Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common human autosomal disorders, affecting approximately 1 per 1000 individuals. It is characterized by formation of multiple abnormal fluid-filled cysts in both kidneys, partly leading to end stage renal failure[1]. ADPKD is genetically heterogeneous with at least three different genes (*PKD1*, *PKD2*, and *PKD3*) responsible for similar phenotypes[2-4]. Abnormality of *PKD1*, which is located on chromosome 16p13.3, is the most common cause of ADPKD, accounting for approximately 85-90 per cent of cases[5], and appears to have a more severe effect with an early age of onset and end stage renal failure[6]. Recent studies have shown that *PKD1* probably occurs from two mutational events, germline and somatic mutations[7,8].

PKD1 has been isolated and characterized (9-12). Its size is 54 kb consisting of 46 exons and its mRNA transcript is composed of 14,148 nucleotides (nt). The predicted protein product, polycystin-1, contains 4,302 amino acids and is proposed to play a role in cell-cell or cell-matrix interaction[11]. Approximately three-fourths of the sequence in the 5' region of *PKD1* is reiterated with about 95 per cent similarity to three highly homologous genes mapped on 16p13.1, whereas, about one-fourth of the sequence in the 3' region is unique[9]. This has complicated the characterization of *PKD1* mutations. Although it is believed that most of the mutations occur in the reiterated region, the majority of mutations identified to date are located within the 3' unique region[9,13-15] and some 82 mutations have been characterized[16]. The rate of mutation characterization has been slow and the number of mutation is too few for analysis of the correlation between type of mutation and variability of the *PKD1* phenotype.

Recently, a long reverse transcription-polymerase chain reaction (RT-PCR) method has been developed for amplification and isolation of the entire *PKD1* coding sequence from peripheral blood lymphocytes[17]. This method is useful not only for the characterization of mutations occurring especially in the reiterated region of *PKD1* but also for the analysis of *PKD1* RNA processing and transcripts, without acquisition of kidney tissue. In this report, we describe a deletion mutation in intron 43 of *PKD1* which resulted in abnormal RNA processing involving exon 43 skipping in the *PKD1* transcripts and also reported the development of a method for direct detection of this mutation in affected family members.

MATERIAL AND METHOD

ADPKD Family and Linkage Analysis

The family described in this study (PK009) was one of approximately 50 Thai ADPKD families that have been identified and followed-up in the Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University. ADPKD was diagnosed according to established clinical and genetic criteria, based on the finding by ultrasonic scanning of polycystic kidneys with at least one cyst in one kidney and two or more in the other[18], and the expression of the disease in members in consecutive generations of the family.

Blood samples (10-15 ml) were taken from ADPKD patients and family members with informed consent. Genomic DNAs were isolated from blood samples by standard method[19] for linkage analysis and PCR amplification. Linkage analysis and haplotype characterization were performed by examinations of five polymorphic markers linked to *PKD1* on chromosome 16p, namely, D16S85 (3' HVR), KG8, SM6, 16AC2.5, and SM7[2,20-23].

RNA Isolation, cDNA Synthesis, and PCR

The methods for RNA isolation, full-length cDNA synthesis, long PCR, and nested PCR have previously been described[17]. Briefly, RNA was isolated from the lymphocytes by TRIzol® reagent (Life Technologies), and used for full-length cDNA synthesis with oligo $(dT)_{12-18}$ primer and RNase H-free reverse transcriptase. *PKD1*-cDNA was amplified by long PCR using a pair of primers (TH1F/TH1B; Table 1) and ELONGASE™ Enzyme Mix (Life Technologies) containing thermostable *Taq* and *Pfu* polymerases. The length of PCR product was 13,634 bp (Fig. 1A).

Nested PCRs were performed by using 9 pairs of nested primers to generate 9 overlapping fragments. Sequences of nested PCR primers (SI9F/SI9B and WT5F/WT5B) used in this study are shown in Table 1. The others are available on request. The nested PCR products were analyzed by electrophoresis on 2 per cent LE agarose gel (FMC Corporation) in Tris-borate-EDTA (TBE) buffer. DNA fragments were stained with ethidium bromide, visualized on UV transilluminator, and photographed.

Table 1. Sequences of PCR primers for amplifications of *PKD1*.

Primer	Primer sequence (5'->3')	Nucleotide position	Location	PCR product size (bp)
Primers for long RT-PCR				
TH1F	CTGGGGACGGCGGGGCCATGCG	175-196[a]	5' UTR	13,634
TH1B	GGCCTGGGGCAAGGGAGGATGACAA	13808-13784[a]	3' UTR	
Primers for nested PCR				
SI9F	CTTCAGCACCAGCGATTACGACGTT	11533-11557[a]	Exon 40	1,650
SI9B	AGAAAGTAATACTGAGCGGTGTCCACTC	13182-13155[a]	Exon 46	
WT5F	TTGGCTGGGAGAGTCCTCACAATG	11556-11579[a]	Exon 40	817
WT5B	AGGGAGTCCACACAGGAAGACACG	12372-12349[a]	Exon 45	
Primers for genomic DNA amplification				
SI9.2F	CGGGCCTCTCGCTGCCTCTGCTCACCTCG	50143-50171[b]	Exon 42	563-758[c]
SI9.2B	ACGGACCACTGGCGCACGAAGCGTAGCTG	50822-50794[b]	Exon 44	
SI9.3F	CGGCCTCGCTGCTCTTCCTGCTTTTGGTC	50678-50706[b]	Exon 43	145
SI9.2B	ACGGACCACTGGCGCACGAAGCGTAGCTG	50822-50794[b]	Exon 44	

[a]The nucleotide positions are according to HUM*PKD1*A, GenBank Accession No. L33243(11).
[b]The nucleotide positions are according to HUM*PKD1*GEN, GenBank Accession No. L39891(10).
[c]Size of PCR product is variable due to numbers of variable repeated sequences in intron 42(14).

The nested PCR product, amplified with SI9F/SI9B, was digested with either *Taq* I plus *Bgl* I or *Pvu* II (New England Biolabs) for detailed analysis of cDNA deletion observed.

Amplification of Genomic DNA

Genomic DNA samples were amplified in a total volume of 25 µl containing 200 ng of genomic DNA, 400 nM of each primer (Table 1), 200 µM dNTP mixture, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.25 unit AmpliTaqGold™ (PE Applied Biosystems), 5 per cent DMSO, and 1.0 mM $MgCl_2$. The PCR was initiated at 95°C for 10 min, then conducted for 2 cycles at 94°C for 30 s, 62°C for 30 s, and 72°C for 30-60 s (depended on size of PCR product). It was then continued for 31 cycles with 2°C reduction of annealing temperature every 2 cycles until reaching the final annealing temperature at 48°C, followed by a terminal extension at 72°C for 10 min.

DNA Sequencing

The PCR product, separated on agarose-gel electrophoresis, was purified using QIAquick™ Gel Extraction Kit (QIAGEN). The purified DNA fragment was sequenced using ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM 310 Automated DNA Sequencer (PE Applied Biosystems).

RESULTS

Linkage Analysis

From analyses of five polymorphic DNA markers in the *PKD1* region on chromosome 16p, 14 haplotypes (with 5 additional variants) were found to segregate in PK009 family and the abnormal *PKD1* allele was linked to haplotype A, which consisted of the following alleles: 2.1 kb for D16S85, 123 bp for KG8, 110 bp for SM6, 169 bp for 16AC2.5, and 88 bp for SM7 (Fig. 3).

Long RT-PCR and Nested PCR

Long RT-PCR and nested PCRs were applied to RNA samples prepared from three affected members (III-1, III-4, and IV-6) of PK009 family, the results of which were mostly normal. However, with nested PCR using SI19F/SI19B primer pair, while a single PCR product (1,650 bp) was observed in samples from normal individuals, products with two different sizes, normal (1,650 bp) and

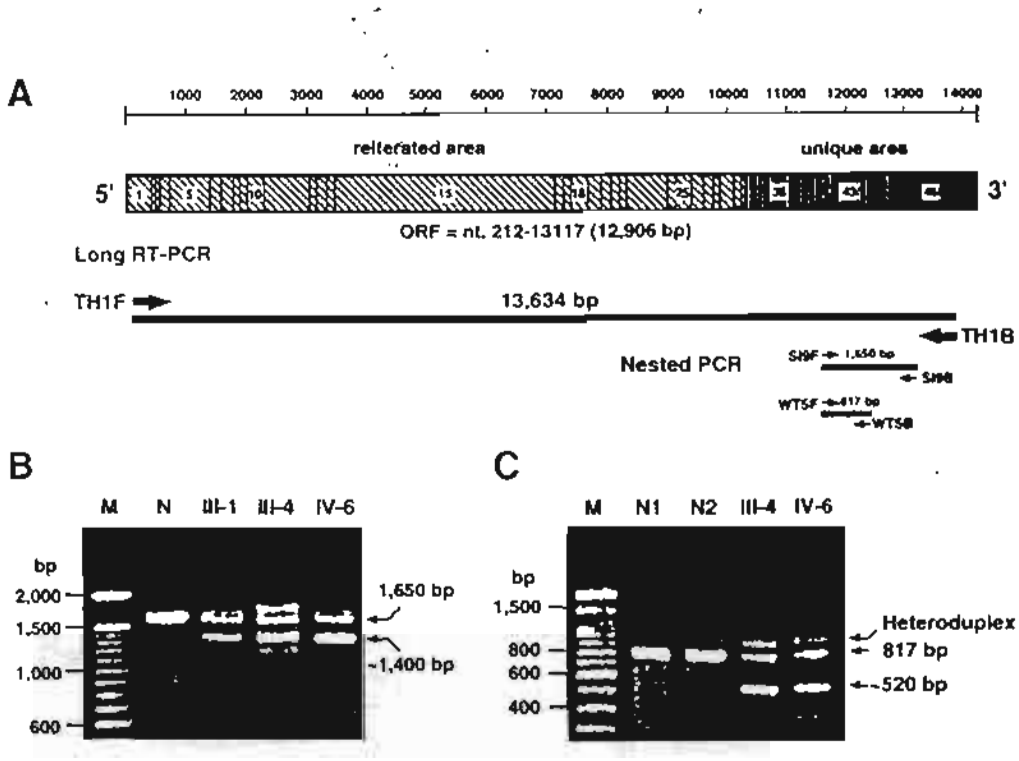

Fig. 1. (A) Diagrammatic representation of the full-length *PKD1* mRNA (upper), long PCR product (middle), and nested PCR products (lower). The reiterated region in the *PKD1* mRNA (exons 1-32) is represented by the hatched area and the unique region (exons 33-46) by the blackened area. Sizes (in bp) and primer pairs (arrows) are shown above and below the long RT-PCR products (long solid horizontal line) and nested PCR products (short solid horizontal lines), respectively. (B) Nested PCR products amplified from long PCR product of *PKD1*-cDNA with the SI9F/SI9B primers from a normal individual (N) and three *PKD1* patients (III-1, III-4, and IV-6) of PK009 family. A single normal PCR product (1,650 bp) was observed in the normal sample while a normal and a shorter (~1,400 bp) products were found in three patients' samples. The uppermost band in the lanes of patients' samples is probably a heteroduplex of the normal and shorter products. Lane M is 100-bp DNA ladder. (C) Nested PCR products amplified from the long *PKD1*-cDNA samples by WT5F/WT5B primers of two normal individuals (N1 and N2) and the two patients (III-4 and IV-6) of PK009 family. A normal PCR product with the size of 817 bp was observed in the two normal samples but a normal (817 bp) and a shorter (~520 bp) fragments were detected in both patients' samples; the top band in both patients' samples was probably a heteroduplex of the normal and deleted DNA strands. Lane M is 100-bp DNA ladder.

shorter (~1,400 bp), were detected in samples from the three affected members (Fig. 1B). In addition, an upper and fainter band of PCR product was also noticed. The presence of a shorter product may have resulted from a partial deletion of *PKD1* cDNA prepared from its mRNA transcript from one allele of *PKD1* in these patients, and upper band might be a heteroduplex of the normal and deleted DNA strands.

To locate the region with possible deletion, nested PCR products amplified by SI9F/SI9B primer pair from a normal individual and a patient (III-4) were digested with either *Taq* I plus *Bgl* I or *Pvu* II. It was found that the deleted region was of 300 bp, located between the first *Taq* I and *Bgl* I sites (or the fourth site of *Pvu* II) (data not shown).

A new pair of primers (WT5F/WT5B ; Table 1 and Fig. 1A) were designed to amplify DNA covering the deleted region. The result of the amplifications showed that two normal samples (N1 and N2) generated a single amplified product with the size of 817 bp, whereas, two patients' sam-

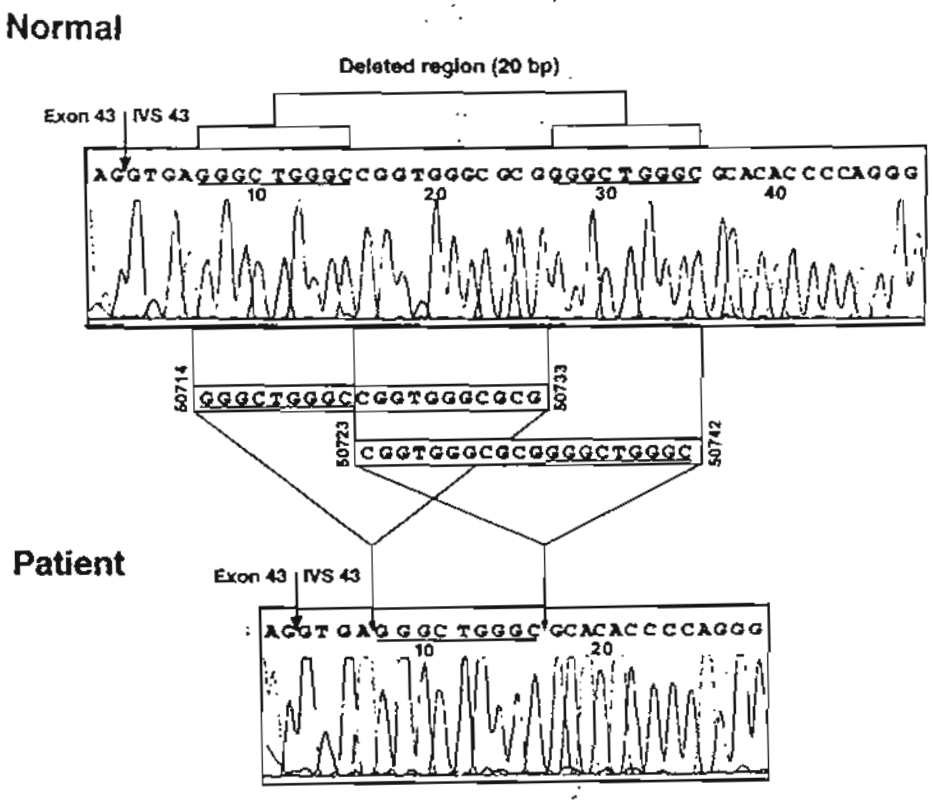

Fig. 2. Results of sequencing analyses of nested PCR products of intron 43 region amplified from genomic DNA samples of a normal control and patient III-4 of PK009 family by SI9.2F/SI9.2B primers. Comparison of the two nucleotide sequences shows a deletion of 20 bp in intron 43. The presence of two direct 9-nt repeat (GGGCTGGGC) (underlined) which are separated by 11 bp in the normal sequence of this intron makes many possibilities of the deletion breakpoint between the nucleotide positions 50714 and 50742 of *PKD1* (GenBank Accession No. L39891). Two examples of possibilities of the 20-bp deletion at the most 5' and 3' ends are shown.

ples (III-4 and IV-6) produced the normal amplified product (817 bp) and a shorter fragment (~520 bp) together with the upper heteroduplex band (Fig. 1C).

Sequencing Analysis of the Shorter Nested-PCR Product

The 520 bp fragment was purified from agarose-gel electrophoresis and subjected to direct DNA sequencing. There was a deletion of 291 bp which corresponded to the total exon 43 sequence of *PKD1* while complete sequences of exons 42 and 44 were still present (data not shown). This finding indicated that the deletion was most likely due to the skipping of exon 43 from the *PKD1*-mRNA transcript.

Analysis of the *PKD1* Gene

The skipping of exon 43 from *PKD1*-mRNA transcript might have occurred from a number of defects in the *PKD1* gene, viz. complete absence of the exon, mutation at the exon-intron junctions, or mutation at the A branch site in the flanking introns 42 and 43. To identify the precise mutation, genomic DNA in this region of *PKD1* was sequenced. Since the genomic region surrounding exon 43 was difficult to amplify by PCR due to the presence of 34-nt repeat polymorphism in intron 42[14], the primary genomic DNA amplification was performed by using SI9F/SI9B primer pair which had been used for nested PCR of *PKD1* cDNA (Table 1). A PCR product with the length of 2,351-2,546 bp was produced and used in nested PCR with SI9.2F/SI9.2B

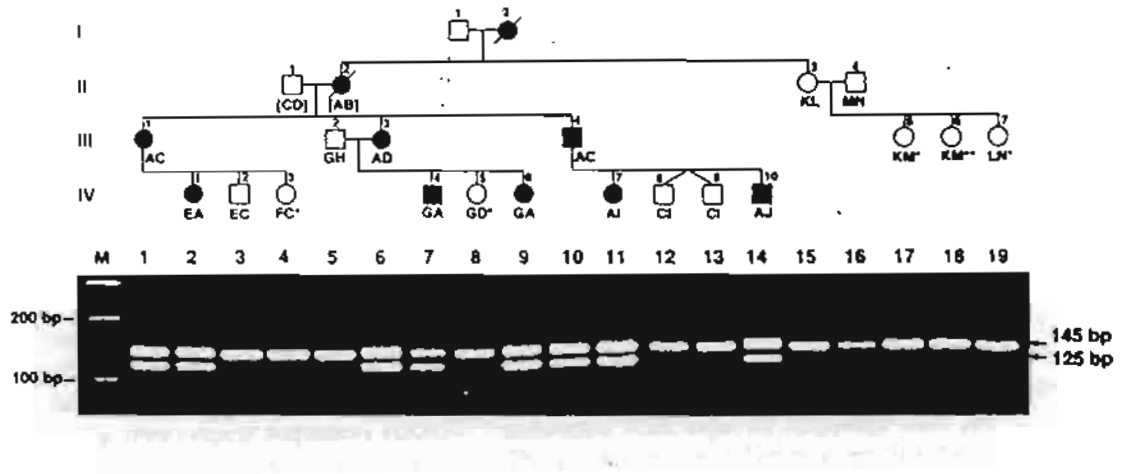

Fig. 3. Detection of the 20-bp deletion in intron 43 of *PKD1* by amplification of genomic DNA samples from members of PK009 family with SI9.3F/SI9.2B primers. Pedigree of the family is shown and haplotyes in the region of *PKD1* on chromosome 16p13.3, determined by using 5 polymorphic DNA markers, are indicated under the symbols. Genomic DNA samples from 19 family members, 8 from affected (filled symbols) and 11 from unaffected (blank symbols) members, were analyzed. DNA samples of the unaffected members produced only the product of the wild-type allele (145 bp) but those of affected members generated the products of both wild-type and mutant (125 bp) alleles. The mutant allele segregated with the haplotype A in the family. The haplotypes with one or two asterisks are variants of the ones without. Lane M is 100-bp DNA ladder.

primer pair specific to sequences in exons 42 and 44 (Table 1). A PCR product with the size of 563-758 bp was generated and sequenced. There was a deletion of 20 bp in intron 43 (Fig. 2), while sequences in other regions were normal. Since there are two 9-bp repeats (GGGCTGGGC) situated 11 bp apart in intron 43 of *PKD1*, it was not possible to locate precisely the deletion breakpoints in this abnormal gene.

Direct Detection of the 20-bp Deletion in Intron 43 in Members of PK009 Family

To perform direct detection of the 20-bp deletion in intron 43 in members of PK009 family, a primer specific to the sequence of exon 43 (SI9.3F) was used together with a primer specific to the sequence of exon 44 (SI9.2B) (Table 1). With this primer pair, genomic DNA sample with normal intron 43 would generate PCR product of 145 bp while that with deleted intron 43 of 125 bp. When genomic DNA samples from all members of PK009 family were amplified, DNA samples of 11 normal members showed only the normal size product (145 bp), whereas, those of 8 affected members displayed 145 bp and 125 bp products (Fig. 3). Heteroduplex band was sometimes observed. The presence of deleted intron 43 in affected members and its absence in unaffected members of PK009 family corresponded with the presence and absence of haplotype A which linked to the abnormal *PKD1* allele (Fig. 3).

