2A), indicating that the Jaws II cells are myeloid-type DCs as previously reported (14, 17, 33). Many reports have described studies on DCs isolated from bone marrow, lymphoid tissues, or peripheral blood by means of the magnetic cell sorting system. In the present study, however, we have used a DC cell line, Jaws II, because a certain proportion of myeloid-type DCs are expected in a cell population.

Obvious differences in quantities of cell surface molecules (MHC class I, MHC class II, CD80, CD86, and CD40) were not observed when Jaws II cells were pulsed with LB, FKB, and WCS of *H. pylori* (Fig. 2B). Jaws II cells were pulsed with each antigen for 48 h at 37°C in the present study. Jaws II cells proliferate slowly, and MHC class II molecules were initially expressed at 12 h (data not shown); therefore, a longer incubation period, as used by another group of researchers (17), was considered to be essential for the Jaws II cells. TNF-α expression has been reported to be closely correlated with the maturation of DCs. A dramatic elevation in the level of TNF-α in the supernatant of the WCS-pulsed Jaws II cells was observed (Fig. 3); hence, WCS pulsing forced a larger proportion of Jaws II cells to mature than pulsing with the other preparations. It is well known that the lipopolysaccharide (LPS) of gram-negative bacteria efficiently directs DCs to mature as well as inducing the expression of proinflammatory cytokines including TNF-α. *H. pylori* LPS, however, has been recognized to be less efficient in such activities (20, 25, 29, 31). In the present study, we purified *H. pylori* LPS and determined its ability to induce the expression of TNF-α and several cell surface molecules including MHC class II antigens, CD80, and CD86. Different from the LPS of other gram-negative bacteria, *H. pylori* LPS failed to induce the expression of those molecules (data not shown). Recently, Kranzer et al. reported that the level of expression of TNF-α was lower in human DC cells pulsed with *H. pylori* WCS than in those pulsed with LB or FKB (19), which contradicts our result. The difference is probably due to the fact that they used human DCs which probably comprised a heterologous DC population rather than myeloid-type DCs selected with CD11b.

H. pylori has evolved to evade not only the innate but also the adaptive immune response, which facilitates bacterial colonization of the stomach, a situation that can cause chronic gastric diseases including atrophic gastritis and peptic ulcers. Chronic persistent infections are one of the best targets of DC-related immunomodulatory therapy. DCs act to organize the host immune system by regulating the functions or interactions of effector cells like $CD4^+$ or $CD8^+$ T cells, macrophages, and B cells. The most significant reduction in the number of colonizing *H. pylori* bacteria was observed in the mice treated with the WCS-pulsed Jaws II cells (Fig. 5A). The 2-log reduction in this number compared to that in the control mice was one of the most impressive effects observed in vivo using C57BL/6 mice, a widely accepted animal model for *H. pylori* infection.

The ability to proliferate as well as production of IFN-γ and

IL-10 was enhanced in naïve T cells exposed to the WCS-pulsed Jaws II cells in vitro (Fig. 4). In addition, a significant simultaneous increase in the expression of IFN-γ and IL-10 was observed only in mice treated with the WCS-pulsed Jaws II cells (Fig. 6A). IFN-γ is a cytokine representative of Th1 cell-mediated immunity, whereas IL-10 is representative of Th2 cell-mediated immunity. Therefore, the functionally up-regulated T-cell population, with a combination of Th1 and Th2 cell-mediated immunity, might play a role in reducing the number of *H. pylori* bacteria colonizing the stomach. Significant systemic rises in IgG, IgA, and IgG1 levels were observed in all the mice treated with *H. pylori*-related antigen-pulsed Jaws II cells (Fig. 6 A and B), and titers of IgG2a in mice of all groups were below or close to the detection limit, regardless of the effect on *H. pylori* (Fig. 6D). This indicates that *H. pylori*-specific antibodies were not associated with the reduction in the number of *H. pylori* bacteria colonizing the stomach, a finding which is supported by several studies (6, 11, 37). Many reports have suggested that postimmunization gastritis caused by proliferated and recruited lymphocytes may contribute to a reduction in the number of colonizing *H. pylori* bacteria (13, 24); however, the effect would be limited in the present study because gastritis scores for the mice with significant *H. pylori* reduction were not significantly higher than those for the negative control (Fig. 5B). Taken together, it could be that some unknown effects of the functionally up-regulated T-cell population, other than lymphocyte recruitment or the involvement of a specific antibody, helped to reduce the number of *H. pylori* bacteria colonizing the stomachs, probably through a combination of Th1 cell-mediated and Th2 cell-mediated immunity.

