ed (CER) cells. Briefly, ca. 3.0×10^4 focus-forming units (ffu) RV suspension was incubated with a serial dilution of the Fab preparation at 37 C for 60 min, then trypsinized CER cells (ca. 6.0×10^5) were added and incubated at 37 C for 48 hr in 96-well tissue culture plates (Corning Coaster). After being fixed and washed, RV-infected cells were directly stained with fluorescent isothiocyanate (FITC)-labeled anti-RV nucleocapsid murine monoclonal antibody stocked in our laboratory and counted microscopically.

Determination of RV-binding capacity. The capacity of Fab preparations to bind RV was determined by ELISA. Briefly, an Fab preparation was reacted with the purified RV virions or RVG-protein coated onto ELISA plates at ca. 1.25 μg/well, for 1 hr at 37 C. After vigorous washing, anti-human IgG (Fab-specific, alkaline phosphatase-conjugated, 1:1,000 diluted) was reacted and the reaction was visualized with phosphatase substrate and OD_{405} reading was determined. A volunteer's serum (diluted 1:200 with PBS) and BSA (500 μg/ml) were used as the positive and negative control, respectively.

Co-immunoprecipitation. Co-immunoprecipitation was performed to determine RV proteins with which Fab preparations interacted. Briefly, 200 μl culture fluids of RV strain CVS were reacted with 20 μl portions of Fab preparations at 4 C overnight, then 2.5 μl of goat anti-human IgG $F(ab')_2$ antibody (Pierce) was added. Immunoprecipitates were collected by adding 20 μl of protein G conjugated-sepharose beads (Amersham Biosciences) and subsequent centrifugation ($5,000\times g$, 30 min). After vigorous washing, the collected immunocomplexes were resuspended in PBS and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, and analyzed by western blotting as described in our previous work (17). Anti-RVG-protein specific monoclonal antibody 4-2 (1:5,000 diluted) and Anti-RVN-protein specific monoclonal antibody Gifu 8-1 (1:5,000 diluted) stocked in our laboratory were used as the primary antibodies and peroxidase conjugated goat anti-mouse IgG (MP Biomedicals, 1:2,000 diluted) was used as the secondary antibody.

DNA sequencing of Fab clones. Sequence analysis of the Fab V_κ and V_H segments was performed on an automated DNA sequencer with the Fluorescence Dideoxynucleotide Terminator Cycle Sequencing Kit using *Taq* DNA polymerase (Perkin-Elmer). Primers 5′-ACAGCTATCGCGATTGCAGTG-3′ (LC1) and 5′-CACCTGATCCTCAGATGGCGG-3′ (LC4) were used for sequencing V_κ segments and primers 5′-ATTGCCTACGGCAGCCGCTGG-3′ (HC-1) and 5′-GGAAGTAGTCCTTGACCAGGC-3′ (HC-4) were used for sequencing V_H segments (15). Software Vector NTI (InforMax) was used for sequence analysis. The DNAPLOT software program (MRC Center for Protein Engineering) was used to search for human immunoglobulin homologues in the database.

Results

RV Neutralizing Antibody Titers of Sera from 6 Volunteers

Neutralizing antibody titers of sera from 6 volunteers against RV strain Nishigahara were determined by RFFIT on BHK-21 cells. With sequential vaccinations, the titers of sera taken at 2 weeks after the last boost increased in the range from 3,162 (volunteer 6) to 22,387 (volunteer 3), with volunteers developing 1.4-fold (volunteer 3) to 100-fold (volunteer 5) higher neutralizing antibody titers (Fig. 2). The range of RV neutralizing antibody titers was considered to be high enough to construct a combinatorial Fab antibody library.

Construction of Human Combinatorial Fab Antibody Library

Approximately 350-bp DNA products were yielded by PCR for amplifying variable regions of κ light chain (V_κ) or heavy chain (V_H). The 350-bp constant domains of κ light chain (C_κ) or heavy chain (C_H1) were amplified using pCom3XTT as the template. Complete κ chain DNA products or Fd region products were constructed by an overlap assembly PCR yielding ca. 750-bp products in each reaction. In the final amplification of full-length Fab DNA products, ca. 1,500-bp products were created. A library of approximately 7.0×10^7 *E. coli* transformants was constructed using pComb3X plasmids containing full-length Fab PCR products (Fig. 1).

Panning of the Phage Library

Firstly, the constructed library was panned in five successive rounds against RV virions immobilized directly in an ELISA plate. The number of harvested phages decreased during the 1st to 3rd panning cycles, then increased, finally reaching ca. 1.0×10^7 colony forming units (cfu) of *E. coli* infected with the eluted phage. Similarly, the same library was panned in five successive rounds against purified RVG-protein coated directly on a 35-mm tissue culture dish in an attempt to enrich clones displaying RVG-protein-specific Fab. The number of harvested phages reached ca. 1.1×10^8 cfu of *E. coli* infected with the eluted phage. After the fifth panning, phagemid pools were isolated from the two separately enriched libraries, with which the Top

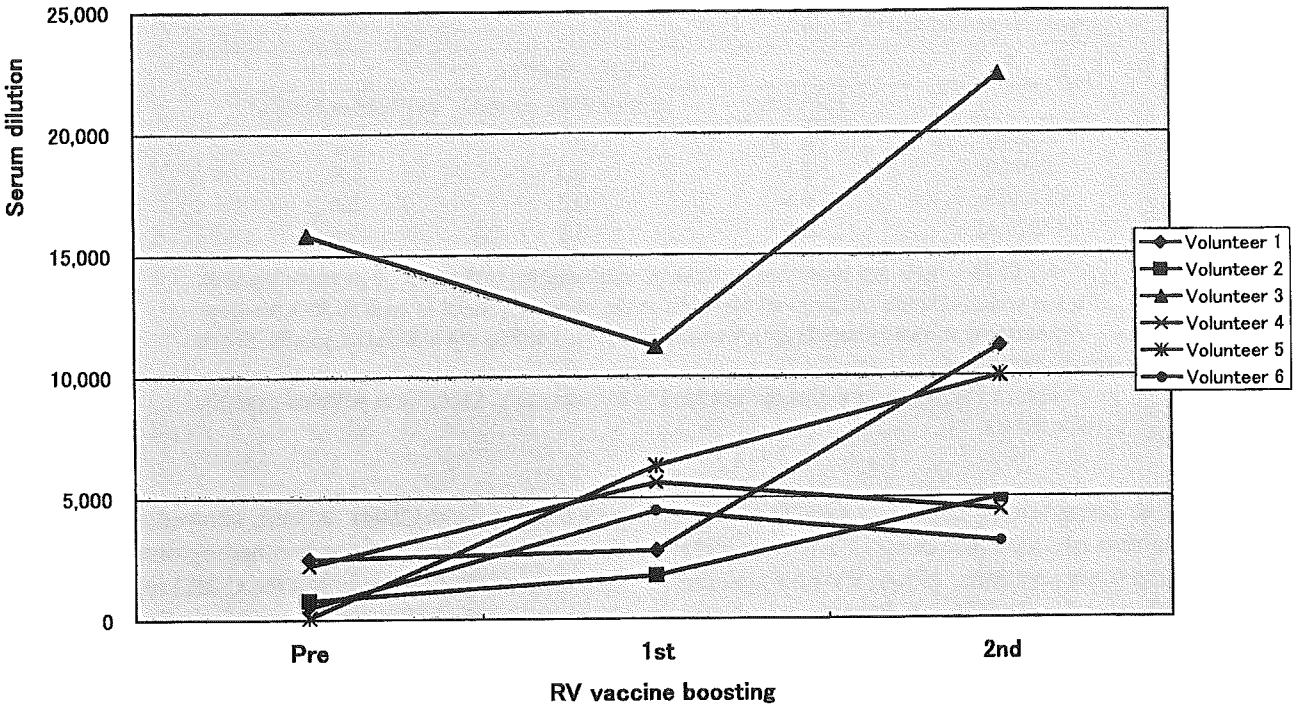

Fig. 2. A profile of developed neutralizing antibody titers of 6 volunteers against rabies virus (RV) (Nishigahara strain) is depicted. The volunteers were boosted 2 times with RV vaccine (HEP Flury strain) at a 2-week interval. At 2 weeks after each boosting, the volunteers were bled and the antibody titers were determined by the rapid fluorescent focus inhibition test (RFFIT) using BHK-21 cells. The titer is expressed as the reciprocal of the endpoint serum dilution demonstrating a 50% reduction in the number of fluorescent foci. Each volunteer had a background of RV vaccination.