DISCUSSION

An analysis of *PKD1*-mRNA transcripts from three patients in PK009 family showed that the truncated *PKD1* cDNA observed in the initial nested PCR screening (Fig. 1B and C) was due to skipping of the entire 291 nt sequence of exon 43, while the sequences of exon 42 and 44 were still intact in the abnormal transcript. Further analysis of *PKD1*-genomic DNA by direct sequencing revealed a 20-bp deletion in intron 43 of the patient's *PKD1* gene (Fig. 2) while the sequences of other critical regions, such as splice donor and acceptor sites close to exon 43 and branch sites in the flanking introns, were not changed. Thus, the observed 20-bp deletion in intron 43 was most likely to be the cause of exon 43 skipping in the patient's mRNA transcript. A PCR method for amplification of DNA region covering

intron 43 was used to examine the deletion in DNA samples from 19 members of PK009 family. The expected 20-bp deletion in intron 43 was found in all samples from 8 affected members but was not detected in the 11 unaffected members (Fig. 3). In addition, the deletion segregated in complete linkage to the haplotype A as characterized by using 5 polymorphic DNA markers in the *PKD1* region (Fig. 3). All the evidence clearly supported the 20-bp deletion in intron 43 of *PKD1* as being the disease mutation in this family.

Two 9 bp direct-repeat sequences (GGGC TGGGC) separated by 11 nucleotides were present in *PKD1* intron 43 (Fig. 2). Thus, the deletion might have occurred from a misalignment between the two direct repeat sequences in this region of two *PKD1* alleles and interchromosomal recombination during meiosis. Since the length of deleted nucleotides (20 bp) was equal to the sum of one repeat (9 bp) and the joining part (11 bp), and one repeat sequence still remained in the deleted allele, the exact position of the recombination or deletion breakpoint could not be determined. As the deletion did not involve the nearby exon, it would not be detected by exon analysis of genomic DNA.

Mutations causing exon skipping usually involve either splice donor or splice acceptor site at 3' and 5' consensus sequences of the intron. Two reported mutations of *PKD1* resulting in exon 39 and exon 44 skipping are IVS39+1G>C and IVS44+1G>C substitutions, respectively[9,24]. However, the intron 43 deletion leading to exon skipping reported here did not affect those functionally important sites. One explanation for the 20 bp-deletion in intron 43 causing exon 43 skipping may be that the truncated intron is too short for proper spliceosome formation and correct RNA splicing process. The minimal length of intron in human genes for correct *in vivo* splicing is not known. In an *in vitro* study to determine a minimal intron length using rabbit beta-globin gene constructs in HeLa cells, it was found that the correct splicing required six 5' and twelve or more 3' intron nucleotides with at least 80 inner nucleotides[25]. The shortest *PKD1* intron is intron 19 with a length of 66 bp[11]. The length of the mutated intron 43 is 55 bp.

Deletions of 18 and 20 bp in intron 43 of *PKD1* have been reported[26]. In each case, two different transcripts, either with deleted-intron sequence retained or with a 66-nt deletion due to activation of a cryptic 5' splice site, as well as the transcripts with exon 43 skipping, were produced. These findings are different from this study where the major defective transcript contained exon 43 skipping. It should be noted that in this study fresh peripheral blood lymphocytes were used for RNA isolation while lymphoblastoid cell lines were used in the previous work.

The deletion of 291 nucleotides of exon 43 in *PKD1*-mRNA transcript will lead to an in-frame deletion of 97 amino acids at positions 3904-4001 in polycystin-1. This deleted peptide region is located between the second half of the 7th transmembrane (TM) domain and the first one-third of the 9th TM domain of the proposed model of polycystin-1[27]. Since the deletion of this region has resulted in the disease phenotype, it is of importance to the function of polycystin-1.

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Construction of infectious dengue 2 virus cDNA clones using high copy number plasmid

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Abstract

Procedures for cloning entire dengue serotype 2 virus genome in the multiple cloning site of a commercially available high copy number plasmid are described. The 10.7 kb viral RNA genome was reverse transcribed, amplified as three overlapping DNA fragments and successively ligated into pBluescript II KS, which contains the colE1 origin of replication. When propagated at room temperature (20–25°C) under low level of antibiotic selection, the full-length recombinant plasmid was stable upon serial passages in two common *Escherichia coli* strains employed. Under the same culture conditions the whole dengue cDNA sequence was transferred successfully to another high copy number plasmid, pGem 3Z. Following in vitro transcription and lipofectin-mediated transfection, capped RNA transcripts derived from the plasmid initiated virus replication in C6/36 mosquito cells and BHK-21 cells within 3–4 days of transfection. Upon subsequent expansion in C6/36 cells, dengue viruses derived from the first- and eighth-plasmid passages achieved similar titers as the parent virus. They were also indistinguishable from the parent virus by the criteria of replication kinetics in mosquito and mammalian cell lines, and size and reactivity of selected viral proteins as detected with polyclonal and monoclonal antibodies. The cloning scheme and resultant recombinant plasmids based on high copy number cloning vectors allows greater flexibility in manipulation of dengue viral genome when compared with previous attempts employing low-copy number counterparts.

Keywords: Flavivirus; cDNA; High copy number plasmid; Expression

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1. Introduction

Following the first successful construction and propagation of full-length cDNA clone of a dengue serotype 4 virus in *Escherichia coli* (Lai et al., 1991), subsequent attempts in cloning dengue serotype 2 genome had met with variable success. In an extreme case, full-length cDNA was generated by in vitro ligation of subgenomic segments and employed directly as template for synthesizing infectious RNA transcripts without being able to propagate serially as recombinant plasmid clone in *E. coli* (Kapoor et al., 1995). In another case, the full-length clone had to be assembled first in yeast before transferring into *E. coli*, but the frequency of which *E. coli*-derived clones gave rise to infectious virus was less than those derived originally from yeast (Polo et al., 1997; Puri et al., 2000). In two attempts in which construction and propagation of complete dengue serotype 2 cDNA sequence were accomplished directly in *E. coli*, a common strategy was the use of low copy number plasmids as cloning vectors (Kinney et al., 1997; Gualano et al., 1998). Similar to several viral systems, instability of dengue cDNA sequence remains a major obstacle in obtaining and propagating full-length cDNA clone in *E. coli* (Boyer and Haenni, 1994).

Currently, the use of low copy number plasmid and, in some cases, selected bacterial strains only alleviated partially dengue genome instability problem as rearrangement of viral sequences still occurs during successive propagation in *E. coli* (Lai et al., 1991). Moreover, low copy number plasmids, due to their generally large sizes and the presence of relatively high number of restriction enzyme recognition sites, are quite cumbersome to manipulate. In order to take advantage of high yield and ease of manipulation of high copy number plasmids, we introduced simple modifications to the cloning procedures and were able to utilize high copy number plasmid in the construction and propagation of entire cDNA of a dengue serotype 2 virus genome in *E. coli*. Subsequent expression and testing revealed that plasmid-derived viruses were indistinguishable from parental virus in their antigenicity and replication kinetics.

2. Materials and methods

2.1. Dengue virus, cell lines, antibodies and plasmids

Dengue virus strain 16681, a serotype 2 virus isolated from a fatal case of dengue hemorrhagic fever in Bangkok, was kindly provided by Drs Bruce Innis and Ananda Nisalak, Department of Virology, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand. It was serially propagated at 29°C in the C6/36 mosquito cell line using Leibovitz's L15 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 10% tryptose phosphate broth and glutamine–penicillin–streptomycin solution. Virus stock was stored in 20% fetal bovine serum at −70°C. BHK clone 21 cells were maintained in MEM supplemented with 10% fetal bovine serum and glutamine–penicillin–streptomycin solution in 5% CO_2 in humidified air at 37°C. Ps clone D cell line was maintained in Leibovitz's L15 medium supplemented with 10% fetal bovine serum, 10% tryptose phosphate broth and glutamine–penicillin–streptomycin solution at 37°C. A pool of high-titered sera taken at convalescence from patients with dengue hemorrhagic fever at Siriraj Hospital, Bangkok, was used as anti-dengue polyclonal antibody. Monoclonal antibodies specific for dengue E glycoprotein (4G2, 3H5), prM glycoprotein (2H2, 4C1), and NS1 protein (1B2, 1A4) (Henchal et al., 1982, P. Malasit, unpublished results) were used in the form of ascites or concentrated culture supernatant. Cloning vectors, pBluescript II KS and pGem 3Z, were distributed by Stratagene (La Jolla, CA, USA) and Promega (Madison, WI, USA), respectively.

2.2. Long reverse transcription-polymerase chain reaction

Virions present in 80 ml of C6/36 culture fluid (approximate titer 10^8 pfu/ml) were pelleted by spinning culture supernatant at 210 000 × *g* at 4–6°C for 2 h and resuspended in 140 μl of supernatant. Genomic RNA was extracted by employing RNAeasy mini kit (QIAGEN, Hilden,

Germany) according to the manufacturer's protocol and stored at −70°C until use. Complementary DNA synthesis was accomplished by using RNAseH⁻ reverse transcriptase (Superscript II, Gibco BRL), 0.5 mM dNTP and 100 pmol each of the B-1, B-2 and B-3 oligonucleotide primers which were designed for the amplification of three overlapping regions of genome (Table 1). Following reverse transcription at 45 or 56°C for 1 h, reaction mixture was heated at 94°C for 4 min, cooled and digested with RNAseH (Gibco BRL) at 37°C for 1 h. The resultant cDNA was extracted with phenol–chloroform–isoamyl alcohol (25:24:1) mixture once, precipitated in 0.3 M sodium acetate with 95% ethanol, washed with 70% ethanol, dried, resuspended in diethyl pyrocarbonate-treated water and stored at −70°C until use. For long polymerase chain reaction, different amounts of each cDNA preparation were tested separately against various concentration of Mg^{2+} for successful amplification. Typically, a mixture of Taq and Pyrococcus species GB-D DNA polymerases (elongase enzyme mix, Gibco BRL) was employed in 50-μl reaction containing 0.2 mM each dNTP, 50 pmol of corresponding forward and backward primer pairs (F-1 and B-1, F-2 and B-2, F-3 and B-4, Table 1) and the combinations of two buffer mixes in order to obtain Mg^{2+} at the final concentration of 1.5–1.6 mM. The cycling parameter consisted of initial denaturation of the cDNA-primer mixture at 94°C for 50 s and subsequent 30–35 cycles of denaturation (94°C, 30 s), annealing (55–68°C; depending on the primers, 30 s) and extension (68°C, 5–10 min) carried out in a thermocycler (model 480, PE Biosystems, Foster City, California, USA). Amplified products were electrophoresed in 0.8% agarose gel and visualized by ethidium bromide staining.

2.3. Cloning of subgenomic fragments

Three overlapping subgenomic fragments of strain 16681 were cloned individually into a high copy number plasmid, pBluescript II KS by employing the following steps: (1) for cloning the region 5′ end-4497, amplified product from the F-1 and B-1-primed reaction was digested with

Table 1
Oligonucleotide primers for reverse transcription, amplification and site-directed mutagenesis

Region	Designation	Range[a]	Sequence (5′–3′)	Introduced sequence/restriction site
5′ End-4997	F-1	1–22	CTCGCTAGCATTTAGGTGACACTATAGA GTTGTTAGTCTACGTGGACCG	SP6 promoter (underlined), *Nhe*I (bold)
	B-1	4997–4972	CCACTCCTTGTAACAACACCATTACC	
4166–7977	F-2	4166–4190	CTCTCTAGAGGATGGTGAGCATTTTAGC CAGTTC	*Xba*I (bold)
	B-2	7977–7943	CGGGATGAAGAAAACGTCAACTCCACTT TGAAGAC	
7377-3′ End	F-3	7377–7400	GGGTCTAGAAGGACTACATGGGCTCTGT GTGAG	*Xba*I (bold)
	B-3	10723–10692	GGTCTAGAACCTGTTGATTCAACAGCAC CATTCCATTTTC	*Xba*I (bold)
	B-4	10723–10684	CTCGGTACCTCTAGAACCTGTTGATTCAA CAGCACCATTCCATTTTCTGGCGTTC	*Kpn*I, *Xba*I (bold)
Pst I (nt 402)	MS-402	397–417	TCTGCCGGCATGATCATTATG	A402C mutation (underlined), *Nae*I (bold)
	MR-402	407–387	ATGCCCGGCAGATCTGCGTCTC	A402C mutation (underlined), *Nae*I (bold)

[a] Number represents base position in the genome of strain 16681 according to Blok et al. (1992) and Kinney et al. (1997).

*Nhe*I (5′ end, introduced in the F-1 primer) and *Kpn*I (nt 4497) and then ligated into *Xba*I- and *Kpn*I-cut plasmid; (2) for cloning the region nt 4166–7977, amplified product from the F-2 and B-2-primed reaction was treated with Pfu DNA polymerase (Stratagene) to ensure blunt 3′ end, digested with *Xba*I (5′ of nt 4166, introduced into F-2 primer) and ligated into *Xba*I- and *Eco*RV-cut vector; (3) for cloning the region nt 7737-3′ end, amplified product from the F-3 and B-4 primed reaction was digested with *Nru*I (nt 7737) and *Kpn*I (3′ end, introduced in the B-4 primer) and ligated into plasmid which was digested with *Eco*RV and *Kpn*I. Ligation mixtures were transformed into chemically competent *E. coli* strains DH5αF′ and STBL2 (Gibco BRL) with the supplementation of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) during 90-min shaking at room temperature to allow for phenotypic lag. Bacterial transformants were then spread onto agar plate containing LB medium (Sigma, St Louis, MO, USA) and 25 μg/ml ampicillin and incubated at 25°C for 2–3 days. Size and orientation of cDNA insert within the cloning vector were determined by restriction enzyme digestions and separation in 0.8% agarose gel.

To construct a larger clone containing the region nt 4166-3′ end, cDNA sequence spanning nt 7844-3′ end in plasmid derived from the cloning step (3) was cut out by digesting with *Stu*I (nt 7844) and *Kpn*I (3′ end, introduced in the B-4 primer) and then ligated into recombinant plasmid from the step (2) which was digested similarly. The resultant cDNA clone containing the region nt 4166-3′ end was designated the 3′ half-genome whereas the other one derived from the cloning step (1) and contained the region 5′ end-4497 was the 5′ half-genome.

2.4. Construction of full-length cDNA clone of strain 16681 in high copy number plasmid

The full-length cDNA clone of strain 16681 was constructed in pBluescript II KS by ligating the 6.2 kb *Kpn*I fragment encoding the region nt 4497-3′ end from a 3′ half-genome into the *Kpn*I site at the position 4497 of viral sequence of a corrected 5′ half-genome. To minimize deletion and/or rearrangement of viral sequence from the resultant plasmid, transformation of ligated product into *E. coli* and all subsequent incubation were done at 20–25°C. Following three days of incubation at this low temperature, distinctively large and small bacterial colonies were observed at a proportion of 1:1.6. When both types of transformants were grown in 2 ml volume under low level of amplicillin selection, analysis of plasmid DNA by *Hin*dIII and *Xba*I digestion and gel electrophoresis revealed that none of the large STBL2 transformant colonies retained full-length cDNA sequence whereas approximately half of the small colonies did (Table 2). The rest of small colonies contained single 6.2 kb 3′ half-genome sequence insert but in the inverse orientation, or, more rarely, two tandem copies of insert. The density of bacterial growth in 2 ml liquid media as observed visually and the amount of resultant plasmid DNA in the latter groups of transformants were generally higher than the ones with correct, full-length sequence, indicating possible inhibitory effect of full-length dengue sequence on bacterial growth or plasmid replication. When strain DH5αF′ was used instead of STBL2, the proportion of small colonies with full-length cDNA sequence was even higher (Table 2). Depending on the duration of incubation and resultant cell density, plasmid yield derived from either STBL2 or DH5αF′ growth in 100 ml LB liquid medium ranged from 15 to 55 μg of plasmid DNA.

2.5. Stability assay

Bacterial transformants containing the full-length cDNA clone were streaked onto LB agar plate containing 25 μg/ml ampicillin and incubated at 25°C for 2–3 days until isolated colonies were clearly identified. Plates were kept at 4°C for a minimum of 7 days and then approximately 20 colonies were picked and allowed to grow at 25°C in liquid LB medium containing 25 μg/ml ampicillin for 2 days. The size and orientation of the cDNA insert were next determined by digesting plasmid DNA with *Xba*I and *Hin*dIII and separating the fragments electrophoretically in 0.8%

Table 2
Colony morphology of *E. coli* transformants and retention of full-length dengue cDNA sequence[a]

Experiment	Plasmid	*E. coli* strain	Proportion of *E. coli* colonies with:			
			Full-length, functional cDNA		Full-length, non-functional cDNA	
			Large colony	Small colony	Large colony	Small colony
1	pBluescript II KS	STBL2	0/16	12/27	0/16	15/27
2	pBluescript II KS	STBL2	0/40	13/30	0/40	17/30
3	pBluescript II KS	DH5αF′	0/9	11/13	0/9	2/13
4	pBluescript II KS	DH5αF′	0/40	28/40	0/40	6/40
5	pGem3Z	STBL2	0/20	20/20	na[b]	na[b]

[a] Following the ligation of the 6.2 kb *Kpn*I fragment containing the region nt 4497-3′ end into the *Kpn*I-digested 5′-half genome and transformation of indicated *E. coli* strains, resultant recombinant clones were scored for the presence of either full-length and functional cDNA sequence (the 6.2 kb *Kpn*I fragment was inserted in the correct orientation into 5′-half genome) or the full-length but non-functional cDNA sequence (the 6.2 kb *Kpn*I fragment was inserted in the inverse orientation) and compared with their colony size.

[b] Not applicable.

agarose gel. Two bacterial clones that contained full-length cDNA insert in the proper orientation were streaked onto LB-ampicillin agar plate and the growth-storage-growth testing cycle repeated for 4–8 rounds.

2.6. Nucleotide sequence analysis

Amplified products or plasmid clones were purified by ion exchange spin column chromatography (QIAGEN) and 250–500 ng were employed in each sequencing reaction using dRhodamine dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Electrophoretic separation and detection were carried out on an automated sequencer (model 310, Applied Biosystems). A list of oligonucleotide primers employed for sequence analysis is available upon request.

2.7. In vitro transcription, transfection and detection of virus replication

Full-length cDNA clone was cut at the 3′ end by digesting with *Xba*I, treated with 100 μg/ml proteinase K to destroy RNAse, extracted with phenol–chloroform–isoamyl alcohol (25:24:1) mixture once, precipitated in 0.3 M sodium acetate with 95% ethanol, washed with 70% ethanol, dried and suspended in diethyl pyrocarbonate-treated water. In vitro transcription was performed in a 20 μl reaction containing 1 μg linearized cDNA, 80 mM Hepes–KOH (pH 7.5), 32 mM $MgCl_2$, 2 mM spermidine, 40 mM dithiothreitol, 5 mM each of GTP, CTP and UTP, 1.5 mM ATP, 3.5 mM $m^7G(5')ppp(5')A$ cap analog (New England Biolabs, Beverly, MA, USA) and 20–40 U SP6 RNA polymerase (Gibco BRL) or 2 μl SP6 RNA polymerase–pyrophosphatase mix (SP6 Ribomax kit, Promega). Following incubation at 40°C for 4–6 h, the reaction mixture was either used directly for transfection, or was treated with RNAse-free DNAse and purified using the RNAeasy mini kit (QIAGEN) before transfection.

Transfection of C6/36 and BHK cell lines was carried out by mixing 5–10 μl of in vitro transcription product with 5 μl lipofectin (Gibco BRL) in 1 ml L15 or MEM media before adding onto a twice-washed confluent cell monolayer in a 35 mm plastic dish. Following a 4-h incubation at room temperature (C6/36 cell) or at 37°C (BHK cell), RNA–lipofectin mixture was removed and the maintenance media containing 2% fetal bovine serum was added. Transfected cell monolayer was returned to incubation at 29°C (C6/36 cells) or 37°C in 5% CO_2 in humidified air (BHK cells) for an additional 2 days. Determination of virus

protein within transfected cells by the 5-step peroxidase–antiperoxidase method was performed as described previously (Sittisombut et al., 1995). Plaque assay in Ps clone D cells for the determination of infectious virus titer and kinetic of replication in vitro was according to Avirutnan et al. (1998).

2.8. Immunoblot analysis

Culture supernatant of C6/36 cells infected with parent 16681 strain, first-passage and eighth-passage plasmid-derived viruses were centrifuged at 25 000 × *g* for 90 min at 4°C. Viral pellet was dissolved in SDS-containing loading buffer without boiling, electrophoresed in 5–15% polyacrylamide gel in the absence of reducing agent (dithiothreitol) and blotted onto nitrocellulose membrane using a semidry blotting apparatus. Detection was performed with pooled, convalescent human sera or murine monoclonal antibodies against E, prM and NS1 proteins and appropriate horseradish peroxidase-conjugated anti-IgG antibodies and SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA) followed by exposure to X-ray film, scanning and printing of the digitized image.