The up-regulated T-cell functions were induced only by exposure to the WCS-pulsed Jaws II cells. Potential explanations include that (i) an unknown molecule released from the cytoplasm or the periplasm of *H. pylori* is displayed by the MHC and might play a role, (ii) a larger proportion of the appropriate MHC, that is, an MHC molecule with a processed peptide, was induced on the WCS-pulsed Jaws II cells than on the other cells, (iii) an antigen-presenting molecule other than the MHC might play a role, and (iv) programmed cell death ligand, a costimulatory molecule that inhibits T-cell activities on DCs, was substantially expressed on the surface of LB- or FKB-pulsed Jaws II cells and, as a consequence, T-cell proliferation was suppressed.

The DC-related immunomodulatory therapy described here would be applicable to the eradication of microbial agents capable of evading host immunity and, consequently, causing chronic persistent infections.

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Genetic analysis of dog rabies viruses circulating in Bangkok☆

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Abstract

The genetic diversity of the rabies virus glycoprotein (G) gene isolated from individual rabid dogs (inter-hosts) and within a single infected dog (intra-host) has been analyzed in an effort to better understand selective pressures and population shifts among rabies viruses circulating in Bangkok. Comparison of individual master sequences among inter-hosts revealed that the dog virus isolates circulating in Bangkok were phylogenetically closely related. The ectodomain of the glycoprotein was highly conserved among the virus isolates. Furthermore, the genetic diversity of the G gene within an intra-host was assessed by comparing the cloned sequences in the virus population. The comparisons revealed that rabies virus circulating in an intra-host consisted of closely related heterogenous populations with minor substitutions at nucleotide (0.19%) and amino acid levels.

Keywords: Rabies virus; Thai dog rabies; Glycoprotein gene; Quasispecies

1. Introduction

Most RNA viruses consist of a complex mixture of closely related but nonidentical genomes, known as quasispecies. They provide a significant adaptation advantage for the rapid selection and emergence of a new variant in a changing environmental condition (Holland et al., 1992; Smith et al., 1997; Domingo et al., 1998). In the rabies virus infection cycle, the virus passes through multiple cell types: non-neuronal tissues at the bite wound, neuronal tissue, salivary gland, and other cells within the host. It is then transmitted to another host, usually by a bite. Virus is thus exposed to several environmental changes. Benmansour et al. (1992) showed the existence of heterogenous populations in street rabies virus. Such heterogenous populations remained in a dynamic equilibrium but this equilibrium could rapidly change in a new environment. Morimoto et al. (1998) showed that a mouse-adapted rabies strain also consisted of variants with different genotypical and phenotypical properties and that changes in the host environment rapidly resulted in shifts in the dominant variant. Each RNA virus genome exhibits a high degree of sequence variation. Therefore, the genome sequence is represented by ensemble of most frequently present nucleotide; a master sequence. Accumulated shifts in a pool of heterogenous populations can lead to changes in the master sequence which depended on geographical location or the animal species of the isolate. This could establish a new virus variant.

In the Americas, rabies enzootic cycles are independently maintained in animal species, notably, canines, foxes, skunks, raccoons, and bats (Smith et al., 1995; Krebs et al.,

☆ Nucleotide sequence data reported in this paper are available in the GenBank/EMBL/DDBJ databases under the accession numbers AB195175–AB195210.