Table 2. Rabies virus neutralizing activity of Fab preparations

Reagent	Dilution	Focus counts (% reduction)[a]
Virus control	—	46
RV vaccinated serum control	1:1,024	0 (100)
Fab preparations:		
Di6D3[b]	1:2	58 (0)
EP1C6[c]	1:2	51 (0)
EP5G3	1:2	11 (76)
	1:4	37 (20)
GD2D12	1:2	20 (57)
	1:4	27 (41)

Rapid fluorescent focus inhibition tests (RFFIT) using strain CVS (challenge virus standard) were performed. Data shown in focus counts are the average of duplicated tests. The Fab preparation EP5G3 and GD2D12 was expressed and prepared from a clone isolated from the phage pool panned against RV virions and the purified RVG-protein, respectively.

[a] % reduction was calculated as (number of foci of virus control−number of foci in each reaction)/number of foci of virus control.

[b] Human Fab preparation that did not bind to RV was used. Di6D3 was selected from the same library.

[c] Human Fab preparation that bound to RV shown in ELISA was used.

10 nonsuppressor *E. coli* strains were transformed for the expression of soluble Fabs. Twenty bacterial clones from the library panned against RV virion and 112 bacterial clones from the library panned against the RVG-protein were selected as soluble Fab-producing clones. Those 132 soluble Fab-producing *E. coli* cultures were expanded to 50 ml to prepare Fab preparations (see "Materials and Methods"). Fifteen Fab antibody preparations out of 132, showing a comparatively high ELISA titer against anti-human IgG $F(ab')_2$, were selected for examination for RV-neutralizing activity.

RV-Neutralizing Activities

Fifteen Fab antibody preparations were examined for neutralization activity against RV strain CVS. The Fab preparation EP5G3, which was isolated from the phage pool panned against RV virions exhibited neutralizing activity against RV strains CVS with a reduction in the infected cell count of 76% at dilution of 1:2, and of 20% at 1:4. The Fab preparation GD2D12, a clone isolated from the phage pool panned against the purified RVG-protein, also exhibited neutralizing activity against the same strain with a 57% reduction at 1:2 and 41% reduction at 1:4 (Table 2). Representative neutralizing activities of two Fab preparations are shown in Fig. 5. The FITC-stained cells infected with RV incu-

Table 3. Binding capacity or Fab preparations to rabies virus virions and purified rabies virus G protein as determined by ELISA

Fab preparation	ELISA titer[a] to:		
	RV virion	purified RVG-protein	anti-human $F(ab')_2$[b]
Di6D3[c]	0	0	2.2
EP1C6	2.1	0.2	2.4
EP5G3	0.8	0.2	2.4
GD2D12	0.2	0.2	1.8

[a] All ELISA titers are presented as (titer of each sample)−(the titer of negative control). Bovine serum albumin (500 μg/ml) was used as the negative control in each ELISA reaction.

[b] Reactivity to anti-human $F(ab')_2$ was determined to confirm the amount of soluble Fab in each preparation.

[c] Human Fab preparation that did not bind to RV was used. Di6D3 was selected from the same library.

bated with Fab preparations EP5G3 or GD2D12 were reduced in number and their size of foci. The remaining 13 Fab antibody preparations, either RV-non specific clones represented by Di6D3, or RV-specific clones represented by EP1C6 (Table 3), failed to show any neutralizing activity against the same RV strain (Table 2).

Determination of RV-Binding Capacity and Antigen Specificity of RV-Neutralizing Fab Preparations

ELISA titers of the RV-neutralizing Fab preparation EP5G3 and GD2D12 against RV virions were no more than 0.8, whereas the Fab preparation EP1C6, a RV-non neutralizing Fab preparation, showed a higher titer (Table 3). ELISA titers against the purified RVG-protein were no more than 0.2 among the preparations tested. The binding activities of the Fab preparation EP5G3 and GD2D12 were analyzed by the co-immunoprecipitation to determine RV proteins with which Fab preparation EP5G3 or GD2D12 interacts. A ca. 67-kDa band similar to the molecular weight of the RVG-protein was detected in a reaction with the Fab preparation EP5G3 or GD2D12 when the SDS-PAGE gel-separated immunocomplexes were reacted with the anti-RVG-protein monoclonal antibody (Fig. 3, lane 2 and lane 3). However, no bands in the reactions were detected when the immunocomplexes were reacted with the anti-RVN-protein monoclonal antibody (Fig. 3, lane 5 and lane 6). Those results indicated that the RVG-proteins were incorporated in the immunocomplexes precipitated in a reaction with the Fab preparation EP5G3 or GD2D12.

Sequence Analysis

Sequence analysis of the V_κ and V_H DNA inserts of the two RV-neutralizing Fab clones were performed and compared. The sequences in the heavy chain complementary-determining region 3 (CDR3) critical for antigen binding, and CDR1 as well, showed a greater difference than the sequences in other regions between the two clones. The sequences in the CDR2 region, however, were identical (Fig. 4). A sequence similarity search of the available human immunoglobulin genes was conducted to determine the specific germ line origin of those RV-neutralizing Fab clones. Both EP5G3 and GD2D12 used a V_H sequence homologous to the V_H3 family. EP5G3 used a V_κ sequence homologous to the V_κIII family whereas GD2D12 used a gene homologous to the V_κII family. The nucleotide sequences of heavy and light chains of EP5G3 or GD2D12 have been deposited in DDBJ under accession numbers AB190182, AB190183, AB190184, and AB190185, respectively.

Discussion

In this study, we have constructed a combinatorial phage Fab antibody library using RNAs from peripheral blood lymphocytes of 6 rabies vaccinated volunteers. The constructed phage library was selected against purified RV virions or purified RVG-protein for the isolation of phages displaying RV-neutralizing human Fab antibody. Among selected clones, two Fab preparations EP5G3 and GD2D12 revealed neutralizing activities against RV strain CVS when assayed in RFFIT. In the co-immunoprecipitation analysis, the RVG-proteins were revealed to be incorporated in the immunocomplexes precipitated in reactions with both Fab preparations.