2.9. Site-directed mutagenesis

To mark the plasmid-derived virus, the middle *Pst*I site (nt 402) among the three sites (nt 212, 402 and 1535) present within the genome of 16681 was abolished by employing MS402 and MR402 oligonucleotides (Table 1) in a PCR-based site-directed mutagenesis scheme (QuikChange, Stratagene). A plasmid subclone containing the region nt 1–1547 was annealed with the two oligonucleotides and amplified for 30 cycles (denaturation 94°C, 45 s; annealing 65°C, 45 s; extension 72°C, 10 min) using Pfu DNA polymerase. Amplified products were digested with *Dpn*I (New England Biolabs) to destroy bacteria-derived plasmid templates and then transformed into *E. coli*. Bacterial transformants containing plasmid with the desired mutation were identified by the simultaneous loss of a *Pst*I site and the gain of a *Nae*I site. The 1.3 kb *Pst*I fragment containing the region nt 212–1535 was then digested out of the mutant plasmid, purified and inserted into the *Pst*I-digested 5′-half genome.

3. Results

3.1. Sequence analysis and correction of the 5′ and 3′ half-genomes

Two plasmid clones each of the 5′ and 3′ half-genomes were subjected to nucleotide sequence analysis. Comparison with sequences published previously (Blok et al., 1992; Kinney et al., 1997) and with sequences derived from corresponding PCR products revealed a 5′-half genome clone which differed at only three positions from those reported by Kinney et al. (1997) (Table 3). Two of these differences, however, were also present in the second clone and the corresponding PCR product; quite likely, they were not errors which were incorporated during reverse transcription or polymerase chain reaction, but represented variations among substrains of 16681 which were propagated in different laboratories. Only the third (nt 4340) might result from an error during the construction, necessitating the correction by substituting with an unaltered region from the second clone. Sequence analysis also revealed a 3′-half genome clone, which contained only five base differences from published sequence (Kinney et al., 1997). Again, only one difference (nt 10561) found in this clone was not detected in the second clone or PCR product. Further comparison with several other 3′ half-genomes, however, revealed that this change together with another one at nt 10403 represented one of the two mosaic sequences: the first pattern-10403T, 10561C-was found in four out of six clones and PCR product whereas the second pattern-10403A, 10561A-was present in only two out of six clones. Because these nucleotide differences and mosaic sequence were likely to be present in the parent virus and would not be deleterious for virus replication, this 3′-half genome was then employed in the construction of full-length clone without any correction.

3.2. Stability of full-length cDNA clones

Following the construction and identification of full-length cDNA clones based on pBluescript II KS, we investigated the stability of these clones in *E. coli* by subjecting STBL2 and DH5αF′ transformants to growth-storage-growth testing cycles in parallel. Analysis by restriction enzyme digestion revealed that almost all of individual STBL2 colonies were able to retain full-length dengue sequence through all four cycles (Table 4). The proportion of DH5αF′ transformants with full-length sequence was more variable, especially in later cycles of the first two experiments. With strict adherence to low temperature for growth in liquid medium, however, maintenance of full-length dengue sequence in DH5αF′ was similar to STBL2 in the third experiment.

Table 3
Sequence differences between cDNA clones derived from two substrains of dengue serotype 2 virus strain 16681[a]

Gene	Base position	Nucleotide		Amino acid		Note
		CDC	CMU	CDC	CMU	
NS1	2943	A	C	Lys	Asn	Present in 2/2 clones and PCR product
NS2B	4308	C	T	Val	Val	Present in 2/2 clones
	4340	G	G/A	Ser	Asn	A4340 was found in 1/2 clone, but not PCR product
NS3	4530	A	G	Val	Val	Present in 2/2 clones
NS5	8155	G	A	Ala	Thr	Present in 2/2 clones and PCR product
	8571	C	T	Val	Val	Present in 2/2 clones
3′ Non-translated Region	10331	A	G	–	–	Present in 6/6 clones and PCR product
	10403	T	T/A	–	–	T10403, C10561 pattern was found in 4/6 clones and PCR product
	10561	A	C/A	–	–	A10403, A10561 pattern was found in 2/6 clones, but not in PCR product

[a] Variations at the positions 2943, 4308 and 4340 reported in this study (CMU sequence) were determined from two 5′-half genomes, the rest were from two to six 3′-half genomes. The CDC sequence (Kinney et al., 1997) was from GenBank accession number U87411.

Table 4
Stability of pBluescript II KS-based full-length dengue cDNA clone in *E. coli*

Round no.	*E. coli* strain	Proportion of colony with full-length cDNA		
		Experiment 1	Experiment 2	Experiment 3
1	STBL2	18/18	9/10	20/20
	DH5αF′	17/17	9/9	17/20
2	STBL2	19/19	20/20	19/20
	DH5αF′	11/18	14/20	20/20
3	STBL2	19/20	20/20	20/20
	DH5αF′	10/13	5/16	20/20
4	STBL2	20/20	20/20	20/20
	DH5αF′	11/18	0/20	24/24

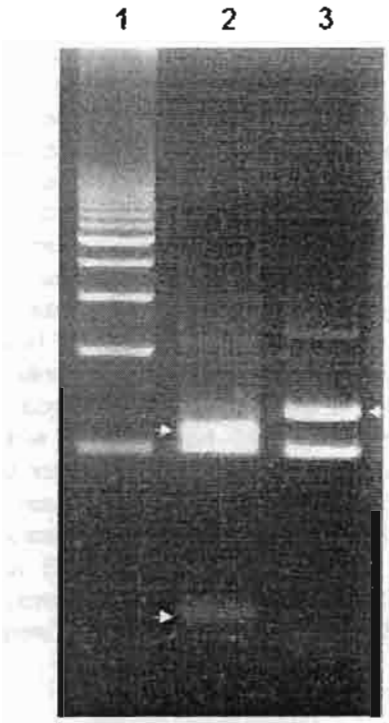

Fig. 1. Comparison of the *Pst*I digestion pattern of the parent virus and the *Pst*I-marked plasmid-derived virus. Genomic RNA was extracted from culture supernatant of 16681 parental virus and the *Pst*I-marked plasmid-derived virus and the region nt 134–2504 amplified by reverse transcription-polymerase chain reaction. The amplified products were purified, digested with *Pst*I, separated in 1% agarose gel, and stained with ethidium bromide. Lane 1, DNA marker (1 kb Molecular Ruler, Bio-Rad, Hercules, CA, USA); lane 2, 16681 parental virus; lane 3, *Pst*I-marked plasmid-derived virus. Left arrows indicate two *Pst*I fragments (1133 bp and 190 bp) derived from *Pst*I digestion at three positions (nt 212, 402 and 1535) of parent virus genome. Right arrow indicates the 1323 bp *Pst*I fragment resulting from a loss of the middle *Pst*I site at nt 402 in the *Pst*I-marked plasmid-derived virus.

To rule out the possibility that stable retention of full-length dengue genome is unique to pBluescript II KS, the entire cDNA was transferred to another high copy number plasmid, pGem 3Z. Analysis of *E. coli* transformants also revealed good retention of dengue genome in this plasmid (Table 2, experiment 5). Similar to pBluescript II KS, the retention was observed only in pGem 3Z transformants displaying small colony morphology.

3.3. Expression and kinetics of replication

A full-length cDNA clone of strain 16681 was restricted at the 3′ end with *Xba*I, digested with proteinase K and employed as template for in vitro transcription using SP6 RNA polymerase. Lipofectin-mediated transfection of either unpurified, or DNAse-treated, RNA affinity column-purified capped transcripts into C6/36 mosquito cells and BHK cells resulted in replication of dengue virus in transfected cells as evident by strongly positive cytoplasmic staining of virtually all cells with the monoclonal antibody 4G2 in a 4-step peroxidase-antiperoxidase staining and indirect immunofluorescence assay after 3–4 days of transfection. When plasmid-derived resultant virus was propagated one to two times in C6/36 cells and quantitated by plaque assay in Ps clone D cell line, its mean titer was similar to that of parent virus (16681 parent, 8.64×10^8 pfu/ml, $n = 2$; plasmid-derived virus, 3.24×10^8 pfu/ml, $n = 2$).

To ensure that the plasmid-derived virus was not a laboratory contaminant, a point mutation A402C, which abolished the middle among the three *Pst*I sites in the viral genome, was introduced into the 5′-half genome and incorporated subsequently into a second full-length clone and resultant virus. When viral RNA was reversed transcribed and amplified, testing for this *Pst*I site revealed that it was indeed absent from the second plasmid-derived virus (Fig. 1).

Comparison of replication kinetics was performed with the *Pst*I-marked, plasmid-derived virus against parent virus by infecting Ps clone D and C6/36 cell lines in parallel and collecting culture medium for plaque assay for 1 and 2 weeks, respectively. Using the multiplicities of infection (moi) which were previously determined for each cell line to allow slow increase of resultant viruses during a period of several days, the multiplication of the plasmid-derived virus was found to be indistinguishable from the parent virus in both cell lines (Figs. 2 and 3). To further investigate the fidelity of virus derived from pBluescript-based clone following repeated passages in *E. coli*, DH5αF′ transformed with the *Pst*I-marked, full-length cDNA plasmid was sub-

jected to eight growth-storage-growth cycles and then the plasmid was used to generate capped RNA transcript in vitro. Following transfection and two passages of plasmid-derived virus in C6/36, enumeration by plaque assay revealed that the mean infectious titer (1.52×10^9 pfu/ml, $n = 2$) and replication kinetics of the high passage, plasmid-derived virus were comparable to both parental and low passage viruses (Figs. 2 and 3).

To examine the preservation of antigenicity of structural proteins during passages of full-length cDNA clone in *E. coli*, the size and antigenicity of two glycosylated virion proteins, E and prM, of both low passage and high passage plasmid-derived viruses were compared with parent virus in immunoblot analyses. Testing with pooled, convalescent sera and monoclonal antibodies specific for E (4G2 and 3H5) and prM (2H2 and 4C1) revealed identical immunoreactive bands corresponding to glycosylated E and prM proteins from the three virus preparations (Fig. 4). In addition, two monoclonal antibodies (1B2, 1A4) specific for dengue NS1 protein reacted equally well in an indirect immunofluorescence assay with C6/36 cells infected separately with the three viruses (data not shown).

4. Discussion

By simply incubating bacterial transformants at reduced temperature and selecting with low concentration of ampicillin, it was possible to clone full-length cDNA genome of a dengue serotype 2 virus in a commercially available high copy number plasmid. The cDNA was quite stable upon propagation in two *E. coli* strains tested and was readily transferred into another high copy number plasmid. Following expansion in C6/36 mosquito cells, first-passage and eighth-passage plasmid-

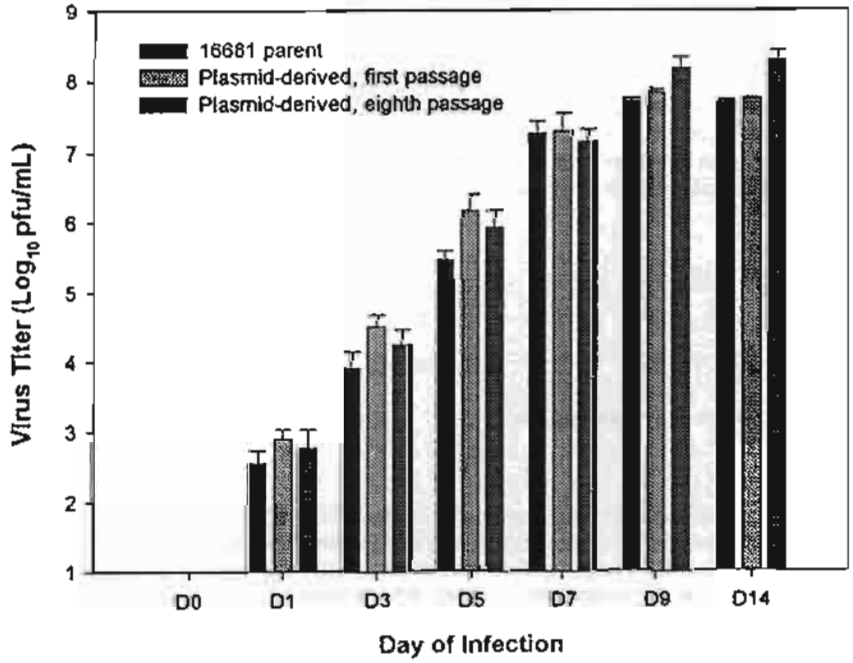

Fig. 2. Comparison of replication kinetics of plasmid-derived 16681 and parent viruses in mosquito cells. Confluent C6/36 cells in T25 flask were infected with plasmid-derived viruses and parent virus at the moi of 0.001 at room temperature overnight. The moi was previously determined to allow slow increase of resultant viruses during a period of several days. Culture media was then removed and replaced with L15 medium supplemented with 1.5% fetal bovine serum. Virus titer was determined at various days after infection by plaque assay in Ps clone D cells and expressed as $\log_{10}$ pfu/ml. Data bars represent mean and S.E. of virus titers derived from three separate experiments.

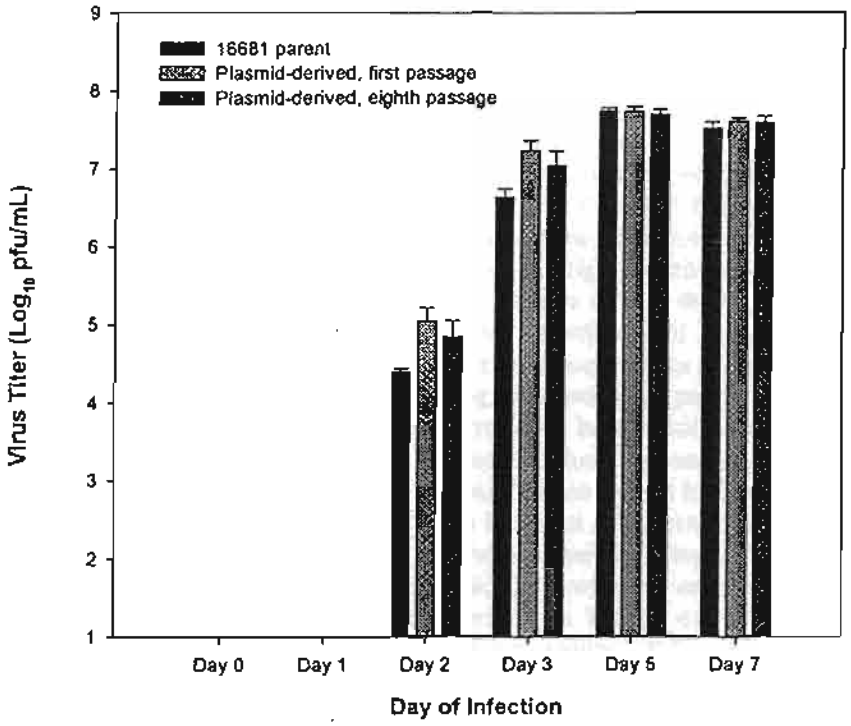

Fig. 3. Comparison of replication kinetics of plasmid-derived 16681 and parent viruses in mammalian cells. Confluent Ps clone D monolayer in T25 flask was infected with plasmid-derived viruses and parent virus at the moi of 0.01 at 37°C for 2 h. Culture media was then removed and replaced with L15 medium supplemented with 1.5% fetal bovine serum. After seven days of infection there were extensive cell death and detachment of Ps cell monolayer in infected cultures. Virus titer was thus determined at various days within a week of infection by using plaque assay in Ps clone D cells and expressed as $\log_{10}$ pfu/ml. Data bars represent mean and S.E. of virus titers derived from three separate experiments.

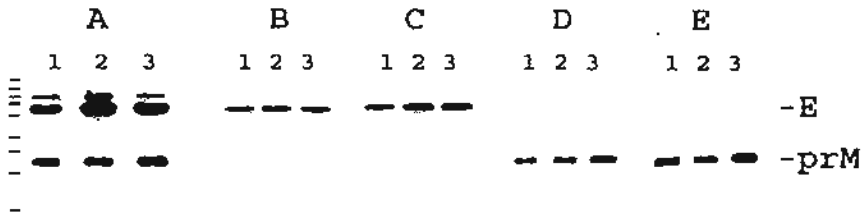

Fig. 4. Immunoblot analysis of E and prM proteins. C6/36 culture supernatant (100 μl) taken after infection with parent 16681 strain (lane 1), first-passage and eighth-passage plasmid-derived viruses (lanes 2 and 3, respectively) were centrifuged at 25 000 × *g* for 90 min. Viral pellet was dissolved in SDS-containing loading buffer without boiling, separated in 5–15% polyacrylamide gel and blotted onto nitrocellulose membrane. Detection was performed with pooled, convalescent human sera (panel A), two monoclonal antibodies against E protein, 4G2 (panel B) and 3H5 (panel C), and two monoclonal antibodies against prM protein, 2H2 (panel D) and 4C1 (panel E) followed by appropriate horseradish peroxidase-conjugated anti-IgG antibodies and chemiluminescent substrate. E and prM proteins were indicated. Mobility of molecular weight markers (benchmark prestained protein ladder, Gibco BRL, containing the 194, 120, 87, 64, 52, 39, 26, 21 and 15 kDa bands) are indicated on the leftmost lane.

derived viruses replicated in mosquito and mammalian cell lines with the same kinetics as the parent strain. Both preparations of plasmid-derived viruses were indistinguishable antigenically from parent virus when tested by immunoblot analysis with convalescent sera and a

panel of monoclonal antibodies directed against two glycosylated virion proteins. These results clearly demonstrate the feasibility of cloning whole dengue virus serotype 2 genome in high copy number plasmids and propagating them directly in *E. coli*.

It is not yet clear how reduced temperature and low antibiotic selection affects recombination machinery of *E. coli* allowing the retention of dengue sequence in high copy number plasmid. These conditions do not prevent completely rearrangement and/or deletion of the viral sequence, but allow a large fraction of host cells to maintain complete viral genome. When structural gene region of hepatitis C virus, another member of Flaviviridae, was cloned in an expression vector and propagated in *E. coli* at 37°C, a high frequency of point mutations and small deletions of the viral sequence was observed (Forns et al., 1997). It was proposed that spurious translation of virus sequence in *E. coli* cells might result in product that was toxic to the host and was selected against by frequent mutations (Forns et al., 1997). It is possible that toxic product was also generated when dengue sequence was cloned in high copy number plasmid and propagated in *E. coli*. At 25°C the efficiency of which toxic product was generated from dengue sequence might be less than at 37°C, resulting in reduced selective pressure against intact dengue sequence at this lower temperature. This possibility agrees well with a previous observation that a large proportion of full-length dengue cDNA clones, although displaying appropriate restriction enzyme digestion pattern, was not capable of initiating viral replication after they were propagated in *E. coli* at 37°C (Polo et al., 1997). Toxicity of viral product generated in *E. coli* may also explain our observation that *E. coli* harboring full-length dengue cDNA sequence (the 3′ half-genome sequence was inserted in the correct orientation) did not grow as well as the ones in which the 3′ half-genome sequence was inserted in the inverse orientation.

In addition to the use of low incubation temperature and antibiotic selection, we adopted several technical approaches that were particularly helpful. First, the use of thermostable DNA polymerase mixture for polymerase chain reaction not only enables amplification of long cDNA stretches but also reduces base incorporation error (Barnes, 1994; Cheng et al., 1994), thus allowing us to clone entire viral cDNA sequence in three overlapping segments with minimal sequence alteration. Second, sequence analysis of at least two 5′ and 3′ half-genomes in parallel and comparison with corresponding regions of PCR product help to distinguish erroneous bases introduced into cDNA during in vitro manipulations from variations and mosaic sequences, which invariably occurred as a result of genetic drift during repeated viral passages. Third, correction of erroneous base in subgenomic clones prior to the final ligation step ensures that the resultant full-length clone will be highly similar, if not identical, to the most prevalent virus present in the population chosen for cloning (Kinney et al., 1997). Because corrective manipulation is easier to perform with known errors at the level of subgenomic clones, these steps help in lowering technical difficulty and reducing delays inherent to the manipulation of full-length clone in addition to avoiding the uncertainty of blind correction.