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2002). Spillover infection, in which a variant is transmitted into another species including humans, has frequently and sporadically occurred but has rarely established itself as a new enzootic cycle (Bourhy et al., 1999; Badrane and Tordo, 2001; Holmes et al., 2002). In Asian countries, an enzootic cycle of rabies is maintained principally in owned or community and stray dogs. Molecular epidemiology studies of dog rabies were performed in Thailand, the Philippines, and Sri Lanka (Susetya et al., 2003; Nishizono et al., 2002; Arai et al., 2001; Nanayakkara et al., 2003). The phylogenetic analysis indicated that the virus variants were closely related to the geographical origin (Holmes et al., 2002). There are only very limited studies of wildlife rabies in Asian. Genetic analysis of sylvatic rabies virus in Sri Lanka suggested that it represented spillover of dog rabies (Arai et al., 2001; Nanayakkara et al., 2003). The existence of sylvatic enzootic cycles other than among bats remains largely unknown. It is however now clear that bat rabies is present in the Philippines and Thailand (T. Hemachudha and B. Lumlertdacha, unpublished). Thailand is a dog rabies endemic country with a large population of owned and stray dogs. Immunization coverage of this population is still inadequate and dog rabies remains a persistent danger. Having a solid public health infrastructure and active research facilities, the Bangkok metropolitan region proves an ideal environment for more detailed molecular studies of the rabies virus among dogs. We therefore decided to investigate the glycoprotein (G) gene diversity of the rabies virus circulating in dogs and to investigate the correlation between rabies G gene and adaptation and evolution of rabies virus.

The nucleoprotein (N) gene has been extensively studied as a tool to elucidate patterns of geographic distribution of rabies virus variants (Smith et al., 1992; Badrane and Tordo, 2001). In this study, we analyzed variations in the G gene among rabies virus isolates from dogs in Bangkok. The envelope glycoprotein is responsible for receptor binding and viral invasion into the host cells (Thoulouze et al., 1998; Tuffereau et al., 1998). It is a main target for the immune response (Wiktor et al., 1973; Cox et al., 1977; Macfarlan et al., 1984). Antigenic shifts of the G protein have an impact on virus invasion and pathogenicity (Dietzschold et al., 1983; Seif et al., 1985). Variations in the G gene might provide the potential source for a variant that might escape host defenses and adapt to a new environment. The genetic relationship and variation of the G gene was examined not only among isolates from different individuals but also within a single individual.

2. Materials and methods

2.1. Source of viral specimens

The diagnostic laboratory of the Queen Saovabha Memorial Institute acts as the main facility for examining rabies suspected human and animal specimens in the central region of Thailand. The origin and history of each animal is recorded. Diagnoses were made using the fluorescent antibody test (FAT) on impression smears from hippocampus and brain stem. FAT-positive samples were stored at −80 °C for further study. Ten samples (DG109, 437, 466, 469, 474, 476, 491, 500, 515, 609) collected during 2002–2003 were randomly selected for gene analysis.

2.2. Preparation of RNA and RT-PCR amplification

Rabies virus RNA was isolated from rabid dog brains using the RNeasy Maxi kit (Qiagen) according to the manufacturer's protocol without passage through mouse or cell culture. The RNAs prepared from the brains of 10 rabid dogs were subjected to a one step RT-PCR (Qiagen) procedure using a primer set of G5a: 5′-CCATCATGAC-CACCAAGTC-3′ (position 923–941) and G3-stop: 5′-TTACAGCTTGGTCTCACC-3′ (position 1558–1575) for G-3 fragment (653 base-length) (G gene encodes position 1–1575; as shown in Fig. 2). After reverse transcription with 50 °C for 30 min, PCR was performed 94 °C for 1 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, and then 72 °C for 10 min. The PCR products were purified using Qiagen MinElute PCR purification Kit (Qiagen) followed by DNA sequencing. The sequences were shown to be the master sequence of the individual isolates. To construct full-length cDNA of G gene, another primer set of G5-start: 5′-AGGAAA-GATG(A/G)TTCC(G/T)CA-3′ (position −7 to 11) and G3a: 5′-GATTT(A/G)TAGTGAGCATCAGC-3′ (position 1030–1049) was used for G-5 fragment (1056 base-length).

2.3. TA cloning of full-length PCR products from DG476 virus

For clonal sequence analysis of quasispecies, the PCR products of G gene in DG476 virus was cloned into plasmids to compare the cloned sequences. In this step, the G-5 and G-3 fragments were combined by performing the second round PCR with G5-start and G3-stop primers to get full-length G-cDNA (1582 base-length). The PCR conditions were 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, and then 72 °C for 10 min with Takara ExTaq polymerase that had a proof reading activity (Takara Shuzo Co., LTD., Japan). A total of 60 cycles of PCR were done to get the full-length PCR products. The full-length PCR products were cloned into vector pCRII-TOPO using TOPO TA cloning Kit (Invitrogen Co., CA). Twelve recombinant clones were picked up, and the plasmid DNAs were purified using Qiagen Plasmid Mini Kit (Qiagen). In each clone, 685 base-length (positions 81–765 of G gene) of G-5 fragment and 621 base-length (positions 955–1575 of G gene) of G-3 fragment were sequenced (as shown in Fig. 4).