Sequential boosting of the recruited volunteers with the rabies vaccine was considered to be essential in order to isolate Fab preparations with RV-neutralizing activities in that it was not easy to isolate antigen-binding Fabs from an individual with a high anti-tetanus toxoid titer who had not been boosted recently (16, 18). Some volunteers showed comparatively high serum-

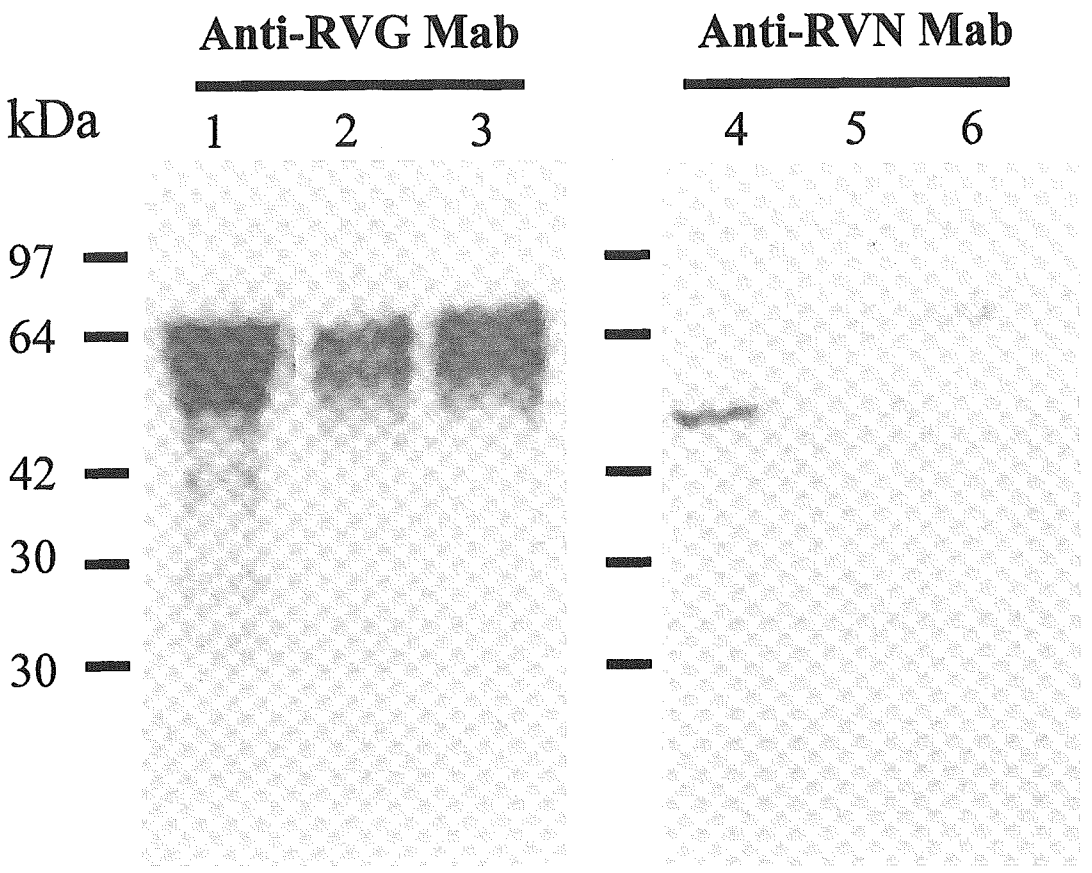

Fig. 3. Co-immunoprecipitation was performed to determine rabies virus (RV) proteins with which Fab preparations interacted. The result of the subsequent western blotting analysis is shown. In the co-immunoprecipitation, RV strain CVS were reacted with Fab preparations EP5G3 or GD2D12, then goat anti-human IgG F(ab')$_2$ antibody was added. Immunoprecipitates were collected by protein G conjugated-sepharose beads and subsequent centrifugation. The immunocomplexes were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, and analyzed by western blotting. Anti-RVG-protein specific monoclonal antibody 4-2 (Anti-RVG Mab, 1:5,000 diluted) and Anti-RVN-protein specific monoclonal antibody Gifu 8-1 (Anti-RVN Mab, 1:5,000 diluted) were used as the primary antibodies and peroxidase conjugated goat anti-mouse IgG (1:2,000 diluted) was used as the secondary antibody. Culture fluid of RV strain CVS was used as a control for demonstrating a band corresponding to the RVG-protein (lane 1) or the RVN-protein (lane 4). Immunocomplexes precipitated with the Fab preparation GD2D12 were applied in lane 2 and lane 5, and immunocomplexes precipitated with the Fab preparation EP5G3 were applied in lane 3 and lane 6. Note that a 67-kDa band corresponding to the molecular weight of the RVG-protein was observed specifically in the reactions with two Fab preparations when reacted with Anti-RVG Mab (lane 2 and lane 3); however, no bands were detected in the reactions when reacted with Anti-RVN Mab (lane 5 and lane 6).

neutralizing antibody titers against RV strain Nishigahara in the pre-boosting period (Fig. 2). However, antigen-specific lymphocytes with a higher concentration of specific mRNA were expected to be elicited by sequential boosting, in which the isolation of Fabs with RV-neutralizing activity would be more likely.

Among 15 Fab preparations tested, EP5G3 and GD2D12 exhibited neutralizing activity against RV strain CVS when assayed in RFFIT. To our knowledge, this is the first report describing the isolation of human monoclonal Fabs with neutralizing activities against a RV strain assayed *in vitro*, using the combinatorial phage display library method. Neither preparation was able to shut off the RV infection even at the highest concentration (Table 2). One possible interpretation would be that the amount of genuine Fab molecules included in Fab preparations was not large enough; hence, we tried to purify Fab molecules from the EP5G3 or GD2D12 clone so as to increase the Fab concentration. A histidine tag was inserted in the carboxy-terminal of the Fd product in the pComb3X vector for efficient protein purification; however, several attempts to purify Fab molecules were not successful using a metal affinity resin column kit (Clontech) or an automated column chromatography system (Acta Prime, Amarsham), or protein G affinity column aiming at

(A)

	FR1	CDR1	FR2	CDR2
EP5G3	- - VMTQSPATLSLSPGERA TLSC	RASQSVRNYLA	W YQQKPG QA P RL L I Y	GASN RAT
GD2D12	EL· · · · · ·LS·PVTL·QP· SI · ·	·S · · · LI HS NGNTYLN	· F· · R· · · S · ·R · · ·	KV·D ·DS

	FR3	CDR3	FR4
EP5G3	GI PARFSGSG SGTDFT L T I SSL E PED F A VYY	CQQR SDWPLS	FGQG T KL E I KR T
GD2D12	·V·D· · · · · · · · · · · · · K··RV · A· · VG · · ·	·L · GTH · ·RT	· · · · · · · · · · · · ·

(B)

	FR1	CDR1	FR2	CDR2
EP5G3	GPAWEVPETLPVQPLDSPSV	AMACT	GVRQAPGKGLEWVA	VISYDGSNKYYADSVKG
GD2D12	VVQPGRSLR·SCAASGFTFS	SY · MH	W· · · · · · · · · · · · ·	· · · · · · · · · · · · · · · · ·

	FR3	CDR3	FR4
EP5G3	RFT I SRDNSKNTLYLQMNSLRAEDTAVYYC	AKDSNS GS Y Y AH DL G Y- - - -	WGQGTLVTV-
GD2D12	R· ·	·R · KALAA R RGYYY Y YGTDV	· · · · · · · · ·S

Fig. 4. Alignment of amino acid sequences of RV-neutralizing Fab monoclonal antibodies. The amino acid sequences of the two Fab monoclonal antibodies recovered by repertoire cloning were compared. (A) Sequences of V_κ segments. (B) Sequences of V_H segments. The framework regions (FR1–4) and complementarity-determining regions (CDR1–3) are shown. The dash symbol is placed where an amino acid deletion occurred and an identical amino acid is represented by a dot.

trapping the Fd portion of Fab molecules (9). It is notable that the sizes of foci were reduced in the neutralizing assays with the Fab preparation GD2D12 or EP5G3 (Fig. 5), indicating that the cell-to-cell spread of RV was somewhat interfered with by those Fab preparations but not enough to abolish focal formation completely (3).