An advantage in using high copy number plasmid as cloning vector is a greater flexibility in the manipulation of viral sequence. When the entire genome of strain 16681 is cloned into the chosen *Xba*I–*Kpn*I sites of pBluescript II KS, at least 30 restriction enzyme recognition specificities, which are present only once or twice in the viral genome but absent from the plasmid, are available for manipulating viral sequence. In comparison, two low copy number plasmids that were employed previously in the cloning of dengue serotype 2 genome, pBR322 (Lai et al., 1991) and pWSK29 (Wang and Kushner, 1991; Gualano et al., 1998), would allow only 17 and 25 specificities, respectively, to be used. Although the latter plasmid contains multiple cloning site that is very similar to pBluescript II, the exchange for low copy origin of replication results in acquisition of a large DNA segment making a number of restriction enzyme specificities in the viral sequence unusable. Enhanced flexibility in manipulating dengue cDNA sequence, which resulted from cloning viral genome in high copy number plas-

mid as reported herein, will be helpful in studying biology of dengue virus, especially in the delineation of structure-function relationship of virally encoded proteins and non-coding viral sequence in the future.

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CORRESPONDENCE

Mutation causing exon 15 skipping and partial exon 16 deletion in factor VIII transcript, and a method for direct mutation detection

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Summary. A splicing defect with 201 nucleotide deletion in the factor VIII transcript due to IVS15 + 1G > T mutation inactivating this donor splice site and activating a cryptic acceptor splice site in exon 16 was identified in a severe haemophilia A patient. Allele specific amplification (ASA) method was successfully developed for direct detection of this mutation.

Keywords: factor VIII gene; factor VIII transcript; haemophilia A; mutation detection; splicing defect; Thai.

Mutations of the factor VIII (FVIII) gene are heterogeneous; the common mutations are gene inversion, missense, and nonsense alterations [1,2]. RNA splicing defects are less common and have usually been identified from nucleotide changes at the donor or acceptor splice sites of the gene. Their true effects on splicing of the mRNA transcript have, however, rarely been substantiated. The presence of illegitimately transcribed FVIII mRNA in the peripheral lymphocytes [3–7] provides an opportunity for *ex vivo* analysis of its splicing defect. We have analysed the FVIII mRNA from haemophilia A patients to investigate its splicing defects. A splicing defect in the FVIII transcript, due to a mutation at the donor splice site in intron 15 of the gene, resulting in exon 15 skipping and partial exon 16 deletion, was observed in a patient of one family. The effect of this mutation was found to be greater than that one would predict from only the effects of the site where the mutation occurred. It is an example of a mutation that not only abolishes the functions of both donor and adjacent acceptor splice sites but also activates a nearby cryptic acceptor splice site, revealed by the mRNA study. The allele-specific amplification (ASA) method was developed for specific and direct detection of this mutation in members of the family.

Eighteen Thai haemophilia A patients from unrelated families were studied. Blood samples (~ 10 mL) from the patients were collected with informed consent. RNA was prepared from lymphocytes and DNA from the remaining white blood cells by standard procedures. The FVIII gene inversion was excluded by Southern blot hybridization. Full-length FVIII cDNA was synthesized from mRNA and amplified by polymerase chain reaction (PCR). The amplified cDNA was fractionated into overlapping fragments by nested PCR [4]. The details of PCR and nested PCR conditions have previously been described in our work [7]. The PCR products were examined by agarose gel electrophoresis. Restriction endonuclease analysis of the abnormal PCR product was performed by using *Ava*II, *Hae*III, *Pst*I, and *Sau*3AI, and the digested DNA fragments were examined by agarose gel electrophoresis. The regions suspected to carry the mutation were amplified from

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the patient's genomic DNA for sequencing analysis. The nucleotide sequences of amplified cDNA and genomic DNA fragments were analysed by automated sequencing.

The ASA method was invented for direct detection of the observed mutation. Primers for mutant (5′ TTCCACTGTCCCTAACCCAA 3′) and wild-type (5′ TTCCACTGTCCCTAACGCAC 3′) alleles were coupled with a common primer (5′ GGATGTGAGG CATTTCTACC 3′). The two allele-specific primers had a different nucleotide at their 3′ termini (underlined), but were complementary to those in the corresponding allele. Two destabilizing mismatch nucleotides were deliberately introduced at the fourth and ninth positions (also underlined) to increase its discrimination power and specificity in amplification [8]. A pair of internal control primers (5′ AACACTCCAGTCTGCCATATCACC 3′ and 5′ CCTGCATCATTTGTGGATTGTGAC 3′) were also included in the reaction. ASA was performed for 40 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 35 s.

The result of FVIII mRNA analysis showed that a cDNA fragment from one out of 18 haemophilia A patients had a shorter length (~ 1000 bp) than that of the normal one (1217 bp) (Fig. 1A), while this and other fragments in the remaining patients were found to be normal. The restriction endonuclease analysis indicated that the deleted region located in the sequences of exon 15 and 16 (data not shown). The sequencing analysis of this fragment demonstrated a deletion of 201 bp involving the entire sequence (154 bp) of exon 15 and a 47-bp region of exon 16 (Fig. 1B). To investigate the primary molecular defect, the regions of exons 15 and 16 and their exon/intron boundaries were amplified from the patient's genomic DNA to analyse by sequencing method. The result showed a single nucleotide substitution, G to T at the first position of donor splice site of intron 15 (IVS15 + 1G > T) (Fig. 1C). This mutation was also observed in an affected younger brother by sequencing analysis (data not shown). Because the mutation did not change a restriction site of any known restriction endonucleases, the ASA technique was therefore developed for direct mutation analysis. The ASA method was used to detect this mutation in the family members (Fig. 1D) and unrelated normal individuals including 10 males and 50 females (a total of 110 chromosomes) to also prove its specificity to the disease. The mutation was found in the DNA samples from the patient, the mother and affected younger brother but was not observed in those from the father or unaffected younger brother, nor in the samples from

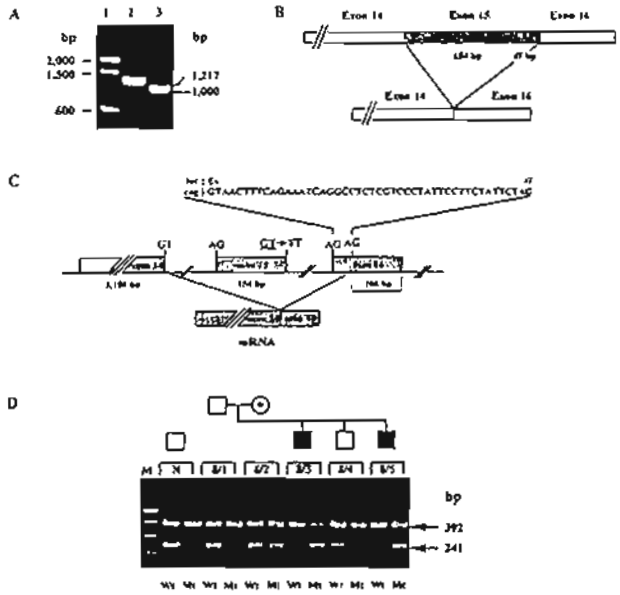

Fig. 1. (A) Amplified factor VIII-cDNA fragments from a normal individual (lane 2) and the haemophilia A patient (lane 3), which is ~ 200 bp shorter. The primers for amplification this fragment were F8N5A (5′ ACCCACCAGTCTTGAAACGC 3′) complementary to the sequence in exon 14 and F8N5B (5′ TCCATATTGTCCTGAAGCTG 3′) complementary to the sequence in exon 20. Lane 1 is a 100-bp ladder. (B) Diagram depicted from sequencing data of the patient's cDNA fragment showing deletion of entire exon 15 (154 bp) and first part of exon 16 (47 bp) (shaded area); exon 14 and the remaining exon 16 were joined. (C) The mutation in the patient's factor VIII gene. The donor splice site of intron 15 was altered from GT to TT and the cryptic splice site in exon 16, where AG followed a polypyrimidine track, was activated. The abnormal mRNA is shown underneath. (D) Direct mutation analysis by the ASA method in the family members. The pedigree is shown above the gel. DNA sample from each individual was amplified for both wild-type (Wt) and mutant (Mt) alleles, generating the same product size of 241 bp. Primers amplifying exon 26 of the factor VIII gene, producing a 392-bp product, were added for internal control amplification. The normal control (N), the father (8/1), and unaffected younger brother (8/4) showed only the product of the wild type. The patient (8/3) and affected younger brother (8/5) had only the mutant product. The mother (8/2), who was a carrier, had both products.

normal individuals, indicating that it was a disease mutation.

The result of our study has demonstrated that the mutation at the donor splice site in intron 15 (IVS15 + 1G > T) of the FVIII gene caused the skipping of the entire 154 nucleotide sequence of exon 15 and the deletion of the first 47 nucleotides of exon 16, which could not be predicted from the result of mutation study of this gene without the mRNA analysis. The deletion of 201 nucleotides in the FVIII mRNA would result in a missense alteration of a codon at the position 1721 (AGG > AGC) leading to

substitution of arginine by serine (R1721S) and in-frame deletion of the sequence encoding 67 amino acids (from positions 1722–1788) in the A3 domain of the FVIII protein. This abnormal FVIII protein would have a severe structural change and loss of its function because a highly conserved region is deleted, which is supported by the finding of severe deficiency of FVIII:C (< 1%) in the patient and in his affected younger brother. Although the same mutation was previously reported in a severe case of haemophilia A [9], its effect on the FVIII mRNA has never been examined. Our study has provided more data and insight into its novel effect on the FVIII transcript as described.

This IVS15 + 1G > T mutation directly inactivates the donor splice site of intron 15, affects the utilizations of the acceptor splice site of intron 14 and the acceptor splice site of intron 15, and activates a cryptic splice site in exon 16. Examination of the sequence data in the region of exon 16 shows a long polypyrimidine track followed by an AG dinucleotide at positions of 46–47, the characteristic feature of an acceptor splice site (Fig. 1C). It is interesting that when the donor splice site of intron 15 approximately 1.3 kb away was mutated, this cryptic acceptor splice site was activated and was more preferable than the usual acceptor splice sites of introns 14 and 15. This led to exon 15 skipping and partial exon 16 deletion, instead of exon 15 retention or solely exon 15 skipping. The fact that the normal cDNA or other fragment was not detected in the patient's sample indicated that the cryptic acceptor splice was used exclusively. The activation of this cryptic acceptor splice site has also been noticed in a case of severe haemophilia A due to a missense (R1781H) mutation in exon 16 of the FVIII gene, resulting in the absence of the first 47 nucleotides of exon 16 in approximately 80% of the processed FVIII mRNA [10]. From this as well as our finding, it is possible that the acceptor splice site in intron 15 of the FVIII gene is highly sensitive to inactivation by nucleotide alterations either in the nearby region, or in the donor splice site region (even though the latter is 1.3 kb away). The cryptic acceptor splice site in exon 16 is relatively more active than the usual acceptor splice site when the latter is affected by a nucleotide change. However, calculation and comparison of scores between the native and the cryptic splice sites based on nucleotide weight tables [11] did not give the explanation for this preferential selection. The selection of the cryptic acceptor splice site may be influenced by the flanking nucleotide sequences of both native and cryptic splice sites. More data on the analysis of mutations in this region of the gene, combined with the result from an FVIII mRNA study similar to that which has been presented in this report, would provide more insight into the mechanism controlling the selection of splice signal in the eukaryotic cells.

The ASA method developed for direct detection of the mutation can be used for determination of carrier status in other female members of this family. It has also served as an example for the development of this method as a molecular diagnostic tool for direct mutation analysis of the FVIII gene in other haemophilia A families, especially large families with many at-risk female members.

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Engagement of Na,K-ATPase β3 subunit by a specific mAb suppresses T and B lymphocyte activation

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Keywords: lymphocyte inhibition, mAb, Na,K-ATPase β3 subunit, retroviral cloning system

Abstract

In order to identify new molecules involved in regulation of T cell proliferation, we generated various mAb by immunization of mice with the T cell line Molt4. We found one mAb (termed P-3E10) that down-regulated the *in vitro* T cell proliferation induced by CD3-specific OKT3 mAb. The P-3E10 mAb was also able to inhibit IFN-γ, IL-2, IL-4 and IL-10 production of OKT3-activated T cells. The antigen recognized by P-3E10 mAb is broadly expressed on all hematopoietic as well as on all non-hematopoietic cell lines tested so far. Within peripheral blood leukocytes, the P-3E10 antigen was detected on lymphocytes, monocytes and granulocytes. Human umbilical vein endothelial cells (HUVEC) also scored positively. By evaluating the effect of P-3E10 mAb on these cell types we found that it also inhibited anti-IgM-induced B cell proliferation. However, it did not block growth factor-mediated proliferation of HUVEC, and spontaneous proliferation of SupT-1, Jurkat, Molt4 and U937 cell lines. Moreover, it did not influence phagocytosis of human blood monocytes and granulocytes. Biochemical analysis revealed that the P-3E10 antigen is a protein with a mol. wt of 45–50 kDa under non-reducing and 50–55 kDa under reducing conditions. By using a retroviral cloning system, the P-3E10 antigen was cloned. Sequence analysis revealed the P-3E10 antigen to be identical to the β3 subunit of the Na,K-ATPase.

Introduction

Highly orchestrated cooperation of stimulatory and suppressive immune pathways is required in order to defeat pathogens without causing harm to self tissues. Under certain circumstances, such as autoimmunity or hypersensitivity, the immune system fails to reach this harmony and becomes rather a threat than a favor. In such cases, therapy is directed to suppress autoreactive responses and thus to restore the natural balance of the immune system. Characterization of the molecular mechanisms underlying negative immune regulation is supposed to provide targets for clinical interventions (1–4). The interaction between T cells and antigen-presenting cells (APC) seems to be a key event leading to activation of the cellular branch of immunity. Examples of molecules and their ligands involved in this interaction include CD4–MHC class II (5), CD8–MHC class I (6) and CD28/CTLA-4–CD80/CD86 (7–9). Therefore, the most suppressive therapeutic agents being developed target those molecules on the surface of both T cells and APC which are essential for regulation of T cell activation (10,11). For instance, immunosuppressive agents specific to the TCR complex molecules CD3, CD4 or CD8 as well as to the co-stimulatory molecules and their ligands CD28–CD80, CD152–CD86 or CD40–CD154 have been designed and used with success (10–12). In addition, some molecules not directly involved in T cell activation also appear to be a reasonable target for immunosuppressive treatment, e.g. P-glycoprotein (13).

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In an attempt to identify new molecules involved in regulation of T cell proliferation, we generated hybridomas from BALB/c mice immunized with the T cell line Molt4 and screened them for the ability to inhibit T cell activation. From among others, we selected one mAb (termed P-3E10) that down-regulated the T cell proliferation induced by immobilized CD3-specific mAb OKT3 as well as production of IFN-γ, IL-2, IL-4 and IL-10 *in vitro*. To identify the molecule recognized by this mAb, we cloned the encoding cDNA using a retroviral expression cloning system (14–16) and found that it is identical to the Na,K-ATPase β3 subunit.

Methods

Cells, reagents and antibodies

All human hematopoietic and non-hematopoietic cell lines used in this study were maintained in RPMI 1640 medium supplemented with 10% FCS (Gibco, Grand Island, NY), 40 μg/ml gentamicin and 2.5 μg/ml amphotericin B in a humidified atmosphere of 5% CO_2 at 37°C. Phoenix packaging cells, an ecotropic retroviral packaging cell line developed by Nolan *et al.* (14), were maintained in DMEM supplemented with 10% FCS.

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors by Ficoll-Hypaque density-gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden).

The CD99 mAb MT99/3 (IgG2a isotype), CD147 mAb M6-1E9 (IgG2a isotype) and the mAb KLH (IgG2a isotype) specific for the keyhole limpet hemocyanin molecule were generated by us [(17,18) and unpublished data]. The mAb MEM-188 (CD56; IgG2a isotype), MEM-M6/2 (CD147; IgG2a isotype) as well as the mAb against human α-fetoprotein AFP-01 (IgG1 isotype) were kindly provided by Dr V. Horejsi (Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic). Mouse IgG2a, UPC 10, was purchased from Sigma-Aldrich (St Louis, MO). The mAb 4G2 (IgG2a isotype) specific for E protein of dengue virus was kindly provided by Dr P. Malasit (Medical Biotechnology Unit, Mahidol University, Bangkok, Thailand). The IgG isotype mAb were purified by using a Protein A-coated Sepharose column (Zymed, San Francisco, CA) according to the methods described elsewhere (19).

Hybridoma production

mAb P-3E10 was generated by immunization of a female BALB/c mouse 3 times i.p. at 1-week intervals using 1×10^7 Molt4 cells. Then, the mouse was boosted i.v. using 1×10^6 cells. Splenocytes were collected and fused with P3-X63Ag8.653 myeloma cells by standard hybridoma fusion techniques using 50% polyethylene glycol and HAT medium selection. The IgG2a isotype of the mAb was determined using an isotyping ELISA kit (Sigma-Aldrich).

Proliferation assay for lymphocytes

Each culture was set up in a flat-bottom 96-well plate (Nunc, Roskilde, Denmark) in a final volume of 200 μl/well. Triplicate aliquots of 1×10^5 or 5×10^5 PBMC were activated using immobilized CD3 mAb OKT3 (20 ng/ml or 1 μg/ml; Ortho Pharmaceuticals, Raritan, NJ) or soluble goat anti-human IgM antibody (10 μg/ml; Hyland Diagnostics, Deerfield, MA) respectively in the presence or absence of various concentrations of tested mAb. The cultures were incubated for 3 days in a 5% CO_2 incubator at 37°C and then 1 μCi/well of [^{3}H]thymidine (Amersham Pharmacia Biotech, Freiburg, Germany) was added. The culture was incubated for an additional 18 h before harvesting. Incorporated radioactivity was counted in a liquid scintillation counter (MicroBeta; Wallac, Turku, Finland).

Proliferation assay for cell lines

For the cell line proliferation assay, triplicate aliquots of 1.5×10^4 cells were cultured with 0.5 μCi/well [^{3}H]thymidine (Amersham Pharmacia) with or without 2.5 μg/ml P-3E10 mAb. The cultures were incubated for 3 and 5 h in a 5% CO_2 incubator at 37°C. Then the culture was harvested and the incorporated radioactivity was counted in a liquid scintillation counter (Wallac).

Proliferation assay of human umbilical vein endothelial cells (HUVEC)

HUVEC were isolated by collagenase digestion. Briefly, human umbilical veins were flushed with Ringer's lactate and then incubated with 0.5 mg/ml collagenase type II (Sigma-Aldrich) at 37°C for 30 min. Detached HUVEC were collected, washed, and then cultured in fibronectin-coated flasks (Nunc, Naperville, IL) using M199 medium that contained 20% supplemented calf serum (SCS; Hyclone, Logan, UT), 25 μg/ml EC growth supplement (Technoclone, Vienna, Austria), 5 U/ml heparin, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml fungizone. Confluent HUVEC were gently trypsinized, seeded onto 24-well plates (1.5×10^4 cells/well) and cultured in the presence of P-3E10 mAb or isotype-matched control mAb at a final concentration of 20 or 1 μg/ml in M199 medium supplemented with 20% SCS (Hyclone). As negative control, the cells were cultured in M199 supplemented with 1% SCS. After 3 days of cultivation, cells were fixed by methanol and stained by crystal violet. After intense washings, cells were solubilized in 0.5% Triton X-100 and the number of cells was determined by measuring the absorbance at 595 nm using an ELISA reader and a standard curve.

Determination of cytokine production

PBMC (1×10^5) in the presence or absence of various concentrations of tested mAb (in a total volume of 200 μl) were culture in a flat-bottom 96-well plate (Nunc) precoated with mAb OKT3 (1 μg/ml). After incubation at 37°C in a CO_2 incubator for 24 or 72 h, the culture supernatants were harvested.