2.4. DNA sequencing and the phylogenetic tree

The PCR products and the recombinant plasmid clones were sequenced using an ABI PRISM BigDye terminator cycle sequence kit with a model ABI-310 DNA sequencer (Applied Biosystems, CA). A phylogenetic tree was constructed using the neighbor-joining method by Genetyx-Win gene analysis software (GENETYX Co., Tokyo, Japan). Bootstrap probability was calculated using 1000 replicates.

3. Results and discussion

3.1. Genetic diversity of the G gene in rabies virus isolates circulating in Bangkok

Sequence analysis of N and G genes in human rabies virus isolates in Bangkok showed that no amino acid substitutions were in the N protein and the ectodomain (Ecto) of the G protein. All amino acid substitutions in the G protein of the isolates were restricted to the transmembrane (TM) and endodomain (Endo) (Hemachudha et al., 2003). Almost all human patients in Bangkok have been exposed to rabid dogs. In this study, the analysis in genetic diversity of the G gene was extended to dog rabies viruses. Ten FAT-positive brain samples were randomly selected and assessed for genetic variation in the dog rabies viruses. The 3′-half region (G-3 fragment: 653 base-length), including a part of the Ecto and the TM + Endo domains, was amplified and the PCR products were directly sequenced. The sequences were shown to be the master (most frequently represented) sequence of the individual virus isolates.

Phylogenetic analysis of the G gene was performed in 10 dog virus isolates sequenced in this study (DG isolates) and in four human virus isolates (HM65, HM75, HM88, and HM208; reported by Hemachudha et al., 2003), including other three Thai strains and one Malaysian strain which had already been reported in GenBank (Fig. 1). All the 14 Bangkok isolates, including the human ones, were closely related but could be divided into two subgroups. Thai AB052666 strain was an isolate from the northeastern part of Thailand (N. Minamoto, pers. comm.). Pair-wise comparison of the 10 dog virus isolates exhibited more than 95% identical nucleotides and showed differences of eight amino acids or less in the region (out of 211 amino acids). They could be divided into group 1 (DG109, DG437, DG491, DG474), group 2 (DG515, DG469, DG500, DG609, DG466, DG476) (Table 1). One rabies virus isolate was not associated with any distinct clinical manifestations of rabies (encephalitic or paralytic) as had been previously reported for human rabies (Hemachudha et al., 2003) (Fig. 1). In the absence of selective pressure, base substitutions by errors of viral polymerase were evenly distributed through the viral genome. However, variations in the G gene were not

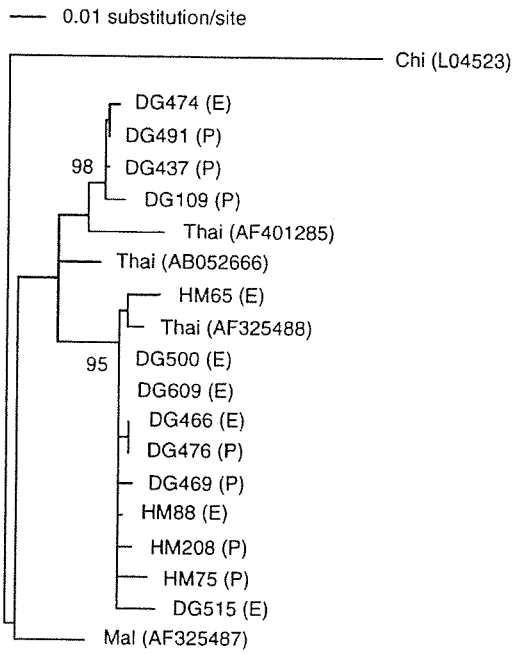

Fig. 1. Phylogenetic tree of the G gene in dog rabies viruses circulating in Bangkok. Genetic sequence distance was determined by the neighbor-joining method. The bootstrap percentages of 1000 replicates were presented for key nodes only. Chinese dog strain (accession no. L04523) was used as an outgroup. Branch lengths were drawn to scale. (E) Encephalitic type and (P) paralytic types of clinical manifestations were shown in each virus isolate. Accession nos. AB195175–AB195184 for DG virus isolates.