ELISA titers of the two RV-neutralizing Fab preparations against RV virions or RVG-protein were less than 0.8 (Table 3). Different from those two clones, the Fab preparation EP1C6, a non-RV neutralizing clone, showed a much higher ELISA titer for RV virions. Taken together, the epitope(s) of RV virions associated with the neutralizing activity of isolated Fab molecules are probably not directly correlated with their binding specificity for RV antigens detected by ELISA. Champion et al. demonstrated, however, that the ability of the monoclonal antibodies they prepared from mouse-human heterohybrids system to neutralize RV strains *in vitro* correlated with their binding specificity for these viruses in ELISA (6). A neutralizing epitope recognized by their antibodies was probably different from the one EP5G3 or GD2D12 recognize, possibly due to differences in preparative methods.

In the co-immunoprecipitation analysis, a band of ca. 67-kDa was specifically observed in lanes with immunocomplex precipitated in a reaction with RV-neutralizing Fab preparation EP5G3 or GD2D12 when anti-RVG-protein monoclonal antibody was used as the primary antibody in the subsequent western blotting analysis (Fig. 3, lane 2 and lane3). However, no bands in the reactions were detected when the immunocomplexes were reacted with anti-RVN-protein monoclonal antibody (Fig. 3, lane 5 and lane 6). Those results indicated that the RVG-protein but not the RVN-protein was incorporated in the immunocomplexes precipitated in reactions with those two Fab preparations. The incorporation of the RVG-protein was Fab dependent, because the ca. 67-kDa band was not detected in the reactions without the Fab but with all other components (data not shown). Considering the results that the two Fab preparations showed neutralizing activity against a RV strain and the fact that the neutralizing activities have been reported to be closely related to the RVG-protein (29), it is most likely that the Fab in each preparation interacted directly with the RVG-protein and was captured by the protein G-conjugated sepharose beads via anti-human IgG $F(ab')_2$.

The phagemid vector pComb3X, provided by Dr. C. Barbas III, was used for construction of the human Fab library. Usage of the vector was advantageous in regards to the following: 1) one-step Fab gene cloning was available (Fig. 1) in place of conventional step-wise gene cloning where the heavy chain and the light chain

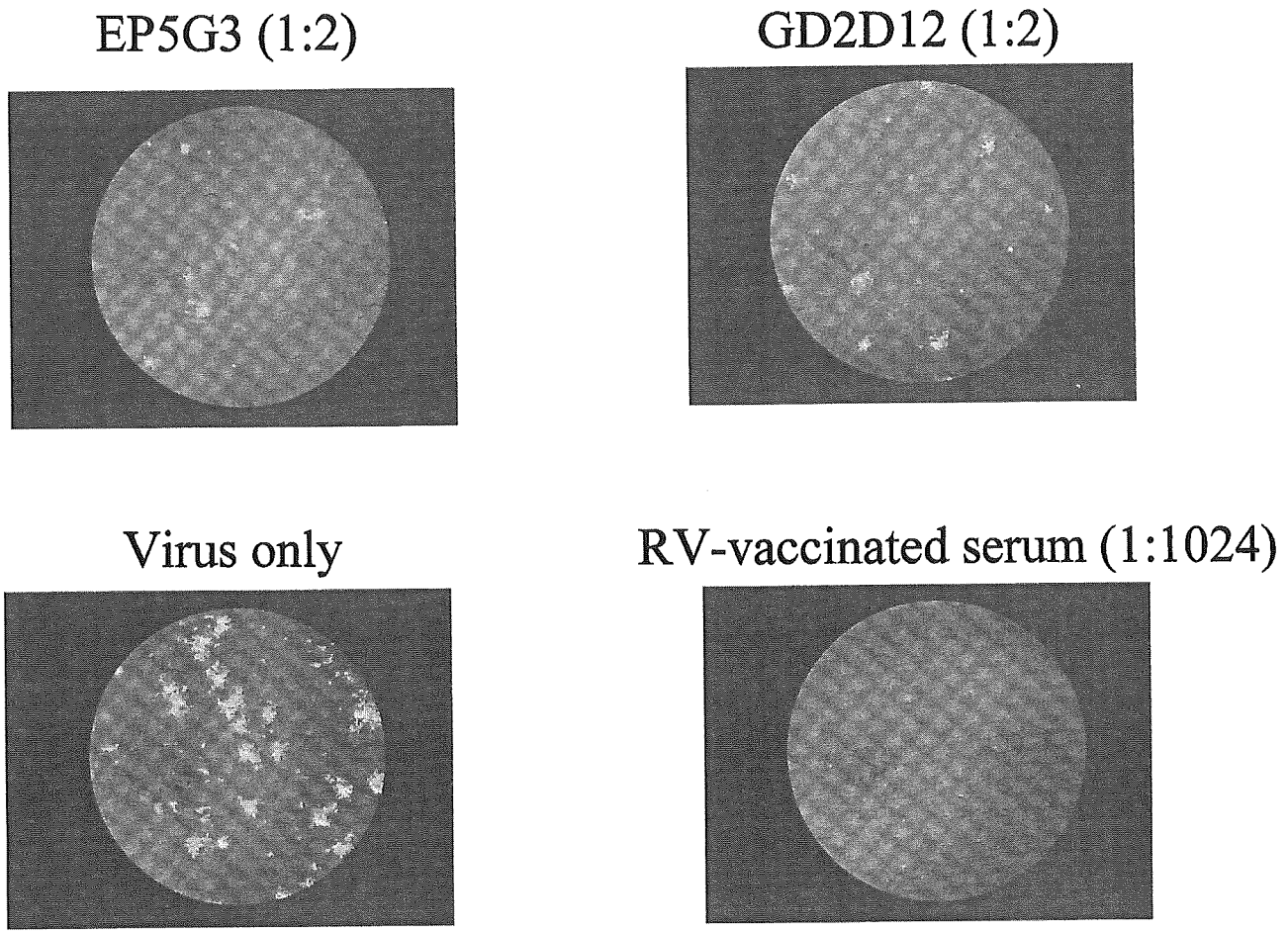

Fig. 5. Representative neutralizing activities of Fab preparations EP5G3 and GD2D12 toward rabies virus (RV) strain CVS, by the rapid fluorescent focus inhibition test (RFFIT). Note that the FITC-stained cells infected with RV treated with Fab preparations EP5G3 or GD2D12 are reduced in number and size of foci.

were cloned sequentially, 2) the gene III removal was not needed in preparing soluble Fab when the *E. coli* Top 10 strain was used, and 3) histidine-tag was available for purifying large-scale Fab samples (21).

A moderate-sized human Fab phagemid library was constructed in the current study. It has been reported that an enormous library was essential to isolate Fabs with biological activity from a human "naïve" library (5, 24). The isolation of Fab with high specificity and affinity for the antigen is possible even if the size of the library is not enormous in the case of an "immune-library," described in the current study, because peripheral lymphocytes with antibody repertoires roughly oriented to an antigen-specific antibody group were used to construct the Fab library.

Ray et al. described two RV-neutralizing scFv-Fc fusion proteins isolated from a human synthetic scFv phage display library (19). The molecules revealed neutralizing activity against the RV PV strain after fusing to the human Fc protein, in a standard *in vivo* neutralization assay where the virus was incubated with the molecules before inoculation in mice. Conversion of the Fab construct EP5G3 or GD2D12 to whole IgG by providing a constant region may confer a more efficient neutralizing activity against a RV strain. Increased valency and possible structural stability would contribute to the improvement of avidity.

We are grateful to Dr. C. Barbas III, the Scripps Research Institute, for providing pComb3X, and pComb3XTT vector.