Cytokines were measured by sandwich ELISA using matched pairs of antibodies. Capture as well as detection antibodies to human IL-10 were obtained form R & D Systems (Minneapolis, MN). For the determination of IFN-γ a mAb (clone 25718.111) from R & D Systems was used as capture antibody and a mAb (clone GZ4) from Roche Diagnostics (Mannheim, Germany) as detection antibody. For IL-2 and IL-4, ELISA kits from Euroclone (Wetherby, UK) were used. Standards consisted of human recombinant material were obtained from R & D Systems. Assays were set up in

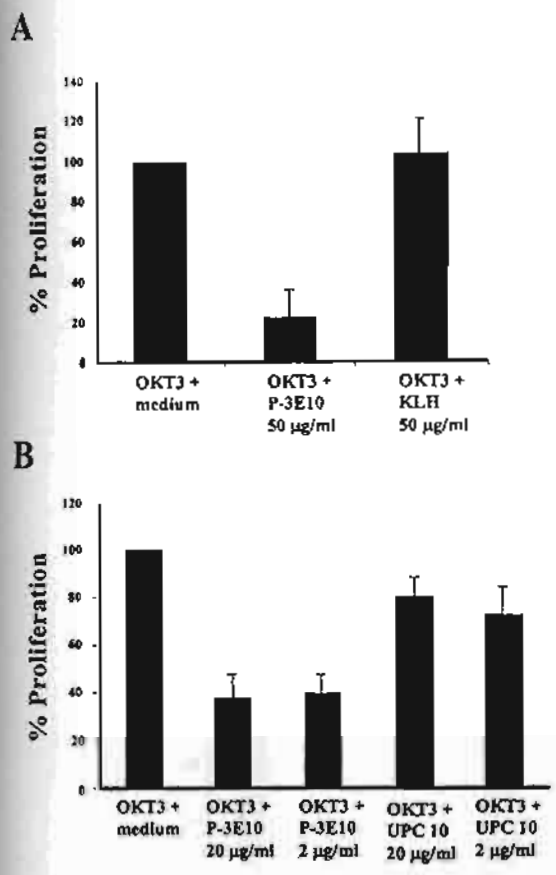

Fig. 1. P-3E10 mAb inhibits CD3-induced T cell proliferation. PBMC were activated with immobilized OKT3 mAb at 20 ng/ml (A) or 1 µg/ml (B) in the presence of the indicated concentrations of P-3E10 mAb, isotype-matched control mAb (KLH and UPC 10) or medium. The bars represent mean ± SD of 10 and six healthy donors for (A) and (B) respectively.

duplicates and performed according to the recommendations of the manufacturers.

Phagocytosis assay

Escherichia coli was grown in LB broth (Gibco) overnight at 37°C. Cells were washed twice and resuspended in PBS. The optical density of the bacterial suspension was measured at 600 nm and adjusted to 2.5. For phagocytosis assay, 100 µl of EDTA-blood was incubated with 25 µl of the bacterial suspension in the presence or absence of 10 µg/ml of P-3E10 mAb or isotype-matched control mAb at 37°C for 30 min. The sample was then smeared on a grass slide and stained with Wright's stain. Phagocytic cells were counted by light microscopy.

Immunofluorescence analysis

mAb binding to cells was analyzed by indirect immunofluorescence using FITC-conjugated sheep $F(ab')_2$ anti-mouse Ig antibodies (Immunotech/Coulter, Miami, FL). To block non-specific FcR-mediated binding of mAb, cells were pre-incubated for 30 min at 4°C with 10% human AB serum before staining. Membrane fluorescence was analyzed on a FACSCalibur (Becton Dickinson, Sunnyvale, CA) flow cytometer. Individual populations of blood cells were gated according to their forward and side scatter characteristics.

Labeling of cells and immunoprecipitation

For surface labeling, PBS-washed cells were biotinylated with Sulfo-NHS-LC-biotin (Pierce, Rockford, IL) (5 mM) for 1 h at 4°C. The reaction was quenched by washing once with 1 mM glycine in PBS and then twice with PBS. Cells (1×10^7) were solubilized in 1 ml lysis buffer (1% NP-40, 50 mM Tris–HCl, pH 8.2, 100 mM NaCl, 2 mM EDTA, 5 mM iodoactamide, 1 mM PMSF and 10 µg/ml aprotinin). Cell lysates were precleared with Protein A–Sepharose beads coated with non-specific mAb. Precleared lysates were then mixed with specific mAb-coated Protein A–Sepharose beads at 4°C for 24 h. After immunoprecipitation and SDS–PAGE, biotinylated proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% skimmed milk in PBS for 1 h at room temperature. The blocked membrane was incubated for 1 h at room temperature with avidin–peroxidase (Dako, Glostrup, Denmark) and the biotinylated proteins were visualized by the chemiluminescence detection system (Pierce).

Retroviral cloning of the P-3E10 molecule

The retroviral library construction was performed as described previously (15,16). In brief, a cDNA from human myeloid KG1a cells was cloned into the retroviral expression vector pBabeMN, kindly provided by G. Nolan (Stanford University).

For transfection of the library, Phoenix cells at 50% confluence were harvested by trypsinization, and 3×10^7 cells were added to a cocktail of 50 ml DMEM, 1% NuSerum (Genome Therapeutics, Waltham, MA), 200 µg/ml DEAE–dextran, 25 µM chloroquine diphosphate and 60 µg of the pBabeMN retroviral library. The cells were kept in suspension for 2 h at 37°C, washed once and cultivated in a 175 cm^2 flask (Nunc) in DMEM containing 10% FCS at 37°C. At 24 h post-transfection the medium was renewed. After an additional 48 h of cultivation at 32°C, the virus-containing supernatant was collected, supplemented with 10 µg/ml hexadimethrene bromide (Sigma) and added to 1×10^6/ml BW5147 mouse thymoma cells in 10 ml RPMI 1640 medium containing 10% FCS.

For the isolation of P-3E10-reactive cells, infected BW5147 cells (4×10^7) were washed with PBS containing 1% BSA and incubated with P-3E10 mAb for 30 min on ice. After another washing step the cells were incubated with goat anti-mouse IgG microbeads (Miltenyi Biotec, Bergish Gladbach, Germany) according to the manufacturer's instructions. After washing, cells were resuspended in 500 µl of MACS sorting buffer (0.5% BSA/2mM EDTA in PBS) and loaded onto MS^+ separation columns (Miltenyi Biotec) for positive selection of P-3E10 transduced cells. The isolated fraction was cultured in RPMI 1640 medium supplemented with 10% FCS. After three rounds of sorting, >95% of the isolated cells stained positively with P-3E10 mAb. Then, single-cell clones were obtained by limiting dilution.

For recovery of the P-3E10 cDNA, total RNA was extracted from the single-cell clone using Tri-Reagent (Sigma). RT-PCR was performed with Stratascript (Stratagene, La Jolla, CA) and

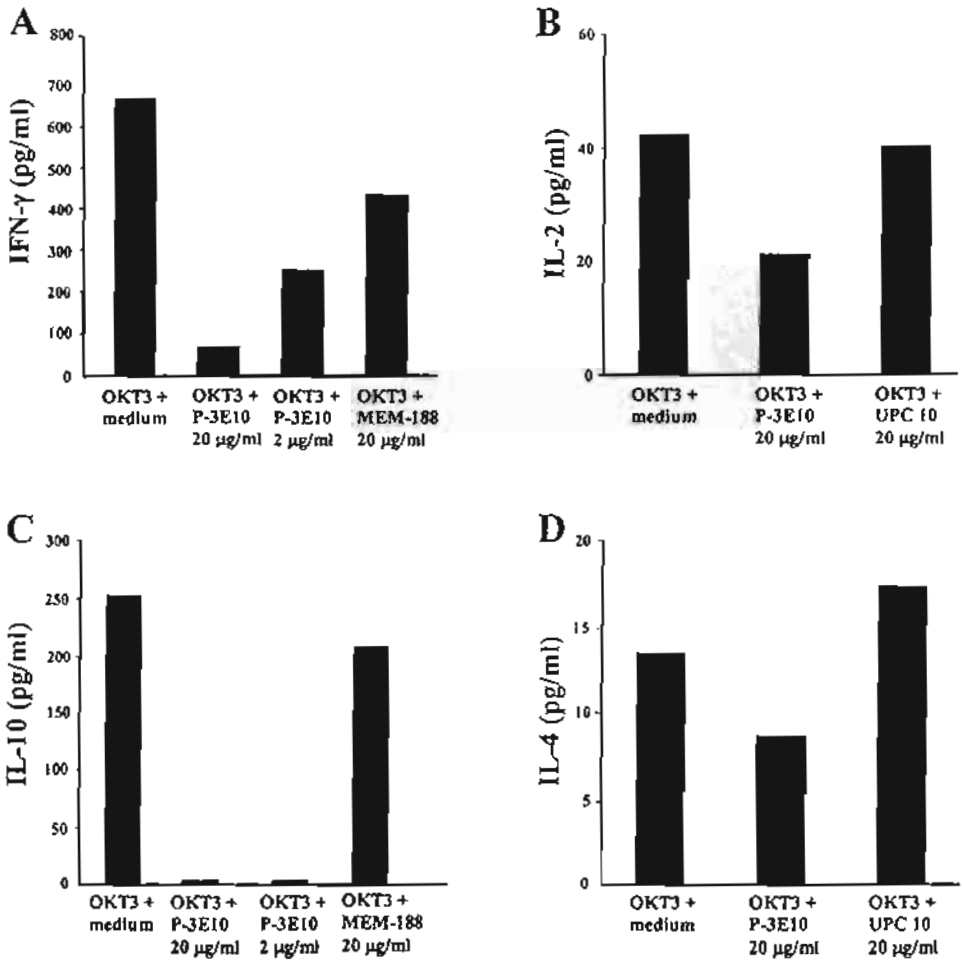

Fig. 2. P-3E10 mAb inhibits cytokine production. PBMC were activated with immobilized CD3 mAb OKT3 in the presence of indicated concentrations of soluble P-3E10 mAb, isotype-matched control mAb or medium alone. The culture supernatants were harvested, and IFN-γ, IL-2, IL-10 and IL-4 were measured by ELISA.

the Advantage-GC polymerase system (Clontech, Palo Alto, CA) using primers flanking the multiple cloning site of the retroviral vector pBabeMN. The PCR was run for 30 cycles (30 s at 94°C, 30 s at 58°C and 4 min at 68°C). The purified PCR product was subcloned back into pBabeMN and transformed into *E. coli* DH5α. The plasmid DNA was isolated using a Qiagen Miniprep column according to the manufacturer's recommendation (Qiagen, Hilden, Germany). To confirm that the isolated plasmid encodes the P-3E10 antigen, we used it to infect BW5147 cells as described above, and analyzed the transductants for P-3E10 expression by indirect immunofluorescence and flow cytometry. The plasmid was sequenced at the VBC-Genomics sequencing facility (Bioscience Research, Vienna, Austria).

Results

mAb P-3E10 inhibits the OKT3-induced T cell proliferation

In an attempt to identify new molecules involved in the regulation of T cell proliferation, various mAb against leukocyte surface molecules were generated using the T cell line Molt4 as an immunizing agent. The mAb were examined for their ability to modulate T cell proliferation in mononuclear cell preparations isolated from peripheral blood. We found that one of the mAb, named P-3E10, inhibited the OKT3-induced T cell proliferation *in vitro*. As shown in Fig. 1, T cell proliferation was significantly inhibited by mAb P-3E10 ($n = 16$). In contrast, isotype-matched control mAb, KLH and UPC 10, had no effect on the response of T cells to immobilized OKT3.

Inhibition of cytokine production of T cells by P-3E10 mAb

PBMC were activated with immobilized OKT3 mAb in the presence or absence of soluble P-3E10 mAb, and IFN-γ, IL-2, IL-10 and IL-4 were measured in the culture supernatants by ELISA. In the presence of P-3E10 mAb, production of all cytokines tested was inhibited (Fig. 2). The isotype-matched control mAb, however, had no such effect.

Cellular distribution of the P-3E10 antigen

To characterize the molecule recognized by P-3E10 mAb, various cell types were stained. All peripheral blood

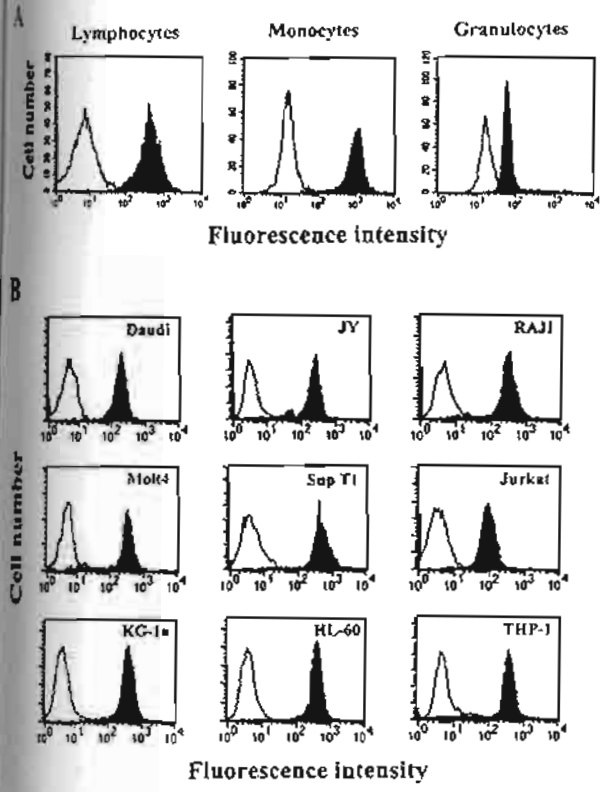

Fig. 3. Distribution of the antigen recognized by the mAb P-3E10 on peripheral blood leukocytes (A) and various hematopoietic cell lines (B). The indicated cells were stained with P-3E10 mAb (open) or AP-01 control mAb (solid) by indirect immunofluorescence. Data is representative of 10 independent donors (A) and three independent experiments (B).

leukocytes (n = 10) including lymphocytes, monocytes and granulocytes were positive with mAb P-3E10 (Fig. 3A). Then we examined expression on hematopoietic cell lines. As shown in Fig. 3(B), all cell lines tested, including B cell lines (Daudi, JY and RAJI), T cell lines (Molt4, Sup T1 and Jurkat) and myeloid cell lines (KG1a, HL-60 and THP-1), were strongly positive with P-3E10 mAb. We also analyzed several non-hematopoietic cells and cell lines, including HUVEC, 293 human embryonic renal epithelial, MCF-7 breast cancer, OVMZ ovarian cancer and TCL kidney cancer cell lines, and found that all were clearly stained by P-3E10 mAb (data not shown). Thus, our results indicate that the P-3E10 antigen is a broadly expressed plasma membrane molecule.

Effect of mAb P-3E10 on non-T cells

Because of the broad expression of the P-3E10 molecule, we tested the effect of the P-3E10 mAb on cells other than T cells. When we treated anti-IgM-induced B cells with 10 μg/ml of mAb P-3E10, similar to the results obtained with T cells, proliferation was inhibited by 65 ± 14% (mean ± SD; n = 3) (Fig. 4). However, in contrast to lymphocytes, the mAb did not block proliferation of HUVEC. Furthermore, it had also no effect on the growth of the hematopoietic cell lines Sup T-1, Jurkat, Molt4 and U937. Moreover, we analyzed the influence

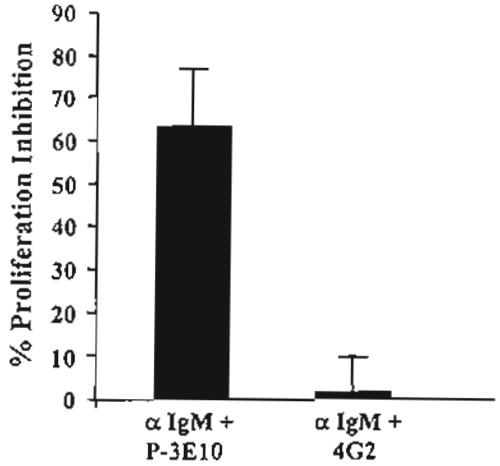

Fig. 4. P-3E10 mAb inhibits anti-IgM-induced B cell proliferation. PBMC were activated with 10 μg/ml of soluble anti-IgM antibody in the presence of 10 μg/ml P-3E10 mAb, isotype-matched control mAb (4G2) or medium alone. The bars represent mean ± SD of percent proliferation inhibition of three healthy donors.

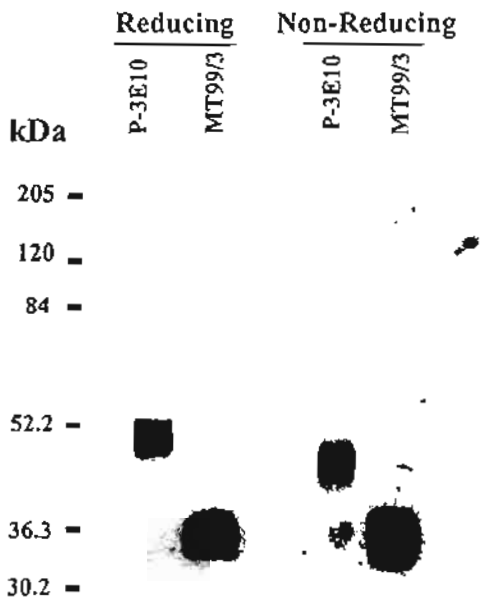

Fig. 5. Biochemical characterization of the cell surface molecule recognized by P-3E10 mAb. SDS–PAGE analysis of immunoprecipitates obtained with either P-3E10 mAb or CD99 mAb MT99/3 from lysates of surface biotin-labeled Sup T1 cells. Electrophoresis was performed under reducing and non-reducing conditions. Molecular markers are shown on the left in kDa.

of P-3E10 on phagocytosis of myeloid cells, monocytes and granulocytes, but no effect was observed. The percentages of monocytes that had phagocytosed *E. coli* in the presence or

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   1                                                                  CCCGTAAC
   9 GAGGAGGTGTTCTCGGCCGTCCCACCCTTCACTGCCGTCTCCGGGCTGCGCCGCCGGAGCCGGGACG
  76 CGCCTCCGCAGCCCTCGCCGCCTCCATCCCCGCGGCCGCAGCTCCTCTCGCCGTCCGCGCGCACACC
     Met Thr Lys Asn Glu Lys Lys Ser Leu Asn Gln Ser Leu Ala Glu Trp Lys     17
 143 ATG ACG AAG AAC GAG AAG AAG TCC CTC AAC CAG AGC CTG GCC GAG TGG AAG
     Leu Phe Ile Tyr Asn Pro Thr Thr Gly Glu Phe Leu Gly Arg Thr Ala Lys     34
 194 CTC TTC ATC TAC AAC CCG ACC ACC GGA GAA TTC CTG GGG CGC ACC GCC AAG
     Ser Trp Gly Leu Ile Leu Leu Phe Tyr Leu Val Phe Tyr Gly Phe Leu Ala     51
 245 AGC TGG GGT TTG ATC TTG CTC TTC TAC CTA GTT TTT TAT GGG TTC CTG GCT
     Ala Leu Phe Ser Phe Thr Met Trp Val Met Leu Gln Thr Leu Asn Asp Glu     68
 296 GCA CTC TTC TCA TTC ACG ATG TGG GTT ATG CTT CAG ACT CTC AAC GAT GAG
     Val Pro Lys Tyr Arg Asp Gln Ile Pro Ser Pro Gly Leu Met Val Phe Pro     85
 347 GTT CCA AAA TAC CGT GAC CAG ATT CCT AGC CCA GGA CTC ATG GTT TTT CCA
     Lys Pro Val Thr Ala Leu Glu Tyr Thr Phe Ser Arg Ser Asp Pro Thr Ser    102
 398 AAA CCA GTG ACC GCA TTG GAA TAT ACA TTC AGT AGG TCT GAT CCA ACT TCG
     Tyr Ala Gly Tyr Ile Glu Asp Leu Lys Lys Phe Leu Lys Pro Tyr Thr Leu    119
 449 TAT GCA GGG TAC ATT GAA GAC CTT AAG AAG TTT CTA AAA CCA TAT ACT TTA
     Glu Glu Gln Lys Asn Leu Thr Val Cys Pro Asp Gly Ala Leu Phe Glu Gln    136
 500 GAA GAA CAG AAG AAC CTC ACA GTC TGT CCT GAT GGA GCA CTT TTT GAA CAG
     Lys Gly Pro Val Tyr Val Ala Cys Gln Phe Pro Ile Ser Leu Leu Gln Ala    153
 551 AAG GGT CCA GTT TAT GTT GCA TGT CAG TTT CCT ATT TCA TTA CTT CAA GCA
     Cys Ser Gly Met Asn Asp Pro Asp Phe Gly Tyr Ser Gln Gly Asn Pro Cys    170
 602 TGC AGT GGT ATG AAT GAT CCT GAT TTT GGC TAT TCT CAA GGA AAC CCT TGT
     Ile Leu Val Lys Met Asn Arg Ile Ile Gly Leu Lys Pro Glu Gly Val Pro    187
 653 ATT CTT GTG AAA ATG AAC AGA ATA ATT GGA TTA AAG CCT GAA GGA GTG CCA
     Arg Ile Asp Cys Val Ser Lys Asn Glu Asp Ile Pro Asn Val Ala Val Tyr    204
 704 AGG ATA GAT TGT GTT TCA AAG AAT GAA GAT ATA CCA AAT GTA GCA GTT TAT
     Pro His Asn Gly Met Ile Asp Leu Lys Tyr Phe Pro Tyr Tyr Gly Lys Lys    221
 755 CCT CAT AAT GGA ATG ATA GAC TTA AAA TAT TTC CCA TAT TAT GGG AAA AAA
     Leu His Val Gly Tyr Leu Gln Pro Leu Val Ala Val Gln Val Ser Phe Ala    238
 806 CTG CAT GTT GGG TAT CTA CAG CCA TTG GTT GCT GTT CAG GTC AGC TTT GCT
     Pro Asn Asn Thr Gly Lys Glu Val Thr Val Glu Cys Lys Ile Asp Gly Ser    255
 857 CCT AAC AAC ACT GGG AAA GAA GTA ACA GTT GAG TGC AAG ATT GAT GGA TCA
     Ala Asn Leu Lys Ser Gln Asp Asp Arg Asp Lys Phe Leu Gly Arg Val Met    272
 908 GCC AAC CTA AAA AGT CAG GAT GAT CGT GAC AAG TTT TTG GGA CGA GTT ATG
     Phe Lys Ile Thr Ala Arg Ala Stop                                      279
 959 TTC AAA ATC ACA GCA CGT GCA TAG TATGAGTAGGATATCTCCACAGAGTAAATGTTGTG
1018 TTGTCTGTCTTCATTTTGTAACAGCTGGACCTTCCATTCTAGAATTATGAGACCACCTTGGAGAAAG
1085 GTGTGTGGTACATGACATTGGGTTACATCATAACGTGCTTCCAGATCATAGTGTTCAGTGTCCTCTG
1152 AAGTAACTGCCTGTTGCCTCTGCTGCCCTTTGAACCAGTGTACAGTCGCCAGATAGGGACCGGTGAA
1219 CACCTGATTCCAAACATGTAGGATGGGGGTCTTGTCCTCTTTTTATGTGGTTTAATTGCCAAGTGTC
1286 TAAAGCTTAATATGCCGTGCTATGTAAATATTTTATGGATATAACAACTGTCATATTTTGATGTCAA
1353 CAGAGTTTTAGGGATAAAATGGTACCCGGCCAACATCAAGTGACTTTATAGCTGCAAGAAATGTGGT
1420 ATGTGGAGAAGTTCTGTATGTGAGGAAGGAAA
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Fig. 6. Nucleotide and deduced amino acid sequence of the cDNA encoding the P-3E10 antigen. Nucleotide positions are indicated on the left and amino acid positions on the right. The putative transmembrane-spanning domain is underlined. The six cysteines are boxed.

absence of P-3E10 mAb were 72 ± 4.6 and 70 ± 6.2; those of granulocytes 56 ± 5.3 and 53 ± 3.8 (mean ± SD: $n = 3$), respectively.