Table 1
Pairwise comparison of nucleotide and amino acid substitutions of the G gene among dog rabies virus isolates circulating in Bangkok[a]

	DG109	DG437	DG491	DG474	DG515	DG469	DG500	DG609	DG466	DG476
DG109	–	5	5	7	14	22	25	25	27	27
DG437	(1)	–	2	4	11	19	22	22	24	24
DG491	(1)	(0)	–	2	11	19	22	22	24	24
DG474	(2)	(1)	(1)	–	13	21	24	24	26	26
DG515	(8)	(7)	(7)	(8)	–	15	16	16	18	18
DG469	(8)	(7)	(7)	(8)	(5)	–	3	3	7	7
DG500	(8)	(7)	(7)	(8)	(5)	(0)	–	0	4	4
DG609	(8)	(7)	(7)	(8)	(5)	(0)	(0)	–	4	4
DG466	(8)	(7)	(7)	(8)	(5)	(0)	(0)	(0)	–	0
DG476	(8)	(7)	(7)	(8)	(5)	(0)	(0)	(0)	(0)	–

[a] The number of the substitutions out of 633 bases (211 amino acids) between the isolates was shown in nucleotide and amino acid (in parentheses).

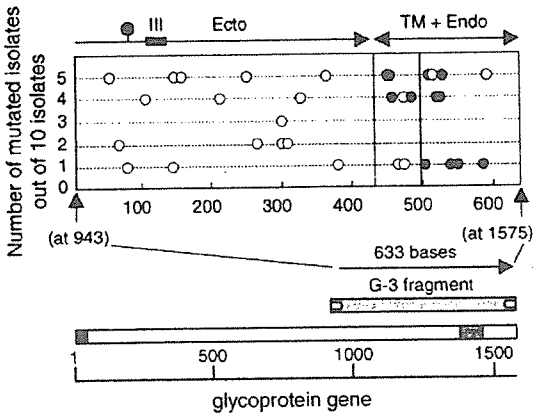

Fig. 2. Distribution of base substitutions in the G gene among 10 dog rabies virus isolates. The PCR products of 3′-half of G gene (G-3 fragment; 653 bases) were sequenced in 10 virus isolates. The position of base substitutions were plotted as synonymous mutation (open circle) and nonsynonymous mutation (closed circle). The potential glycosylation site and the antigenic site III in the ectodomain (Ecto), and the transmenbrane domain (TM) and endodomain (Endo) of G protein were shown on the top.

scattered homogenously through the coding region (Benmansour et al., 1992; Badrane and Tordo, 2001). The difference of genetic constraints between Ecto and TM + Endo domains of the G protein was analyzed by examining the extent and the distribution of sequence variation. The mutated positions of the sequenced region among 10 dog virus isolates were plotted in Fig. 2. As seen in Fig. 1 and Table 1, there were two subgroups among the 10 isolates. Therefore, four or five isolates had frequent base substitutions at the same positions as seen in Fig. 2. There were a total of 33 nucleotide substitutions among the 10 isolates and these were only point mutations. No deletion, insertion, and termination mutations were seen. The number of synonymous nucleotide substitutions (SYN) was 22 and the number of nonsynonymous nucleotide substitutions (NSY) was 11 (NSY/SYN = 0.5). The nonsynonymous substitutions were restricted to the TM + Endo domain of

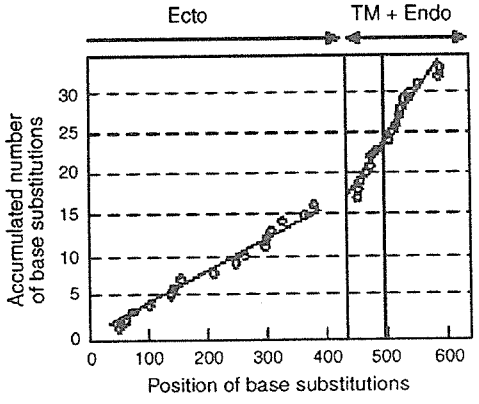

Fig. 3. Analysis of mutation rate in the Ecto and TM + Endo domains. Accumulated number of base substitutions was plotted with the mutated position and compared between endodomain and TM + endodomain among 10 dog rabies virus isolates.