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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.,

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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′-CCATCATGACCACCAAGTC-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′-AGGAAAGATG(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

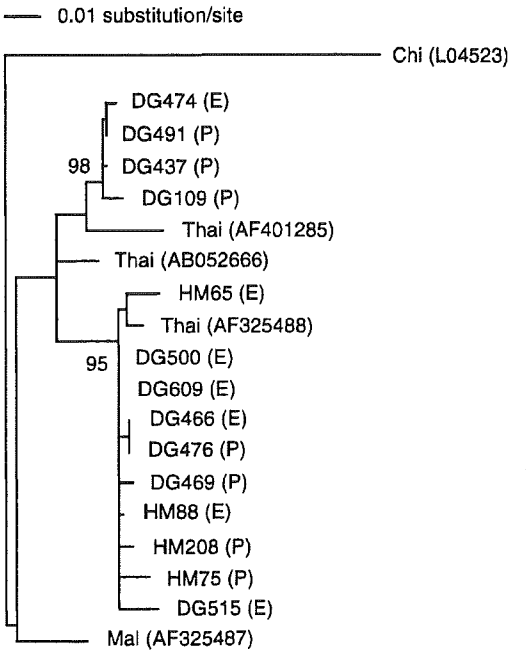

Fig. 1. Phylogenetic tree of the G gene in dog rabies viruses circulating in Bangkok. Genetic sequence distance was determined by the neighbor-joining method. The bootstrap percentages of 1000 replicates were presented for key nodes only. Chinese dog strain (accession no. L04523) was used as an outgroup. 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).

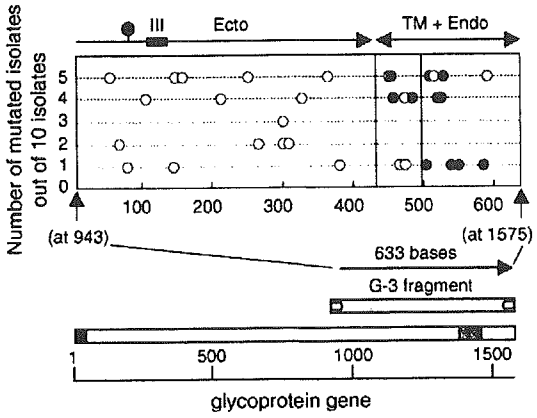

Fig. 2. Distribution of base substitutions in the G gene among 10 dog rabies virus isolates. The PCR products of 3′-half of G gene (G-3 fragment; 653 bases) were sequenced in 10 virus isolates. The position of base substitutions were plotted as synonymous mutation (open circle) and nonsynonymous mutation (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

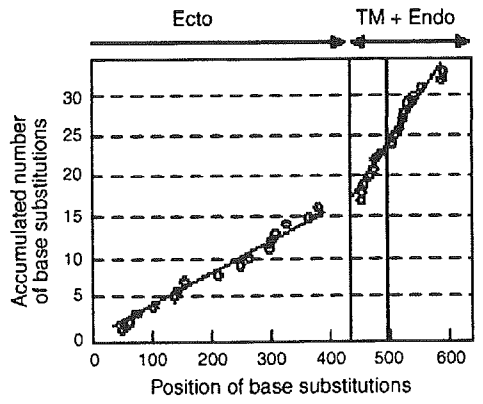

Fig. 3. Analysis of mutation rate in the Ecto and TM + Endo domains. Accumulated number of base substitutions was plotted with the mutated position and compared between endodomain and TM + endodomain among 10 dog rabies virus isolates.

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 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.

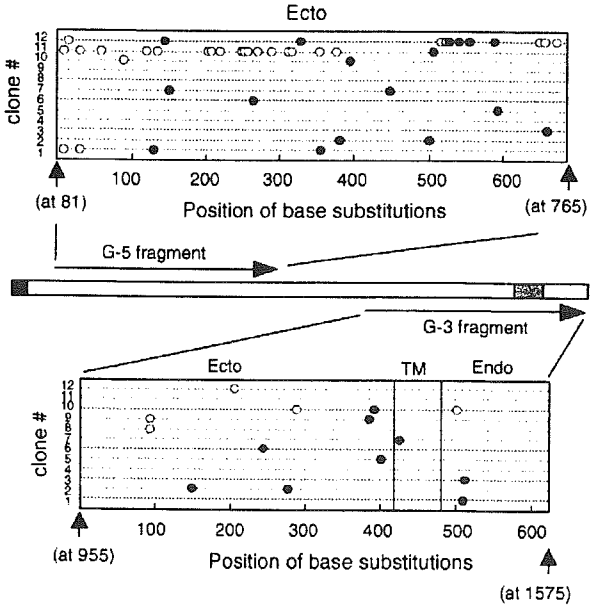

Fig. 4. Mutated positions of 12 clones obtained from DG476 virus isolate. Two parts of G gene were sequenced in each of 12 cloned plasmids (accession nos. AB195185–AB195210). The position of base substitutions was plotted as synonymous mutation (open circle) and nonsynonymous mutation (closed circle).

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.

Acknowledgements

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A novel rapid fluorescent focus inhibition test for rabies virus using a recombinant rabies virus visualizing a green fluorescent protein

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Abstract

Virus-neutralizing antibodies (VNAs) against rabies virus play a major role in protection from rabies. The rapid fluorescent focus inhibition test (RFFIT) has been internationally recognized as a standard in vitro test for measuring the VNA. CVS strain of rabies virus is used as challenge virus and the infected cells are indirectly detected by staining with fluorescein isothiocyanate (FITC)-conjugated rabies antibody which is expensive and high-quality products are often in short supply. In this study, a recombinant rabies virus strain carrying a green fluorescent protein (GFP) gene, rHEP-GFP, was used as a challenge virus in the virus neutralization assay. Expression of the GFP could be readily detected in the infected cells under a fluorescent microscope. This novel RFFIT modification RFFIT-GFP is a neutralization test, and it is based on the sound principle of the standard RFFIT using 96-well plates. VNA titers in 25 human, 18 canine and 15 horse sera have been compared between the RFFIT and RFFIT-GFP methods. The results obtained by the both methods showed good agreement between both methods in all sera investigated (coefficient of correlation, $r = 0.98$). It allowed direct detection of virus by expression of GFP and might be applicable for other viruses. The novel method is convenient, economical and a reliable tool not requiring expensive FITC-conjugated antibody for routine rabies VNA assays.

Keywords: Rabies virus; Recombinant rabies virus; GFP; Virus-neutralizing antibody test

1. Introduction

The measurement of virus-neutralizing antibody (VNA) against rabies virus is an indispensable and important technology to determine the levels of neutralizing antibodies. Without such a test, it is virtually impossible to evaluate new vaccines or administration schedules investigating immunogenicity and efficacy. The rapid fluorescent focus inhibition test (RFFIT) is the most widely method approved by WHO that measures the virus-neutralizing antibody content in the sera (Smith et al., 1996). The reduction of infectious foci in virus-infected cells incubated with serum is detected under a fluorescent microscope by fluorescent antibody staining. The RFFIT has several advantages over the original mouse neutralization test (MNT) which is still widely used in developing countries. It requires less manpower, laboratory and animal care space and is far less time-consuming. A signif-