Biochemical characterization of the surface molecule recognized by mAb P-3E10

To biochemically characterize the molecule bearing the P-3E10 antigen, we performed an immunoprecipitation experiment. As shown in Fig. 5, we were able to precipitate a 50- to 55-kDa protein under reducing conditions from lysates of surface-biotinylated Sup T1 cells using P-3E10 mAb. The protein shifted to 45–50 kDa under non-reducing conditions (Fig. 5) indicating that it contains intramolecular disulfide bonds. In comparison the isotype-matched control mAb MT99/3 (to the CD99 antigen) precipitated, as described previously (17), a protein band of 32 kDa both under reducing and non-reducing conditions.

Molecular cloning of the cDNA coding for the antigen recognized by P-3E10 mAb

A KG1a cDNA library in the retroviral vector pBabeMN and the packaging cell line Phoenix were used to produce ecotropic viruses for transducing the target cell line BW5147. BW5147 transductants expressing the P-3E10 antigen were sorted by P-3E10 mAb using MACS and cloned to single-cell cultures by limiting dilution. One strongly positive cell clone was selected to isolate the P-3E10 cDNA. For this, RT-PCR was performed with the RNA extracted from the clone using primers flanking the multiple cloning site of the retroviral vector pBabeMN. The PCR product was digested with *EcoR*I to remove the plasmid sequences flanking the cDNA. Sequencing of the cDNA revealed a length of 1451 bp with an open reading frame of 840 bp coding for 279 amino acids (Fig. 6). Comparison of the sequence using the BLAST program at the NCBI (Bethesda, MD) resulted in 100% homology to the Na,K-ATPase β3 subunit (20). This molecule is a type II transmembrane protein

with a single transmembrane segment. The predicted extracellular domain contains six cysteine residues, which is in accord with our biochemical data in Fig. 5.

To confirm that the isolated Na,K-ATPase β3 subunit cDNA encodes the P-3E10 antigen, the cDNA was re-ligated via *EcoRI* into the pBabeMN vector and transduced into BW5147 cells, which were subsequently tested for binding of the P-3E10 mAb. The transduced BW cells were specifically stained by P-3E10 mAb, demonstrating that the P-3E10 antigen is indeed a determinant on the Na,K-ATPase β3 subunit.

Discussion

In our study, we prepared a set of mAb reactive with the Molt4 T cell line. The generated mAb were screened for the ability to modulate CD3-induced T cell proliferation. P-3E10 mAb was found to be of interest, as it significantly inhibited T cell proliferation as well as IFN-γ, IL-2, IL-4 and IL-10 production *in vitro*. P-3E10 mAb also inhibited anti-IgM-induced B cell proliferation. However, it had no effect on HUVEC and hematopoietic cell line proliferation, as well as phagocytosis of monocytes and granulocytes. By using a retroviral cloning system we proved that the P-3E10 antigen is a determinant on the human Na,K-ATPase β3 subunit (20).

The Na,K-ATPase is a membrane-associated enzyme responsible for the active transport of Na^+ and K^+ in most animal cells (21–23). By using the energy from the hydrolysis of one molecule of ATP, it transports three Na^+ out in exchange for two K^+ that are taken in. By coupling the hydrolysis of ATP to the movement of Na^+ and K^+ ions across the plasma membrane, the enzyme produces the electrochemical gradient that is the primary energy source for the active transport of nutrients, the action potential of excitable tissues and the regulation of cell volume (21–23). In all tissues, Na,K-ATPase is characterized by a complex molecular heterogeneity that results from the expression and differential association of multiple isoforms of both its α and β subunits. At present, as many as four different α polypeptides (α1, α2, α3 and α4) and three distinct β isoforms (β1, β2 and β3) have been identified in mammalian cells (23). The α subunits are multispanning membrane proteins with a molecular mass of ~100 kDa that are responsible for the catalytic and transport properties of the enzyme. The β polypeptides cross the membrane once, depending on the degree of glycosylation in different tissue, and their molecular mass ranges from 40 to 60 kDa. The β subunit is essential for the normal activity of the enzyme, and it appears to be involved in the occlusion of K^+, and the modulation of K^+ and Na^+ affinity of the enzyme (22,23). In addition, in vertebrate cells, the β subunit may act as a chaperone, stabilizing the correct folding of the α subunit to facilitate its transport to the plasma membrane (22,23).

In immune cells, induction of Na,K-ATPase-mediated K^+ fluxes in mitogen-activated lymphocytes has been reported (24–28). Increase in mRNA encoding the α and β subunits of Na,K-ATPase in phytohemagglutinin-activated lymphocytes was also demonstrated (26,29). Several mechanisms have been proposed to explain the increase in the Na,K-ATPase activity in lymphocyte activation (26,28,30). Prasad *et al.* have argued that the increase in intracellular calcium may lead to a subsequent protein kinase-induced enhancement of Na,K-ATPase enzymatic activity (28). Na,K pump activation, accompanying human lymphocyte blast transformation, also plays a critical role in the expression of IL-2 receptor and initiates IL-2 expression (24,31,32). Inactivation of Na,K-ATPase by specific inhibitors causes inhibition of both mitogen- and antigen-induced lymphocyte activation (31–33).

In the present study we demonstrate that engagement of the Na,K-ATPase β3 subunit by a mAb down-regulates both T and B lymphocyte proliferation as well as production of IFN-γ, IL-2, IL-4 and IL10 of T cells. This is, to the best of our knowledge, the first study to demonstrate that a specific mAb to the Na,K-ATPase β chain is able to block lymphocyte activation. Although, the precise mechanism of this inhibition is unknown, engagement of Na,K-ATPase by P-3E10 mAb may block Na,K-ATPase activation and subsequently suppress downstream events, which in turn lead to the inhibition of lymphocyte activation. Further investigation of the mechanisms of the Na,K-ATPase β3 chain in inhibition of lymphocyte activation may lead to a better understanding of immune regulation, which may provide new avenues for clinical intervention.

Acknowledgements

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Abbreviations

APC	antigen-presenting cell
PBMC	peripheral blood mononuclear cell
HUVEC	human umbilical vein endothelial cell
SCS	supplemented calf serum

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Production of Antibodies by Single DNA Immunization: Comparison of Various Immunization Routes

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ABSTRACT

DNA immunization is a recent vaccination method that induces humoral and cellular immune responses in a range of hosts. Different immunization routes induce a different degree of the immune response. In the present report, we demonstrate that multiple intramuscular immunizations of plasmid DNA encoding various leukocyte surface molecules induced a specific antibody response. In contrast, a single intramuscular immunization could not induce antibody production. To study the induction of antibody response after a single immunization of plasmid DNA, mice were single-dose intramuscularly, intraperitoneally, intravenously and intrasplenically immunized, simultaneously, with the same preparation of plasmid DNA encoding CD147 membrane protein. We observed that only the intrasplenic route induced specific antibody production. The induction of antibody by intrasplenic immunization was confirmed by using plasmid DNA encoding CD54 molecule. By this single-dose DNA intrasplenic immunization, the generated antibodies could be detected in mice up to 6 months. These results suggest that the injected DNA is expressing the relevant protein antigen in the spleen for several months after injection. Our results demonstrate that direct immunization of antigen-encoding DNA into the spleen is a more effective method for induction of antibody production. This finding may support future investigations of DNA vaccination strategies that specifically promote the uptake of plasmid by splenocytes. Intrasplenic immunization may also be helpful in the understanding of the early events of the immune response to DNA vaccine and be useful as an effective route for the induction of immune responses.

INTRODUCTION

DNA IMMUNIZATION refers to the induction of an immune response to a protein expressed *in vivo*, subsequent to the introduction of its encoding DNA. In contrast to classical protein immunization, where antigens are administered, DNA immunization involves the administering of genetic material encoding the antigen. The antigen is, therefore, produced within the cells of the immunized individual and induces the immune responses.[1,2] Several investigators have demonstrated the feasibility of using direct injection of plasmid DNA for the induction of protective immunity against various pathogens[2,3] and the production of specific antibodies.[4–12] Immunization with DNA-based plasmid has been successfully attempted in several tissues by various routes of administration.[1,3] However, the intramuscular injection has been demonstrated as the most efficient route to transfer an aqueous solution of plasmid DNA.[13] This method, however, still results in a low efficiency of gene transfer and considerable variability in gene expression.[14,15] Induction of immune responses by a single intramuscular immunization of plasmid DNA has been demonstrated,[16–18] however, booster(s) were required in several reports.[19–22]

To obtain the appropriate immunization route for a single plasmid DNA injection for the induction of humoral immune response, in the present report we compared various administration routes including intramuscular, intraperitoneal, intravenous, and intrasplenic. We demonstrate here that, for a single plasmid DNA administration, intrasplenic immunization of antigen-encoding DNA is a more effective method in induction of antibody production. The induced antibody could be detected in mice up to 6 months after DNA immunization.

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MATERIALS AND METHODS

Cells and antibodies

Human hemopoietic cell lines, Sup T1 and U937, were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY), 40 μg/mL gentamicin and 2.5 μg/mL amphotericin B in a humidified atmosphere of 5% CO_2 at 37°C. COS7 cells were cultured in MEM (Gibco) containing 10% FBS and antibiotics. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by a Ficoll Hypaque density gradient centrifugation (Sigma-Aldrich Chemical, St. Louis, MO).

Purified CD54 monoclonal antibody (MAb) MT54 and CD147 MAb M6-1D4 were generated in our department.[12,23] Fluorescein isothiocyanate (FITC)-conjugated rabbit F(ab')2 anti-mouse immunoglobulin antibodies was purchased from Dako (Glostrup, Denmark).

Preparation of plasmid DNA

cDNA encoding CD4, CD14, CD45, and CD54 membrane proteins inserted into an eukaryotic expression vector pCDM8 (designated CD4-DNA, CD14-DNA, CD45-DNA and CD54-DNA, respectively) and cDNA encoding soluble CD147-human IgG fusion protein (named CD147-Rg-DNA) were kind gifts from Dr. Hannes Stockinger, University of Vienna, Vienna, Austria. cDNA encoding CD147 membrane protein, named CD147-DNA, was generated in our Department.[24] For large-scale preparation, the plasmid DNA were transformed into *E. coli* MC1061/p3. The plasmid DNA were then isolated from transformed *E. coli* by Qiagen chromatography columns (Qiagen, Hilden, Germany). The plasmid DNA obtained were subsequently resuspended in phosphate-buffered saline (PBS). The concentration and purity of DNA preparation were determined by $OD_{260/280}$ reading. DNA were stored at −20°C, until injected into the mice.

The isolated plasmid DNA were proved for expression of the corresponding proteins by using the COS cell expression system. Indirect immunofluorescent staining of the transfected COS cells with specific MAbs was used for proving the expression of membrane protein. Enzyme-linked immunosorbent assay (ELISA) was used for proving the expression of soluble CD147-IgG fusion protein.

DNA immunization

For intramuscular immunization, BALB/c mice were injected one or five times at 2-week intervals by intramuscular route at the hind legs (100 μg of DNA/dose). For intraperi-

TABLE 1. ANTIBODY RESPONSES IN MICE AFTER 5-DOSES OF INTRAMUSCULAR IMMUNIZATIONS WITH PLASMID DNA ENCODING VARIOUS LEUKOCYTE SURFACE MOLECULES

		Dose of immunization				
DNA	*Pre-immune*	*1*	*2*	*3*	*4*	*5*
CD4-DNA						
Mouse 1	−	−	−	+	+	+
Mouse 2	−	−	+	+	+	+
Mouse 3	−	−	+	+	+	+
Mouse 4	−	−	+	+	+	+
CD14-DNA						
Mouse 1	−	−	+	+	+	+
Mouse 2	−	−	−	−	−	+
CD45-DNA						
Mouse 1	−	−	+	+	+	+
Mouse 2	−	−	+	+	+	+
CD54-DNA						
Mouse 1	−	−	+	+	+	+
Mouse 2	−	−	+	+	+	+
Mouse 3	−	−	+	+	+	+
Mouse 4	−	−	+	+	+	+
CD147-DNA						
Mouse 1	−	−	+	+	+	+
Mouse 2	−	−	−	+	+	+
Mouse 3	−	−	−	−	+	+
Mouse 4	−	−	−	−	+	+
Mouse 5	−	−	+	+	+	+
CD147-Rg-DNA						
Mouse 1	−	−	−	+	+	+
Mouse 2	−	−	−	+	+	+
Mouse 3	−	−	+	+	+	+

Mice were immunized with 5 doses of various plasmid DNA. Sera were collected at pre-immunization and at 2 weeks after the indicated doses of DNA immunization. For detection of CD4 antibody, Sup T1 cells were used as target cells. For detection of CD14 antibody, peripheral blood monocytes were used as target cells. For detection of CD45, CD54, CD147 antibody, U937 cells were used as target cells. The target cells were stained with the collected sera (dilution 1:10) by indirect immunofluorescence and analyzed by flow cytometer. Antibody reactivity: −, negative; +, positive.

neal immunization, 500 μL (100 μg) of plasmid DNA solution was injected into the peritoneal cavity. For intravenous immunization, 100 μL (100 μg) of plasmid DNA solution was injected into the tail vein. For intrasplenic immunization, mice were anesthetized with diethylether. The skin and peritoneum on the left side of the body was open to expose the spleen. Fifty microliters (100 μg) of plasmid DNA were injected into the spleen. The peritoneum and skin were then closed by fine sutures. Blood samples were collected from the immunized mice by tail bleeding at 2-week intervals. Sera were separated and stored at −20°C.

Immunofluorescence analysis

To determine antibodies in the sera, indirect immunofluorescence was carried out. To block nonspecific Fc-receptor-mediated binding of antibody, cells were pre-incubated for 30 min at 4°C with 10% human AB serum before staining. Blocked cells were then incubated for 30 min at 4°C with tested sera. After washing, cells were incubated with FITC-conjugate for 30 min. Membrane fluorescence was analyzed on a FACSCalibur (Becton Dickinson, Sunnyvale, CA) flow cytometer.

DEAE-dextran transfection of COS7 cells

Plasmid DNA were transfected into COS7 cells by the DEAE-dextran transfection method.[24] Briefly, 1×10^6 COS7 cells were transfered to 6-cm tissue culture dishes (NUNC, Roskilde, Denmark) on the day before transfection. Cells were incubated with 2 mL of MEM containing 250 μg/mL DEAE-dextran (Sigma), 400 μM chloroquine diphosphate (Sigma), and 2 μg DNA for 3 h at 37°C. Supernatant was removed and cells were treated with 10% dimethyl sulfoxide (DMSO) in PBS for 2 min at room temperature. Cells were then cultured in MEM containing 10% fetal bovine serum (FBS) overnight, washed once, and recultured with the same medium for another 2 days to allow expression of the corresponding proteins.

RESULTS

Production of antibodies by intramuscular immunization of plasmid DNA

To produce antibodies by injection of antigen-encoding plasmid DNA, intramuscular immunization was used as inoculation routes. In this study, plasmid DNA encoding CD4, CD14, CD45, CD54, CD147 membrane protein or secreted CD147-human IgG fusion protein was prepared by using Qiagen chromatography column. All plasmid DNA obtained were tested for its expression by COS cells transfection. The plasmid DNA encoding membrane proteins were to be able to express the corresponding proteins on COS cell membrane as determined by immunofluorescence technique (data not shown). The CD147-Rg DNA was able to produce soluble CD147-IgG fusion protein in culture supernatant of transfected COS cells as determined by ELISA (data not shown).

Mice were intramuscularly immunized with the plasmid DNA for 5 times at 2-week intervals or with a single injection. Sera collected from each mouse were first screened for the presence of specific antibodies by staining of cells expressing the

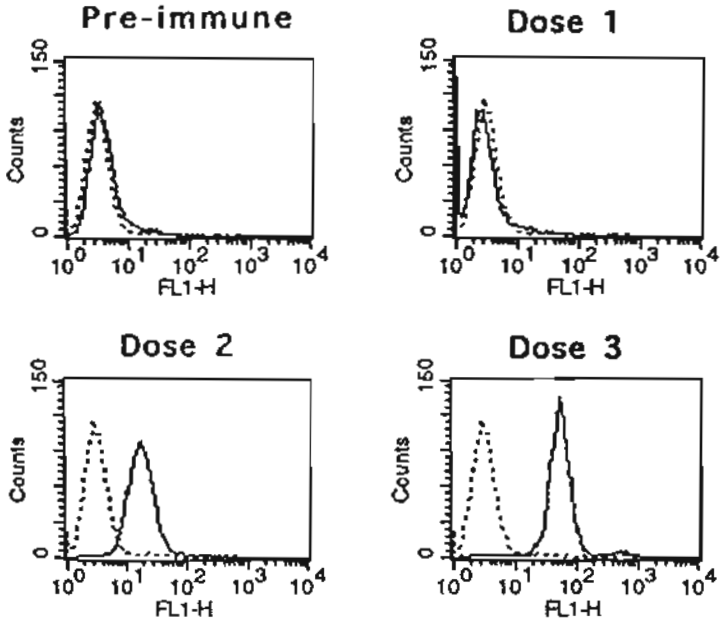

FIG. 1. FACS analysis of anti-CD147 antibodies generated by DNA immunization. Mouse was immunized with plasmid DNA encoding CD147 membrane protein. Sera were collected at pre-immunization and at 2 weeks after the indicated doses of DNA immunization. U937 cells were stained with indicated sera at 1:10 (solid lines) or without serum (dashed lines) by indirect immunofluorescence assay.

TABLE 2. ANTIBODY RESPONSES IN MICE AFTER SINGLE-DOSE IMMUNIZATION OF PLASMID DNA ENCODING CD147 MOLECULE BY VARIOUS IMMUNIZATION ROUTES

		Weeks after DNA immunization															
Route	*Pre-immune*	*2*	*4*	*6*	*8*	*10*	*12*	*14*	*16*	*18*	*20*	*22*	*24*	*26*	*28*	*30*	*32*
Intraperitoneal																	
Mouse 1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Mouse 2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Intramuscular																	
Mouse 1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Mouse 2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Intravenous																	
Mouse 1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Intrasplenic																	
Mouse 1	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Mice were immunized with single-dose plasmid DNA encoding CD147 molecule by the indicated route. Sera were collected at pre-immunization and every 2 weeks after DNA immunization. U937 cells were stained with sera (dilution 1:10) by indirect immunofluorescence and analyzed by flow cytometer.