G protein. No amino acid substitutions occurred in the potential glycosylation site at position 319, the arginine at position 333, and the antigenic site III responsible for the virulence in the fixed strains. Furthermore, the rate of base substitutions were shown in Fig. 3, in which the accumulated number of substitutions was plotted with the mutated position. It was found that the rate of base substitutions was clearly different between the Ecto and TM + Endo domains. The slope of the regression line of the rate in the Ecto was 0.043, the slope of the regression line in the TM + Endo was 0.113 ($p = 0.009$). In the Ecto, one substitution occurred in every 22 bases on an average; in the TM + Endo domain, one substitution occurred in every nine bases. A higher rate (by 2.5 times) of base substitutions and higher number of nonsynonymous nucleotide substitutions were detected in the TM + Endo domain than in the Ecto. It indicated that there were strong structural and functional constraints on the Ecto in contrast to the TM + Endo domain of the G protein among virus circulating in dogs at Bangkok.

3.2. Genetic diversity of the G gene among population of DG476 virus

Futhermore, genetic diversity of the G gene was assessed for virus variants circulating within a single rabid dog. It is difficult to detect a minor variant of the population by direct sequencing of PCR products described above. The PCR product from a dog rabies virus isolate, DG476, was cloned into T/A cloning vector and the obtained clones were sequenced. The sequence obtained directly from the PCR product of the virus isolate exhibited the master sequence of the virus isolate, and the cloned sequence exhibited an

Table 2
Number of nucleotide substitutions in the individual clone of strain DG476

Clone	G-5 fragment			G-3 fragment		
	S[a]	N[b]	Diversity (%)[c]	S[a]	N[b]	Diversity (%)[c]
#1	2	2	0.58	0	1	0.16
#2	0	2	0.29	0	2	0.32
#3	0	1	0.15	0	1	0.16
#4	0	0	0.00	0	0	0.00
#5	0	1	0.15	0	1	0.16
#6	0	1	0.15	0	1	0.16
#7	0	2	0.29	0	1	0.16
#8	0	0	0.00	1	0	0.16
#9	0	0	0.00	1	1	0.32
#10	1	1	0.29	2	1	0.48
#11	17	1	2.63*	0	0	0.00
#12	6	6	1.75*	1	0	0.16
Total	26	17	0.19[d]	5	9	0.19

[a] Number of synonymous substitutions.
[b] Number of nonsynonymous substitutions.
[c] Percent diversity was the number of total substitutions divided by the number of nucleotide sequenced (684 bases and 621 bases were read in G-5 and G-3 fragments, respectively).
[d] Mean diversity except clones #11 and #12 of G-5 fragment.
* Significance different from the master sequence ($p < 0.05$).

individual variant among heterogenous population of a virus isolate. Two parts of the G gene, G-5 fragment (Ecto) and G-3 fragment (part of Ecto and TM + Endo domains) of 12 clones were sequenced. The number and position of base substitutions in each clone were compared with the master sequence of DG476 virus isolate (Table 2 and Fig. 4). Among a total of 24 clones analyzed in the two regions, 57 base substitutions were detected as a point mutation. No deletion, insertion, and termination mutations were seen. Some clones (G-5 #11, #12) had more frequent nucleotide substitutions and were distinct from the others which had fewer substitutions. The percent mean diversity in G-3 fragment is 0.19% [14/(621 × 12) = 0.00188]. Except for clones #11 and #12 in G-5 fragment, only 13 base substitutions were present in the 10 clones. The percent mean diversity is 0.19% [13/(684 × 10) = 0.00190]. These values obtained in the G gene were similar to the value (=0.00219) reported by Kissi et al. (1999). After a total of 60 cycles of PCR amplification, the error frequencies were estimated between 2.3×10^{-4} and 5.5×10^{-4} (Wang et al., 2002). This was lower than the mean diversity (1.9×10^{-3}) observed in our samples. Except for clones #11 and #12 in G-5 fragment, eight synonymous nucleotide substitutions (SYN) and 19 nonsynonymous nucleotide substitutions (NSY) were detected in the 12 plus 10 clones. The ratio (NSY/SYN = 2.4) of clonal sequence analysis was higher than that (NSY/SYN = 0.5) of the inter-dogs sequence analysis described above. Nonsynonymous mutations were preponderant in quasispecies populations as seen in a study by Kissi et al. (1999). The amino acid substitutions were