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icantly larger number of samples can be tested at one time. After several comparative studies between the in vitro RFFIT and the in vivo MNT, RFFIT has been recommended as a replacement for the MNT (Meisner et al., 1997). British Pharmacopeia (BP) and United States Pharmacopeia (USP) accepted this technique for evaluating the potency of equine and human rabies immunoglobulin (ERIG and HRIG) preparations (British Pharmacopeia, 2001; United States Pharmacopeia, 2002). The level of VNA titers provides an estimate of vaccine efficacy, an indication of silent disease prevalence in animals and rarely even the ante mortem diagnosis of rabies infection of humans (Phanuphak et al., 1987; Kasempimolporn et al., 1991). WHO and the Office International des Epizooties (OIE) recognized the RFFIT to evaluate and to certify the level of VNA (>0.5 IU/ml) prior to allowing animals to enter rabies-free countries which can significantly reduce quarantine periods (OIE, 1996). A new technique (the RFFIT-GFP method) based on manipulation of the rabies genome and creation of a recombinant virus (rHEP-GFP strain) containing a gene which has the ability to visualize the fluorescence under a fluorescent microscope with a normal blue excitation filter is described in this paper. Previous authors have shown differences in VNA assay levels by using different strains of virus (Barth et al., 1988; Blancou et al., 1989). Taking note of this, VNA titers obtained using RFFIT-GFP with rHEP-GFP strain were compared with those obtained using the established RFFIT with CVS-11 strain (Smith et al., 1996) in human, canine and horse sera obtained from the frozen sample banks at the Queen Saovabha Memorial Institute of the Thai Red Cross Society.

2. Materials and methods

2.1. Virus and cells

Neuroblastoma NA cells of A/J mouse origin were grown at 37 °C in minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). BHK-21 cells were grown at 37 °C in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% FBS.

Challenge virus standard (CVS-11) strain was used in the RFFIT as a challenge virus. The recombinant rescue system from cDNA was based on HEP-Flury strain in this study. To compare the growth characters, the original wild-type HEP-Flury strain (wHEP) and the cDNA-derived strain (rHEP) were used as control. The rHEP was generated from full-length cDNA of wHEP strain, using an improved cDNA recovery method (Inoue et al., 2003). The sequence of the full-length cDNA of the HEP-Flury strain has been deposited in the GenBank database (accession no. AB085828).

2.2. Construction of recombinant rabies virus containing GFP gene and recovery of recombinant virus

Construction of the rabies virus full-length cDNA vector (pHEP-3.0) was described elsewhere (Inoue et al., 2003). A PCR fragment of EGFP cDNA (Clontech Co., CA, USA) was amplified using the following primers: SpB-5 (5′-ATTCGTACGACCATGGTGAGCAAGGGCGAGGAG-3′, *Bsi*WI site and start codon underlined) and SCPs-3 (5′-GTTCTGCAGTTACTTGTACAGCTCGTCCATGCC-3′, *Pst*I site and stop codon underlined). The PCR product was digested with *Bsi*WI and *Pst*I, and inserted between G gene and L gene region in the full-length cDNA vector. The resulting plasmid was designated p3.0-GFP. The recombinant virus was rescued as described elsewhere (Inoue et al., 2003). Briefly, BHK-21 cells were grown overnight to 80% confluency (around 4×10^5 to 6×10^5 cells per well) in 6-well culture plates in D-MEM supplemented with 10% FBS. Culture supernatants were replaced with fresh medium before transfection. The cells were transfected with 2.0 μg of p3.0-GFP plasmid, 0.5 μg of pH-N, 0.25 μg of pH-P, 0.1 μg of pH-L and 0.15 μg of pH-G (pH-N, pH-P, pH-L and pH-G are helper plasmids that express viral N, P, L and G proteins by CMV promoter, respectively), using TransIT LT-1 (Takara Shuzo Co., Japan). After 16–24 h, the cells were washed once and maintained in D-MEM supplemented with 10% FBS for a further 2–5 days. The culture supernatant was transferred onto BHK-21 cell monolayers and incubated for 3 days at 37 °C. The culture supernatant was harvested and titrated for preparation of virus stock for further experiments. The rescued virus generated from the recombinant plasmid p3.0-GFP was designated rHEP-GFP virus. Nucleotide sequence analysis confirmed that GFP gene was inserted in the viral genome, indicating that the rescued virus was correctly derived from the cDNA of p3.0-GFP (data not shown).

2.3. Virus infection and titration

To determine virus yield, monolayers of NA cells in 96-well plates were infected with serial 10-fold dilutions of virus suspensions and incubated at 37 °C as described (Wiktor et al., 1984). At 48-h post-infection, cells were fixed in 80% acetone and stained with FITC-labeled rabies virus N protein-specific antibody (Centocor, Inc., PA, USA). Foci were counted under a fluorescence microscope and calculated as focus forming units/ml (ffu/ml).

2.4. Serum samples

Twenty-five human sera and 18 dog sera were taken from serum bank at Queen Saovabha Memorial Institute. The volunteers had received rabies pre-exposure immunization with PCEC vaccine (Purified Chick Embryo Cell rabies vaccine; LEP-Flury strain, Chiron, India) as part of a prior study. These sera were used with the permission from the volunteers under GCP guidelines. All dogs received rabies vaccination with Rabisin (PV-11 Pitman-Moore strain, Rhone Merieux, France). Fifteen horse serum samples were taken from horses which were regular donors for ERIG production at the Thai Red Cross Horse Farm. These horses received rabies vaccina-

tion with PCEC vaccine 3–4 times annually. Serum samples from volunteers, horses and dogs above 3 months of age who all had never been vaccinated were used as negative controls. All serum samples were stored at −20 °C and were heat-inactivated at 56 °C for 30 min prior to testing.

2.5. Virus-neutralizing test

The standard RFFIT was carried out as modified from Smith et al. (1996) as follows. Briefly, 0.05 ml of serial two-fold dilutions of human or horse serum was prepared in 96-well plates. Thirty to 100 $TCID_{50}$ (50% tissue culture infectious does) of CVS-11 virus was added to each serum dilution and incubated in 5% CO_2 incubator at 37 °C for 90 min. After incubation, 0.05 ml of BHK-21 cells (5×10^5 cells/ml) were added into each well of the serum–virus mixtures, and the culture plates were incubated in CO_2 incubator at 37 °C for 21 h. Low dilutions of canine serum sometimes exhibit toxicity to the cells. Therefore, the previously developed method was applied for canine sample testing as follows. After NA cells (5×10^5 cells/ml) were added to the virus–serum mixtures, cells were allowed to settle and attach to the plate for 1 h. Then, the media containing dog serum was removed and replenished with fresh media. The cultures were incubated in CO_2 incubator at 37 °C for 21 h. All culture plates were fixed with 90% acetone, dried and stained with FITC-labeled anti-rabies antibody (Centocor). Serum titers were determined by serum dilution at 50% fluorescent focus reduction in the infected cell cultures under a fluorescent microscope. The 50% endpoint of antibody content of standard serum and test serum were calculated according to the Spearman–Kärber method (Spearman, 1908; Kärber, 1931). Values were compared with the WHO standard serum and were expressed in international units as IU/ml. The Second International Standard for anti-rabies immunoglobulin (human origin; NIBSC, UK) contained 30 IU of rabies antibodies was diluted to contain 1 IU/ml and then aliquoted sufficient for one test series and stored at −20 °C.