Antibody reactivity: −, negative; +; positive.

corresponding proteins. As shown in Table 1, sera from 5-doses intramuscularly either plasmid DNA encoding CD4, CD14, CD45, CD54, or CD147 immunized mice showed positive reactivity. Plasmid DNA encoding membrane CD147 and secreted CD147 protein induced antibody production in a similar pattern. For an example of flow cytometric analysis, antibody responses of a mouse immunized with plasmid DNA encoding CD147 membrane protein is shown in Fig. 1. In contrast, none of the sera, collected every 2 weeks up to 5 months, from single-dose intramuscularly immunized mice showed positive reactivity (data not shown).

To confirm that the generated antibodies after plasmid DNA immunization were specific for the corresponding proteins, the CD4-DNA, CD14-DNA, CD45-DNA, CD54-DNA, and CD147-DNA transfected COS cells were stained with the positive sera. As predicted, sera obtained from CD4, CD14, CD45, CD54, and CD147 and CD147-Rg-DNA immunized mice reacted to CD4, CD14, CD45, CD54, and CD147 transfectants, respectively, but they did not react to mock transfectants (data not shown). Pre-immune sera of each mouse did not react to any transfectants.

Production of antibodies by a single immunization of plasmid DNA

In an attempt to produce specific antibody by only a single immunization of the encoding plasmid DNA, CD147-DNA were injected into mice by using intraperitoneal, intramuscular, intravenous and intrasplenic routes, simultaneously. As shown in Table 2, by a single intramuscular, intraperitoneal and intravenous route, no specific antibodies were detected. However, sera obtained from a single-dose intrasplenic immunized mouse showed positive reactivity. All positive sera were stained with COS cell transfectants and showed positive reactivity with CD147-DNA transfectants, and negative with mock transfectants. The results indicated that intrasplenic immunization is an effective route for single plasmid DNA injection.

To confirm that single intrasplenic immunization can induce antibody production, CD54-DNA were immunized into six mice. We found that all mice generated specific antibody (Table 3). Interestingly, the generated antibodies could be detected in the immunized mice sera up to several months (Table 3 and Fig. 2).

DISCUSSION

The principle of DNA immunization has been demonstrated in several different animal models. Currently, it is clear that the

TABLE 3. ANTIBODY RESPONSES IN MICE AFTER SINGLE-DOSE INTRASPLENIC IMMUNIZATION OF PLASMID DNA ENCODING CD54 MOLECULE

	Mouse number					
Weeks after immunization	*1*	*2*	*3*	*4*	*5*	*6*
Pre-immune	−	−	−	−	−	−
2	+	−	−	−	−	−
4	+	−	−	−	−	−
6	+	−	−	−	−	−
8	+	−	−	−	−	−
10	+	−	+	−	−	−
12	+	+	+	+	−	nd
14	+	+	+	+	−	nd
16	+	+	nd	+	−	nd
18	+	+	nd	+	−	nd
20	+	+	nd	+	−	nd
21	+	+	nd	+	−	nd
22	+	+	nd	+	−	nd
24	+	+	nd	+	−	nd
26	nd	+	nd	+	−	nd
28	nd	+	nd	+	−	nd

Mice were immunized with single-dose CD54-DNA by intrasplenic route. Sera were collected at pre-immunization and every two weeks after DNA immunization. U937 cells were stained with sera (dilution 1:10) by indirect immunofluorescence and analyzed by flow cytometer.

Antibody reactivity: −, negative; +, positive; nd, not determine.

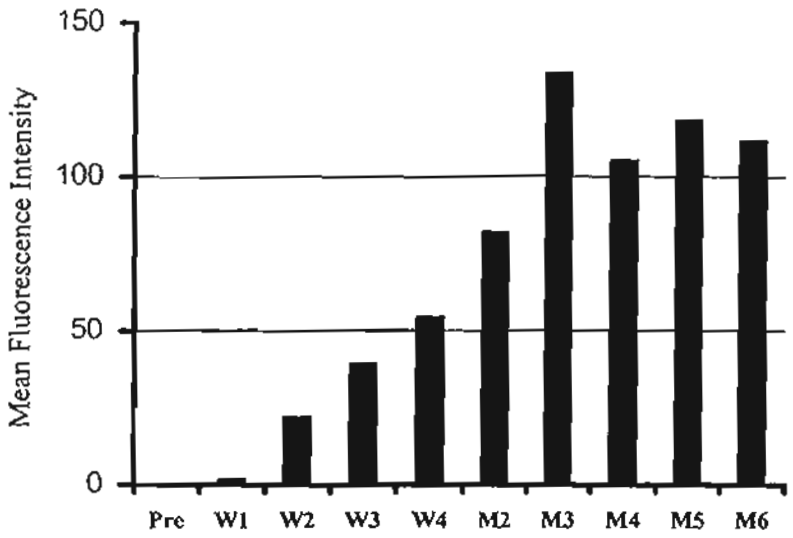

FIG. 2. Antibody response of a mouse intrasplenic immunized with CD54 encoding DNA. Mouse was single immunized on Days 0 and sera from blood drawn periodically were analyzed by indirect immunofluorescence and flow cytometer using U937 cells as antigen. Pre, pre-immune serum; W1-W4, sera obtained from 1–4 weeks after DNA immunization; M2–M6, sera obtained from 2–6 months after DNA immunization.

induction of humoral and cellular immunity is possible with the DNA immunization strategy. DNA immunization, therefore, promises to be an attractive alternative to the classical vaccines.[2,3] DNA immunization was also proven to be a good method for the production of polyclonal antibody as well as for the generation of MAbs.[4–12] This method may provide the best way to produce specific antibodies to proteins that are difficult to purify.

Several DNA delivery methods have been used to introduce plasmid DNA for the induction of immune responses.[13] The most common route to transfer pure plasmid DNA is intramuscularly.[13] In the present report, we found that by using the intramuscular route, antibody production was achieved after multiple immunizations of plasmid DNA expressing various types of leukocyte surface molecules. The specific antibodies were detected after two to five DNA inoculations. In contrast, no mice that obtained a single dose intramuscular immunization generated antibody. Intramuscular injection of plasmid DNA has been widely used with DNA vaccines. In most cases of DNA intramuscular immunization, high titers of antibodies have been found against the expressed protein. Some investigators demonstrated that a single intramuscular immunization of plasmid DNA induced antibody responses,[16–18] however, booster(s) was required in several reports including this report.[19–22] These differences appear to be due to the nature of the particular antigen, the expression vector used and may be due to the skill of the individual administering the DNA.[21,25] When DNA is administered by intramuscular injection, the plasmid DNA is taken up by myocytes. Due to the limitation of diffusion by physical factors such as the organization of the connective tissue, and extent of the extracellular matrix,[14,15] the intramuscular immunization of DNA results in a low efficiency of gene transfer and a considerable variability of gene expression. Therefore, induction of antibody production by intramuscular immunization of plasmid DNA requires booster dose(s).

Injection of protein antigen directly into lymphoid organs such as lymph node or spleen offers some strong theoretical advantages over other injection routes.[26,27] In this type of immunization, the immunogen is concentrated in one region that is specialized in dealing with it. This immunization route has been further used for polyclonal and MAb production purposes.[28–31] In an attempt to induce antibody production by a single-short plasmid DNA inoculation, in the present study, intrasplenic immunization was selected and compared with intramuscular, intraperitoneal and intravenous routes, simultaneously, by using the same preparation plasmid DNA. We found that only the intrasplenic route induced antibody production. Our results confirmed the previous reported possibility of using intrasplenic immunization for the induction of antibody responses by DNA immunization.[11,12] Enhancement of antibody responses by intrasplenic immunization is likely to be related to the fact that the injected plasmid DNA are directly transfected splenocytes, including antigen-presenting cells. The antigens are then expressed and concentrated in the spleen, where the immune responses are initiated. Antigen-presenting cells are thought to play at least three distinct roles in DNA immunization: (1) major histocompatibility complex (MHC) class II-restricted presentation of antigens secreted by neighboring, transfected cells, (2) MHC class I-restricted "cross" presentation of antigens released by neighboring, transfected cells, and (3) direct presentation of antigens by transfected antigen presenting cells themselves.[32] Therefore, injection of DNA into the spleen allows for the direct delivery of antigens to the spleen and the induction of antibody responses occur only with a single-short DNA immunization. In the present report, the induced antibody could be detected in mice up to 6 months after DNA intrasplenic immunization. These results suggest that the injected DNA is still expressing the relevant protein antigen, in the spleen, for several months after injection.

In protein immunization, it was clearly easier to obtain an

antibody response using minute amounts of antigen by intrasplenic route than by the intraperitoneal or intravenous route.[33] In agreement with protein immunization, this study demonstrated that a single injection of antigen-encoding DNA into the spleen induced antibody response better than via an intramuscular, intraperitoneal, or intravenous route. Recently, the use of intrasplenic immunization of plasmid encoding carcinoembryonic antigen (pCEA) for induction of immune responses was reported.[34] Intrasplenic administration of pCEA could induce specific antibody responses and partial immunoprotection against tumor challenge.[34] Our findings, therefore, support future investigations of DNA vaccination strategies that specifically promote the uptake of plasmid by splenocytes. This intrasplenic immunization may also be helpful in the further understanding of early events of the immune response to DNA vaccine as well as for the production of specific antibodies.

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Carrier Detection by DNA Linkage Analysis in Eighty Thai Hemophilia A Families

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Abstract

DNA linkage analysis was performed in Thai hemophilia A families to evaluate its value for carrier detection. Both intragenic and extragenic polymorphic DNA regions of the factor VIII gene, including *Bcl* I-RFLP in intron 18, microsatellites (CA repeats) in introns 13 and 22, and extragenic St14 (DXS 52) VNTR, were amplified by polymerase chain reaction (PCR) before analyses by appropriate electrophoretic procedures. A total of 80 Thai hemophilia A families (48 with a family history and 32 with a sporadic case), containing 349 DNA samples from 90 hemophilia A patients, 143 parents, and 116 relatives, were analyzed. Heterozygosities in the patients' mothers from both families with a family history and with a sporadic case were observed in 71 out of 80 families (88.75%) for all polymorphic DNA markers analyzed. The carrier status could be identified in 36 females and excluded in 44 females. This result indicates that the DNA linkage analysis can be used for carrier detection or exclusion in the majority of Thai hemophilia A families. It should also be useful for prenatal diagnosis in families at risk of hemophilia A, which is part of the prevention and control of this disease.

Key word : Hemophilia A, DNA Linkage Analysis, Carrier Detection, Factor VIII Gene

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Hemophilia A is the most common X-linked recessive bleeding disorder found in all human populations. Its incidence in Thailand is about 1: 20,000[1]. The disease results from the deficiency of factor VIII coagulant activity (F VIII:C) due to the mutations in the factor VIII gene, which is located on the distal region of the long arm of the X chromosome (Xq28). The factor VIII gene, cloned and characterized since 1984[2-4], is a large gene with the length of 186 kb containing 26 exons. The gene encodes factor VIII precursor consisting of 2351 amino-acid residues. The factor VIII protein with 2332 amino-acid residues results from post-translational cleavage of a signal peptide comprising 19 amino acids from the precursor protein[5].

Diagnosis of hemophilia A is generally based on family history, patient's bleeding record, and standard coagulation studies[6]. Among Thai hemophilia A patients, about 60 per cent of the cases were found to be familial and 40 per cent sporadic (unpublished data). The molecular defects of hemophilia A identified consisted of gene inversions, gene deletions, and a large number of different types of point mutations including nucleotide substitutions, deletions, and insertions[7]. Some of these defects such as gross gene deletions and gene inversions are readily identified by DNA hybridization using appropriate DNA probes[7]. However, the majority of defects caused by different types of point mutations cannot be detected by such a method. In the situation that a specific defect of the factor VIII gene has not been characterized in a hemophilia A family, abnormal factor VIII gene tracking in the family is crucial in terms of carrier detection and prenatal diagnosis. This has been possible through DNA linkage study using different kinds of extragenic and intragenic polymorphic DNA markers[6,7].

The restriction fragment length polymorphisms (RFLPs) of enzymes *Bcl* I, *Xba* I, *Bgl* I in the factor VIII gene and of *Bgl* I and *Taq* I at linked loci, DX13 (DXS15) and St14 (DXS52), respectively, detected by DNA hybridizations, in Thai females were previously studied[8]. The *Bgl* I-RFLP was found to be absent in Thais while the frequencies of other polymorphisms were similar in Thais and Caucasians. Availability of the polymerase chain reaction (PCR) technique has made it possible to detect RFLPs in the factor VIII gene and the extragenic variable number of tandem repeat (VNTR) polymorphism linked to the factor VIII gene (St14) without performing DNA hybridization[9]. Highly polymorphic DNA markers, microsatellites or CA repeats, in introns 13 and 22 of the factor VIII gene were described and used for the linkage analysis[10-12]. The frequencies of these two microsatellite markers and their usefulness in DNA linkage analysis in Thai hemophilia A families have not been established. In the present study, the authors report the frequencies of these markers and informativeness of using intragenic *Bcl* I-RFLP, microsatellites in introns 13 and 22, and extragenic St14 (DXS 52) VNTR polymorphism, detected by PCR technique, in the linkage analysis for carrier detection in 80 Thai hemophilia A families.

PATIENTS AND FAMILIES

From October 1993 to July 1997, blood samples from members of 80 Thai hemophilia A families were taken for routine laboratory services and investigations. The families were informed for the factor VIII gene analyses, the results of which would be used as a part of genetic counseling and beneficial to the families. Of these 80 families, 48 families were found to have a family history and 32 families without, described as familial and sporadic, respectively. The total number of individuals examined was 349 and their statuses were indicated in Table 1. Among 90 hemophilia A patients from the 80 families, the disease was found to be severe with F VIII:C <2.5 per cent in 71, moderate with F VIII:C 2.5-5 per cent in 14, and mild with F VIII:C >5 per cent in 5 cases. DNA was prepared from blood samples by the standard proteinase K digestion and phenol/chloroform extraction procedure.

Table 1. Number of hemophilia A patients and family members.

Status	Number
Patient	90
Mother	80
Father	63
Female sibling	72
Male sibling	21
Female relative	16
Patient's spouse	3
Patient's daughter	4
Total	349

METHOD

DNA linkage analyses by using PCR methods

Both intragenic and extragenic polymorphic DNA regions in the factor VIII gene were amplified by the PCR methods as previously described[10-15]. The *Bcl* I-RFLP in intron 18 and the St14 (DXS 52) VNTR were detected by acrylamide gel and agarose gel electrophoresis, respectively. The microsatellites or CA repeats in introns 13 and 22 were analyzed by sequencing gel electrophoresis followed by autoradiography. Nucleotide sequences of primers and references of the methods used are provided in Table 2.

RESULTS

The results of DNA linkage analysis in 80 hemophilia A families using four polymorphic DNA regions including three intragenic and one extragenic marker showed that each marker had variable power (informativeness) to differentiate between the normal and mutant alleles, depending mainly on its degree of polymorphism, which could be demonstrated initially by the state of heterozygosity of the marker alleles in the carrier especially the patient's mother. The result of analysis using *Bcl* I-RFLP showed that the patients' mothers in 23 out of 48 families with a family history (47.9%) and in 9 out of 32 families with sporadic cases (28.1%) were found to be heterozygous. Thus, the overall heterozygosity of the *Bcl* I-RFLP marker in the patients' mothers was 32 out of 80 families (40%) (Table 3). The examples of hemophilia A families heterozygous and homozygous for this marker in the patient's mother are presented in Fig. 1 and 2.

The results of analysis of CA repeats within intron 13 of the factor VIII gene revealed that the patients' mothers in 31 out of 48 families with a family history (64.6%) and in 10 out of 32 families with sporadic cases (31.3%) were heterozygous. The overall heterozygosity of this intron 13 marker was 51.3 per cent (Table 3). The results of analysis of CA repeats within intron 22 demonstrated that the patients' mothers in 28 out of 48 families with a family history (58.3%) and in 10 out of 32 families with sporadic cases (31.3%) were heterozygous. The

Table 2. Polymorphic DNA markers and nucleotide sequences of primers for linkage analyses of the factor VIII gene by PCR methods.

Site	Restriction enzyme		Sequence of primers	Sizes of allele (bp)	References
Intron 18	*Bcl* I	8.1	5'-TAAAAGCTTTAAATGGTCTAGGC-3'	142, 99 + 43	13, 14
		8.2	5'-TTCGAATTCTGAAATTATCTTGTTC-3'		
Intron 13	-	1A	5'-TGCATCACTGTACATATGTATCTT-3'	143 - 155	10, 11, 12
		2A	5'-CCAAATTACATATGAATAAGCC-3'		
Intron 22	-	1B	5'-TTCTAAGAATGTAGTGTGTG-3'	82 - 88	10, 11, 12
		2B	5'-TAATGCCCACATTATAGA-3'		
St14 (DXS 52)	-	St14-R	5'-GGCATGTCATCACTTCTCTCATGTT-3'	660 - >2,000	15
		St14-L	5'-CACCACTGCCCTCACGTCACTT-3'		

Table 3. Per cent heterozygosities of four polymorphic DNA markers in the factor VIII gene region in the patients' mothers in 80 Thai hemophilia A families.

Site	Restriction enzyme	Number of allele	Per cent heterozygosity in the patients' mothers		
			Familial	Sporadic	Overall
Intron 18	*Bcl* I	2	47.9	28.1	40.0
Intron 13	-	7	64.6	31.3	51.3
Intron 22	-	4	58.3	31.3	47.5
St14 (DXS 52) VNTR	-	11	58.3	75.0	65.0 (61.8)*

* with 5% recombination

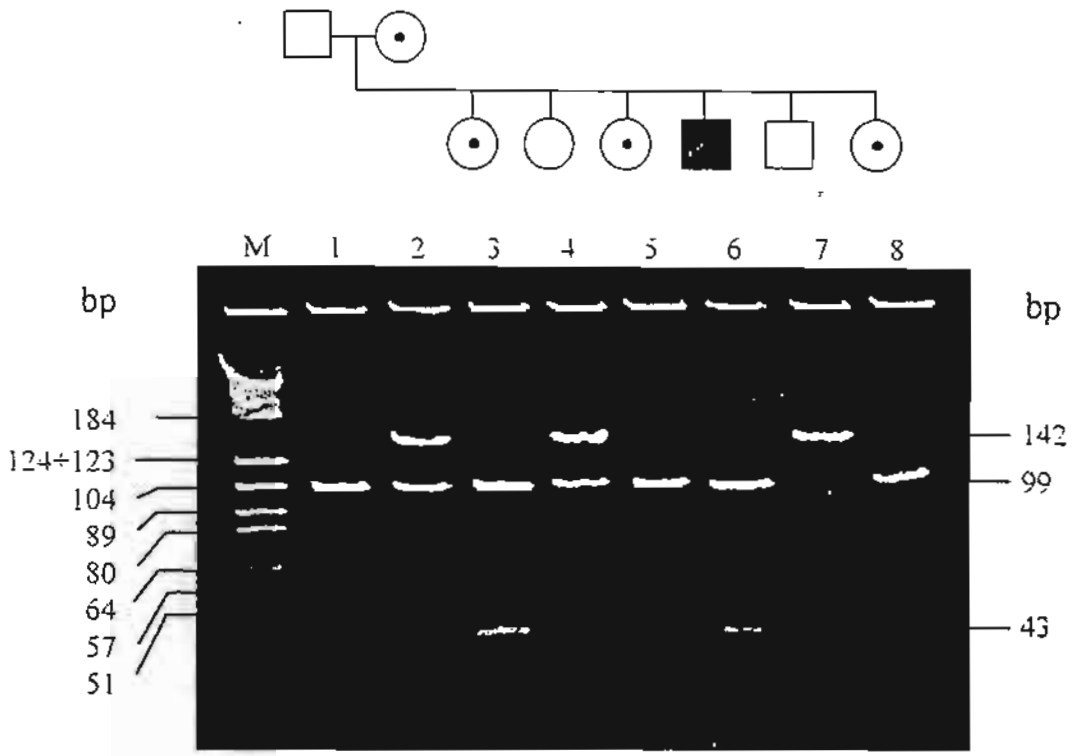

Fig. 1. The DNA linkage analysis by *Bcl* I-RFLP in a hemophilia A family showing the informative result. The mother who was a carrier (lane 2) was heterozygous for the polymorphic alleles with the sizes of 142 bp and 99+43 bp. The patient (lane 6) had the allele with the size of 99+43 bp inherited from the mother. The unaffected son (lane 7) had the allele with the size of 142 bp from the mother. Three daughters (lane 3, 5, 8) were homozygous for the allele with the size of 99+43 bp inherited from the mother and from the father; therefore they were carriers. The other daughter (lane 4) had the alleles with the sizes of 142 bp from the mother and 99+43 bp from the father; therefore, she was a non-carrier.

overall heterozygosity of this intron 22 marker was 47.5% (Table 3). The numbers of the alleles and their frequencies are shown in Table 4.