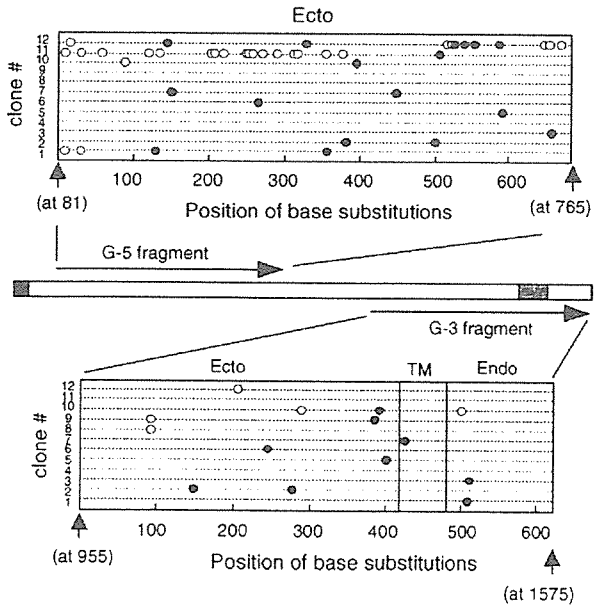

Fig. 4. Mutated positions of 12 clones obtained from DG476 virus isolate. Two parts of G gene were sequenced in each of 12 cloned plasmids (accession nos. AB195185–AB195210). The position of base substitutions was plotted as synonymous mutation (open circle) and nonsynonymous mutation (closed circle).

sporadically seen only in a single clone, suggesting that the mutated population roughly occupied less than 10% (one twelfth) of the population. The clonal sequencing analysis demonstrated that dog rabies virus circulating in Bangkok consisted of heterogenous populations with minor variants at nucleotide and amino acid levels in intra-dog hosts.

3.3. Highly heterogenous clones among population of DG476 virus

The percent mean diversities of clones #11 and #12 in the G-5 fragment were significantly higher than the others, representing 2.63 and 1.75%, respectively (Table 2). There were a total of 18 nucleotide substitutions in clone #11 from the G-5 fragment, of which 17 were synonymous and one was nonsynonymous. On the other hand, there were a total of 12 nucleotide substitutions, of which six were synonymous and six were nonsynonymous in clone #12. In this clonal sequence analysis, the G-5 and G-3 fragments that were separately produced from the first round PCR, were combined as a full-length G cDNA, and were cloned into the plasmid. Therefore, the sequences of G-5 and G-3 fragments obtained in the same plasmid clones originated independently from different genomes within the DG476 population. The sequences of clones #11 and #12 in G-3 fragment exhibited a common low substitution rate. Therefore, higher mutation rates of clones #11 and #12 in G-5 fragment were not due to artifactual misincorporation during the cloning and sequencing procedures. The existence of clones #11 and #12 in the G-5 fragment indicated that highly heterogenous variants existed as a minor population in intra-dog hosts. Clone #11 in the G-5 fragment had many synonymous substitutions in the Ecto and was not different from other clones in protein level. This suggests that it had been accepted, and remained in stable equilibrium among the population. Although a strong genetic constraint was forced on the Ecto during the inter-dog transmission, clone #12 in the G-5 fragment had many nonsynonymous substitutions in the Ecto. Therefore, it is not sure whether the variant will survive in the host. However, when environmental change occurs in the population, the variant might be shifted to dominant population. Alternatively, it might disappear as a disabled population. The existence of this clone might prove to be a potential source of new variants with better fitness for a new environment. Cat rabies in Bangkok is due to spillover infection exposed to rabid dogs (Kasempimolporn et al., 2004). Further studies including cat rabies are needed for better understanding of the spread of epizootic rabies and the cross-species transmission.

In conclusion, dog rabies viruses circulating in Bangkok were phylogenetically closely related and consisted of quasispecies populations with minor variations at nucleotide and amino acid levels in intra- and inter-dog hosts. The G protein is the only viral protein exposed on the surface. The G protein is responsible for the adherence to cellular

receptors and the immunological responses of the infected host. In inter-dog transmission, the ectodomain of the G protein was exposed to structural and functional constraints in comparison with other parts of the G protein. The master sequences of rabies virus isolates in inter-dog hosts were maintained and circulated with only minor variations. However, distinct variant with many mutations existed as a minor population in the intra-dog host.

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