2.6. RFFIT-GFP method

The rHEP-GFP, derived from the HEP-Flury strain, was used as a challenge virus for the VNA assay. The RFFIT-GFP method by counting GFP-expressed foci of infected cells. They were counted using a fluorescent microscopy without acetone-fixation and the conjugate staining step. Briefly, two-fold dilution of standard, negative and test sera were conducted to 96-well microplates. Fifty microliters of 30–60 $TCID_{50}$ of rHEP-GFP were used as challenge dose. The microplates were then incubated for 90 min as serial dilutions of the serum samples. Following incubation, 0.05 ml of BHK-21 (4×10^5 cells/ml) for human and horse sera or NA cell (5×10^5 cells/ml) for dog sera were added to each well. Plates were incubated in 5% CO_2 incubator at 37 °C. The culture plates with dog sera were replenished with fresh media in the same manner as with the standard RFFIT method described above. After 44–48 h incubation, the media were discarded. Then, 0.03 ml of PBS with rhodamine or Evans blue was added in each well for reading without fixation. Expressed GFP was detected under a fluorescent microscope with a blue-excitation filter as FITC fluorescence. VNA titers were also obtained using the FITC-method that was performed after fixation with 90% acetone and stained with FITC-labeled anti-rabies antibody in which fluorescent foci of infected cells was detected in the same manner as with the standard RFFIT. The VNA values obtained with three different methods were compared and analyzed statistically. The validity of each test was accessed by recoding results obtained from back-titration of rabies virus challenge dose with negative control serum and WHO standard serum.

3. Results and discussion

3.1. Growth characteristics of the recombinant rabies virus carrying GFP gene (rHEP-GFP)

The GFP gene was inserted between G and L gene of the virus genome in HEP-Flury strain using a reverse genetics technique. The constructed rHEP-GFP virus expresses an additional GFP mRNA in addition to five viral mRNAs. At first, the effect of the insertion of an additional GFP gene and expression of GFP on growth properties of the recombinant virus was examined. The progeny production was examined in the infected NA cells (Fig. 1A). The virus carrying an additional GFP gene (rHEP-GFP) showed similar growth properties as the parental wild-type virus (wHEP) and cDNA-derived virus (rHEP).

Expression of GFP in the cells infected with rHEP-GFP was shown in Fig. 1C. The GFP was distributed in cytoplasm and the nucleus of infected cells 48 h after infection. On the other hand, viral N antigen was seen exclusively in cytoplasm with granular staining pattern (Fig. 1B). GFP was expressed in the virus-infected cells and was easily detected without fluorescent staining by the use of a blue-excitation filter under a fluorescent microscope appearing as green fluorescence (Fig. 1D). This method created a technology that eliminated the need for a conjugated fluorescent antibody.

3.2. Comparative analysis of VNA titers determined by FITC fluorescence and GFP expression

VNA titers on the same serum were determined by using three different methods. (1) The standard RFFIT using CVS-11 strain as a challenge virus and detection with FITC-conjugated antibody. (2) The FITC-method using rHEP-GFP as a challenge virus and detection with FITC-conjugated antibody. (3) The RFFIT-GFP method using rHEP-GFP as a challenge virus and detection with GFP expression without using FITC-conjugated antibody. Because the accumulation of GFP in infected cells can be read clearly after 35 h, several points of the experimental protocol; the number of input

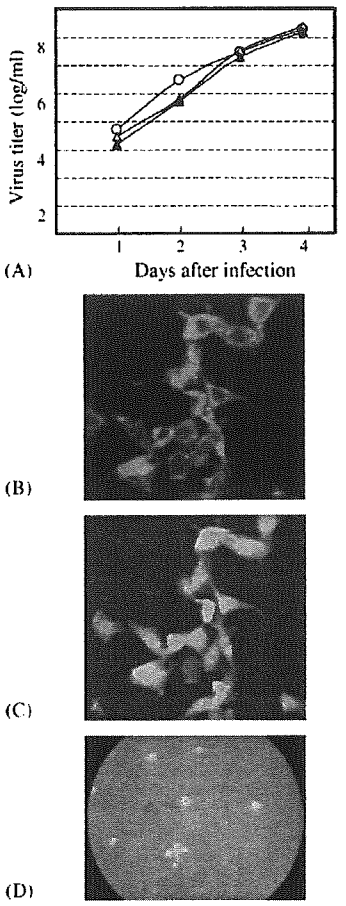

Fig. 1. (A) Viral growth of rHEP-GFP virus. Mouse neuroblastoma NA cells were infected with wHEP (open circle), rHEP (open triangle) or rHEP-GFP virus (closed triangle) at MOI = 0.01. At different time points, supernatants of the culture fluids were harvested, and subjected with virus titration assay. (B, C) Expression of viral N protein and GFP. BHK-21 cells were infected with rHEP-GFP at MOI = 10. At 2 days post-infection, the infected cells were fixed with 4% paraformaldehyde, and subjected with indirect immunofluorescent staining. Expression of viral N protein (B) (staining with rhodamine-conjugated anti-rabbit IgG and rabbit anti-N antibody) and expression of GFP (C) were detected on the rHEP-GFP virus-infected BHK-21 cell under a fluorescent microscope. The pictures of the same field were taken with different excitation/emission filter sets. (D) Foci of GFP-expressed cell in RFFIT-GFP method. NA cells were inoculated with virus–serum mixture in 5% CO_2 incubator at 37 °C for 44 h, then stained with Evans blue without fixation. GFP expression was detected under a fluorescent microscope with excitation/emission filter for FITC without using FITC-conjugated antibody.

cell per well, the multiplicity of $TCID_{50}$ and the incubation time were modified and adapted to 44–48 h incubation time. The optimized condition of the RFFIT-GFP method was described in Section 2. The representative picture of foci by RFFIT-GFP was shown in Fig. 1D.

The values of VNA titers with three different VNA tests were seen in Table 1 for 25 human sera (plotted in Fig. 2) and in Table 2 for 15 horse sera. The results obtained on the same sera with three assays showed good agreement. At first, the values of VNA titers obtained with two different detections

Table 1
Virus-neutralizing antibodies titers (IU/ml) of human sera determined by three different methods

Serum No.	RFFIT	FITC	GFP
1	<0.03	<0.03	<0.03
2	<0.03	0.09	<0.03
3	<0.03	0.11	0.18
4	<0.03	0.06	0.15
5	0.07	0.26	0.26
6	0.10	0.26	0.25
7	0.15	0.30	0.21
8	0.38	0.92	0.96
9	0.39	0.46	0.40
10	0.46	0.92	1.19
11	0.52	0.44	0.52
12	1.00	0.71	0.92
13	1.36	2.18	2.18
14	5.00	5.91	6.73
15	9.31	11.00	13.63
16	9.58	12.00	17.67
17	10.16	15.00	22.93
18	10.16	25.00	26.11
19	13.75	28.47	27.26
20	14.03	15.52	14.23
21	15.00	31.08	28.47
22	15.21	40.26	45.85
23	17.08	16.93	20.13
24	50.00	52.21	56.94
25	250.00	284.70	284.70

using FITC-antibody and GFP expression were compared. Linear regression analysis of the data was performed by the least-square method. Results are shown in Fig. 3A and B. The regression line in human sera is $y = 1.008x + 0.029$ and the coefficient of determination is 0.981. The regression line in horse sera is $y = 0.942x + 0.102$ and the coefficient of determination is 0.983. There is a significant high correlation between the values obtained by two methods in both human and horse samples. Detection by GFP expression was not significant different from FITC-antibody detection. The detection by GFP expression in which rHEP-GFP virus is used as a challenge virus is a reliable alternative method for measuring VNA titer.

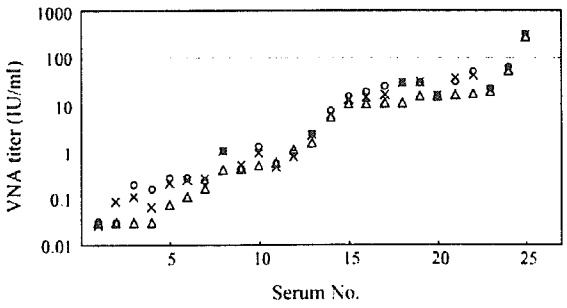

Fig. 2. Virus-neutralizing antibodies titers of human sera determined with three different methods. FITC method using rHEP-GFP virus (cross), RFFIT-GFP (open circle) and the standard RFFIT (open triangle).

Table 2
Virus-neutralizing antibodies titers (IU/ml) of horse sera determined by three different methods

Serum No.	RFFIT	FITC	GFP
1	5.00	3.36	3.22
2	50.00	28.47	32.42
3	262.78	541.70	541.70
4	326.33	417.71	417.71
5	220.97	308.44	295.36
6	441.94	475.68	400.00
7	274.41	541.70	436.20
8	441.94	541.70	475.68
9	185.00	200.00	183.40
10	185.00	113.88	175.62
11	185.81	147.68	135.42
12	211.60	270.85	259.37
13	312.50	766.08	475.68
14	185.00	237.84	200.00
15	240.97	496.74	455.51

3.3. Comparative analysis of VNA titers determined by standard RFFIT and RFFIT-GFP method

The recombinant rabies virus expressing GFP is derived from HEP-Flury strain. Therefore, the rHEP-GFP virus particles are composed of HEP-Flury antigens. The rHEP-GFP virus is used as a challenge virus for VNA assay in the RFFIT-GFP method. On the other hand, the standard RFFIT is performed with CVS-11 strain as a challenge virus. It is well known that values of VNA titer vary depending on the strain of challenge virus (Blancou et al., 1989; Morimoto et sal., 2001). Therefore, the values of VNA titers determined by standard RFFIT and RFFIT-GFP were compared. The seed virus of PCEC vaccine is the LEP-Flury stain, which is closely related to the HEP-Flury strain, on the other hand, the seed virus of Rabisin vaccine is the Pitman-Moore strain, which is closely related to CVS-11 (Sacramento et al., 1992). Therefore, in the RFFIT-GFP, the serum samples of human and horses, which had received PCEC vaccine, were subjected to a homologous strain as a challenge virus (called homologous VNA assay), the serum samples of dogs, which had received Rabisin vaccine, were subjected to a heterologous strain (heterologous VNA assay). In contrast, in standard RFFIT, the human and horse serum samples were subjected to the heterologous VNA assay, the dog serum samples were subjected to the homologous VNA assay. The values of VNA titers in the human and horse sera obtained by standard RFFIT and RFFIT-GFP were statistically analyzed. Linear regression analysis of the data was performed by the least-square method. The result is shown in Fig. 3C. There is a significant correlation between the two assays. The regression line shows $y = 0.932x + 0.239$; coefficient of determination 0.976. The y-intercept is 0.239, which means that the RFFIT-GFP has higher titers than RFFIT in the human and horse sera, which had received PCEC vaccine. Although human sera #1 to #4 had undetectable titers (<0.3 IU/ml) by RFFIT, some of them showed detectable low titers by RFFIT-GFP. A level above 0.5 IU/ml in serum is arbitrarily considered protective (WHO, 1992). Some of the human sera #8 to #11 showed values around 0.5 IU/ml in standard RFFIT and higher ones (above 0.5 IU/ml) in the RFFIT-GFP. It was shown that 0.5 IU/ml in standard RFFIT corresponds to 0.9 IU/ml in the RFFIT-GFP in accordance with the obtained regression line.

Titers of 18 dogs which had received Rabisin vaccine were compared with standard RFFIT and RFFIT-GFP (Table 3) and analyzed statistically (Fig. 3D). In these dog sera, the RFFIT-GFP is a heterologous VNA assay and the RFFIT is homologous. There is a significant correlation between the two assays. The regression line shows $y = 0.891x - 0.123$ with a coefficient of determination of 0.899. The y-intercept is -0.123, which means that the values of VNA titers obtained with the RFFIT-GFP were lower than those with RFFIT. In the dog sera, the value of 0.5 IU/ml with standard RFFIT corresponds to 0.4 IU/ml in the RFFIT-GFP or the value of 0.5 IU/ml with RFFIT-GFP corresponds to 0.6 IU/ml with standard the RFFIT in accordance with the regression line.

In conclusion, there is a significant correlation between standard RFFIT and RFFIT-GFP in all the sera studied in this text. There is a trend in which titers obtained with a homologous VNA assay show higher values than those with the heterologous VNA assay. The VNAs are directed exclu-

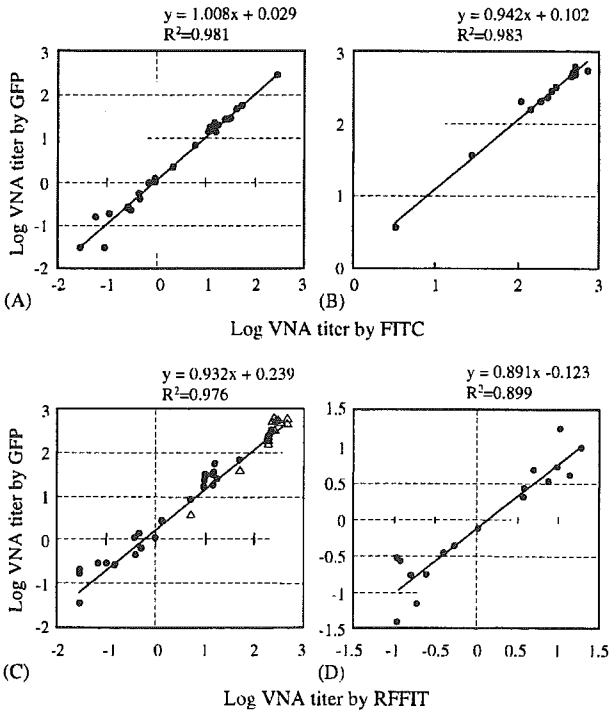

Fig. 3. Correlation of the virus-neutralizing antibodies (VNA) titers in human (A) and horse (B) sera detected with FITC method and RFFIT-GFP. (C) Correlation of the VNA titers in human (closed circle) and horse sera (open triangle) determined by RFFIT and RFFIT-GFP. (D) Correlation of the VNA titers in dog sera detected by RFFIT and RFFIT-GFP.

Table 3
Virus-neutralizing antibodies titers (IU/ml) of dog sera determined by RFFIT and GFP-method

Serum No.	RFFIT	GFP
1	0.11	0.30
2	0.11	0.04
3	0.12	0.27
4	0.16	0.17
5	0.19	0.07
6	0.25	0.18
7	0.25	0.18
8	0.40	0.35
9	0.55	0.44
10	1.06	0.74
11	3.76	2.03
12	3.87	2.73
13	5.03	4.82
14	7.50	3.41
15	9.72	5.26
16	10.59	17.68
17	13.72	4.05
18	19.39	9.64

sively against the surface G protein of rabies virus (Cox et al., 1977). It is suggested that the difference in VNA values between standard RFFIT and RFFIT-GFP is mainly due to the difference in the antigenic structure of G proteins between the challenge virus strain used in the VNA assay and the vaccine strain administrated. This concept has been previously described by others (Blancou et al., 1989; Briggs et al., 1998). Therefore, on adapting RFFIT-GFP method as a routine VNA assay, whether the VNA titer around 0.5 IU/ml is enough protective or not have to be carefully decided in consideration for the characteristic of RFFIT-GFP method studied in this text. It is also the case with the standard RFFIT. The titers near 0.5 IU/ml in the standard RFFIT must be dealt with carefully (Cliquet et al., 1998). The RFFIT-GFP method is a useful and reliable tool for measuring VNA titers. The RFFIT-GFP provides an alternative to the RFFIT and does not require a conjugated antibody for fluorescent staining.

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