The result of linkage analysis using extragenic St14 (DXS 52) VNTR marker showed that the patients' mothers in 28 out of 48 families with a family history (58.3%) and in 24 out of 32 families with sporadic cases (75.0%) were heterozygous. The overall heterozygosity of this marker was 65.0 per cent but when calculated by taking the 5 per cent recombination rate into account, it was reduced to 61.8 per cent. At least 8 alleles were detected; their allele sizes and frequencies are shown in Table 5.

From the results of linkage analysis using all DNA markers, 36 female carriers of hemophilia A were identified in families with a family history. Of these, 33 were patients' siblings and 3 were mothers' relatives from two families. In addition, 27 females in the hemophilia A families were identified to be non-carriers.

In the families with sporadic cases of hemophilia A, although the patients' mothers were heterozygous for the polymorphic DNA markers and one of the alleles in the mother was detected in the patient, the carrier status of female siblings and relatives in the families could not be identified with certainty. The results of linkage analysis were, however, useful for exclusion of the carrier status in the female siblings and relatives who did not carry the same allele as the patients. Thus, among females from the sporadic families, 17 were excluded as being hemophilia A carriers.

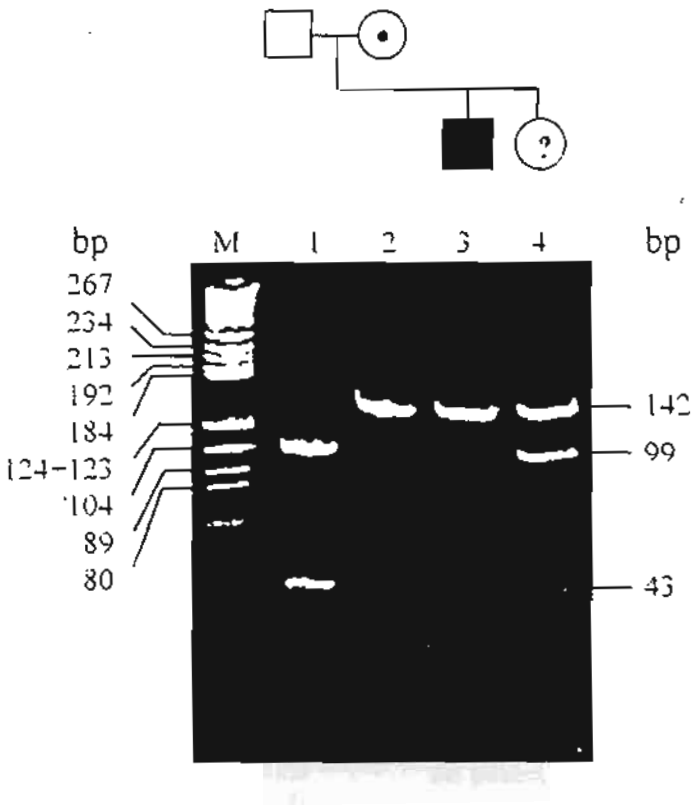

Fig. 2. The DNA linkage analysis by *Bcl* I-RFLP in a hemophilia A family showing the non-informative result. The mother (lane 2) was homozygous for the allele with the size of 142 bp and her hemophilic son also carried one allele with the size of 142 bp. Therefore, it was unable to predict which of the two alleles with the size of 142 bp from the mother was inherited to the daughter (lane 4) who carried an allele with the size of 142 bp from the mother and that of 99+43 bp from the father.

There were 8 females in whom the carrier or non-carrier status could not be identified although they were heterozygous for the polymorphic DNA markers. One was a female sibling of two hemophilia A patients from a family with a family history whose father was not available for the investigation and there were no other male or female siblings in the family (Fig. 3). The other 7 females (2 siblings and 5 relatives of the hemophilia A patients) were found in 5 unrelated families with sporadic cases. Thus, female carriers and non-carriers identified by DNA linkage analysis in 80 Thai hemophilia A families are summarized in Table 6.

DISCUSSION

Several intragenic and extragenic polymorphic DNA regions in the factor VIII gene have been described. RFLPs within the gene including *Bcl* I-RFLP in intron 18[13], *Xba* I RFLP in intron 22[16], *Hind* III RFLP in intron 19[17] were commonly used for carrier detection and prenatal diagnosis. The rates of the female heterozygosity, especially in the patient's mother, of these markers were found to be between 42-48 per cent in various ethic groups and the marker with the highest rate was *Xba* I-RFLP [16]. This polymorphic marker was successfully detected by the Southern blotting method but was difficult to detect using the PCR-RFLP method due to interference of specific amplified product. In the present study, the detection of *Bcl* I-RFLP was carried out because the method has been well established; it is simple and non-radioisotopic with informativeness between 31 per cent and 47 per cent [6]. There was no reason to analyze other RFLP

Table 4. Allele frequencies of polymorphic CA repeats markers in introns 13 and 22 of the factor VIII gene observed in Thai hemophilia A families.

Intron 13		Intron 22	
CA repeats	Allele frequency	CA repeats	Allele frequency
(CA)21	0.017	(CA)18	0.013
(CA)22	0.050	(CA)19	0.353
(CA)23	0.245	(CA)20	0.546
(CA)24	0.399	(CA)21	0.088
(CA)25	0.206		
(CA)26	0.050		
(CA)27	0.017		

Table 5. The allele sizes and frequencies of polymorphic St14 (DXS 52) VNTR marker observed in Thai hemophilia A families.

Allele sizes (bp)	Allele frequencies
660	0.417
1,210	0.243
1,280	0.026
1,315	0.009
1,350	0.115
1,500	0.030
1,550	0.030
1,650	0.047
1,800	0.021
2,000	0.011
> 2,000	0.051

markers since they are normally in linkage disequilibrium with the marker that had been examined and the results would not add more information, as previously reported by Goodeve et al[8]. Using this *Bcl* I-RFLP marker alone, approximately 48 per cent of Thai hemophilia A families with a family history were informative. As it is an intragenic marker and its detection method is simple, it should be the first marker of choice for carrier detection or prenatal diagnosis. However, about half of the families were not informative, and other markers are required for the detection and diagnosis.

The extragenic St14 (DXS 52) VNTR close to the factor VIII gene is a highly polymorphic marker, which should be another marker of choice for the detection. It was previously reported that nearly 90 per cent of females would be heterozygous[18] for this marker. However, the result in the present study showed that heterozygosity in the patients' mothers in the hemophilia A families with a family history was about 58 per cent, much less than was previously reported. The heterozygosity in the patients' mother in the families with sporadic cases was 75 per cent, raising the overall heterozygosity to be the highest of all the markers analyzed. The reason for the difference between the two groups of hemophilia A families is unknown, possibly due to a bias from a smaller number of families with sporadic cases. The crossing over in the region between the factor VIII gene and the marker can occur between 3 per cent and 5 per cent[5] which will partly limit its utility in the carrier and prenatal detection.

Table 6. Female carriers and non-carriers identified by DNA linkage analysis in 80 Thai hemophilia A families.

Status	Number in the families with family history	Number in the families with sporadic case
Carrier	36	-
Non-carrier	27	17
Unknown	1*	7

* No result of DNA analysis from other family members for verification.

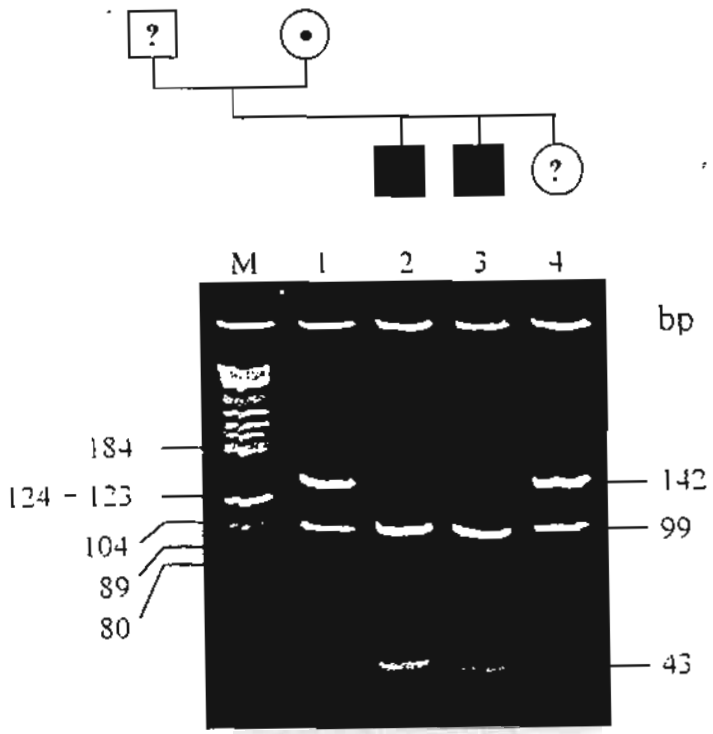

Fig. 3. The DNA linkage analysis by *Bcl* I-RFLP in a hemophilia A family showing the non-informative result, since the father was not available for testing although the patients' mother (lane 1) was heterozygous for the polymorphic alleles with the sizes of 142 bp and 99+43 bp. The allele with the size of 99+43 bp was observed in her two hemophilic sons (lanes 2 and 3). The daughter (lane 4) could have either the allele with the size of 142 bp or 99+43 bp from her mother; therefore, her carrier status could not be identified.

The polymorphic microsatellite (CA repeats) markers within introns 13 and 22 of the factor VIII gene were more difficult to analyze since they required detection of radioactive PCR products using sequencing polyacrylamide gel and autoradiography. Previous studies in various racial groups revealed different numbers of CA repeats and allele frequencies[12,19]. The allele numbers of CA repeats in introns 13 and 22 were found to be 7-11 alleles and 5-11 alleles, respectively. The rate of informativeness were between 58-80 per cent, of which the highest was seen among black Africans[20] and lowest in the Chinese[21]. The present result of the CA repeats study in both introns revealed fewer allele numbers (7 and 4 alleles, respectively) than that previously reported. The informativeness of the analysis using the CA repeats in introns 13 and 22 in the hemophilia A families with a family history were about 65 per cent and 58 per cent, which were higher than that of the *Bcl* I-RFLP marker. The families that were not informative by the *Bcl* I-RFLP marker were informative by these two CA repeat markers. Therefore, the CA repeats analysis in introns 13 and 22 would contribute additional information to the detection.

The DNA linkage analysis is very useful for hemophilia A carrier detection in families with a family history of this disease. However, its informativeness is dependent on the availability of at least one affected male and most importantly heterozygosity of the detected marker in the obligate carrier mother. The examples of DNA linkage analysis for

informative and non-informative families are shown in Fig. 1 and 2. In the circumstance that *Bcl* I-RFLP, which is a bi-allelic marker, is used especially in the family that has only one daughter and one hemophilic son, the father is also required for testing. The authors saw one family in whom the carrier status could not be identified in the daughter because the father was not available for testing (Fig. 3). In the families with sporadic hemophilia A cases, although the result of DNA linkage analysis could not be used for the carrier detection, it was helpful for exclusion of the carrier status. In these families as well as those non-informative families with a family history, analysis of mutation and development of direct methods for mutation detection will be necessary.

In conclusion, the DNA linkage analysis can be used for carrier detection or exclusion in the majority of Thai hemophilia A families. It should also be useful for prenatal diagnosis in families at risk of hemophilia A, which is a part of the prevention and control of this disease.

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การตรวจหาพาหะโรคฮีโมฟีเลีย เอ ในครอบครัวผู้ป่วยไทย 80 ครอบครัว ด้วยวิธีวิเคราะห์ดีเอ็นเอลิงค์เกจ

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คณะผู้วิจัยได้ทำการวิเคราะห์ดีเอ็นเอลิงค์เกจในครอบครัวผู้ป่วยไทยโรคฮีโมฟีเลีย เอ เพื่อประเมินคุณค่าของวิธีนี้ในการตรวจผู้ที่เป็นพาหะ ดีเอ็นเอที่หลากหลายในขนาด ทั้งที่อยู่ภายในและภายนอกยีนแฟคเตอร์แปด ได้แก่ *Bcl* I-RFLP ในอินตรอน 18, microsatellites (CA repeats) ในอินตรอน 13 และ 22, และ St14 (DXS 52) VNTR ซึ่งอยู่ภายนอกยีน ถูกเพิ่มปริมาณด้วยวิธีพีซีอาร์ก่อนการตรวจด้วยวิธีอิเล็คโตรโฟรีซีสตามประเภทของดีเอ็นเอที่ถูกตรวจ จำนวนครอบครัวผู้ป่วยที่นำมาศึกษาทั้งหมดมี 80 ครอบครัว (48 ครอบครัวมีประวัติของโรคภายในครอบครัว และ 32 ครอบครัวไม่มีประวัติ) ซึ่งมีจำนวนดีเอ็นเอทั้งหมด 349 ตัวอย่าง (90 ตัวอย่าง จากผู้ป่วย, 143 ตัวอย่าง จากบิดาและมารดา, และ 166 ตัวอย่างจากญาติพี่น้อง) ผลการศึกษาพบว่าครอบครัวผู้ป่วยฮีโมฟีเลีย เอ จำนวน 71 จาก 80 ครอบครัว (คิดเป็น 88.75%) สามารถตรวจได้ด้วยดีเอ็นเอที่หลากหลายในขนาดหนึ่งชนิดหรือมากกว่า นอกจากนี้ ภาวะที่เป็นพาหะพบในผู้หญิงจำนวน 36 ราย และภาวะที่ไม่ใช่พาหะพบในผู้หญิง 44 ราย ผลของการศึกษานี้แสดงว่าการวิเคราะห์ดีเอ็นเอลิงค์เกจสามารถจะนำไปใช้เพื่อตรวจผู้ที่เป็นพาหะและไม่ใช่พาหะในครอบครัวผู้ป่วยโรคฮีโมฟีเลีย เอ ส่วนใหญ่ได้ วิธีนี้น่าจะเป็นประโยชน์สำหรับใช้ในการวินิจฉัยโรคก่อนกำเนิด ในครอบครัวที่เสี่ยงต่อการให้กำเนิดทารกที่เป็นโรคฮีโมฟีเลีย เอ ซึ่งเป็นส่วนหนึ่งของการควบคุมและป้องกันโรคนี้

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Comparison of four reverse transcription-polymerase chain reaction procedures for the detection of dengue virus in clinical specimens

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Abstract

The sensitivity of dengue virus identification by mosquito inoculation and four reverse transcription-polymerase chain reaction (RT-PCR) procedures (Am. J. Trop. Med. Hyg. 45 (1991) 418 (H); J. Clin. Microbiol. 29 (1991) 2107 (M); J. Clin. Microbiol. 30 (1992) 545 (L); and Southeast Asian J. Trop. Med. Public Health 27 (1996) 228 (Y)) were compared using coded clinical specimens derived from areas in Thailand where all four dengue serotypes circulate. The sensitivity of virus detection in serologically confirmed dengue cases was 54, 52, 60, 79, and 80% for mosquito inoculation, procedures H, M, L and Y, respectively. In comparison to clinical specimens which yielded virus isolates by mosquito inoculation, there was relatively low sensitivity in detecting each of the four dengue serotypes by PCR: procedure H-dengue 4 (25%), procedure M-dengue 3 (73%), procedure L-dengue 1 (70%), and procedure Y-dengue 1 (79%). Dengue virus was detectable by RT-PCR for more days of illness and in the presence of dengue-specific

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antibody when compared to virus isolated in mosquitoes. Procedures L and Y were more sensitive than mosquito inoculation or procedures H and M in detecting all four dengue serotypes in clinical specimens and may be the RT-PCR methods of choice for virus surveillance or research use.

Keywords: Dengue virus; Diagnosis; RT-PCR

1. Introduction

Dengue virus types 1–4, members of the family Flaviviridae, are transmitted between humans by the mosquito vector, principally *Aedes aegypti*. Infection can give rise to a wide spectrum of disease ranging from a mild, self-limited febrile illness to more severe vascular and hemostatic abnormalities known as dengue hemorrhagic fever and dengue shock syndrome (DHF and DSS, World Health Organization, 1997). Dengue infections constitute a significant cause of morbidity and mortality throughout tropical and subtropical regions (reviewed by Halstead, 1988; Henchal and Putnak, 1990; Innis, 1995; Gubler, 1998).

Conventional dengue diagnosis is based on the detection of virus-specific antibody or the isolation and identification of virus from patient serum or plasma. The most commonly used serologic tests include the hemagglutination inhibition (HAI; Clarke and Casals, 1958) assay and the IgM capture enzyme immunoassay (EIA; Innis et al., 1989). However, depending on the specific assay format testing can be time consuming and difficult to quality control. More recently, rapid dengue IgM and IgG detection kits utilizing immunochromatographic technology have become available commercially (Vaughn et al., 1998; Lam and Devine, 1998; Porter et al., 1999; Branch and Levett, 1999; Palmer et al., 1999; Wu et al., 2000; Groen et al., 2000). Nevertheless, all of these serologic assays fail to consistently provide a diagnosis during the acute febrile phase of the illness (Vaughn et al., 1997). In addition, they do not identify the dengue virus type responsible for the infection owing to the high cross-reactivity between antibodies induced by different dengue serotypes (Calisher et al., 1989). The traditional method to determine the infecting virus type is virus isolation in cell culture (Gubler et al., 1984) or live mosquitoes (Rosen and Shroyer, 1985), followed by immunofluorescent staining using dengue type-specific monoclonal antibodies (Henchal et al., 1982, 1983). However, virus isolation takes from days to weeks and the success rate is often low because of several factors such as inappropriate handling of specimens, formation of virus-antibody complexes, and low numbers of viable virus (reviewed by Vorndam and Kuno, 1997; Gubler, 1998).

The polymerase chain reaction (PCR) technique has been applied widely for the rapid and sensitive detection of many infectious agents including dengue viruses. A number of reverse transcription PCR (RT-PCR) procedures to detect and to identify dengue serotypes in clinical specimens have been reported (Deubel et al., 1990; Henchal et al., 1991; Morita et al., 1991; Lanciotti et al., 1992; Chang et al., 1994; Seah et al., 1995a,b; Yenchitsomanus et al., 1996; Meiyu et al., 1997; Sudiro et al., 1998; Harris et al., 1998; Laue et al., 1999; Houng et al., 2001; Callahan et al., 2001). These PCR methods vary somewhat in terms of gene regions of the genome amplified, methods to detect the RT-PCR products, and methods of virus typing. For example, the method developed by Henchal et al. (procedure H, Fig. 1) selectively amplifies the 482 nucleotide sequence in the NS1 region of the dengue virus genome using degenerate primers followed by blot hybridization with type-specific DNA probes (Henchal et al., 1991). The method reported by Morita et al. (procedure M) utilized four pairs of type-specific primers selected from the E-NS1-NS2A-NS2B gene regions to detect simultaneously and identify the dengue types in a single tube (Morita et al., 1991). Lanciotti et al. (procedure L) and, more recently, Yenchitsomanus et al. (procedure Y) employed a similar strategy—an initial RT-PCR using universal dengue primers followed by a type-specific nested PCR (Lanciotti et al., 1992; Yenchitsomanus et al., 1996). However, the two methods

differ in the gene regions amplified (C-prM region for procedure L and E region for procedure Y). Applicability of these PCR procedures on clinical specimens has been investigated to a certain extent (Henchal et al., 1991; Morita et al., 1991; Lanciotti et al., 1992; Yenchitsomanus et al., 1996); nevertheless, the procedures have never been compared in a diagnostic laboratory setting.

The results are now described of a multi-center study to evaluate the relative sensitivity and specificity of the four PCR procedures described above using the same set of coded clinical specimens from Thailand, where all 4 dengue serotypes were prevalent. Standard dengue serology and virus isolation by mosquito inoculation were used as methods of reference. Except for procedure L, all other PCR procedures were undertaken in the laboratories that either developed the procedures (procedure H and Y) or had a published report based on the procedure (procedure M; Maneekarn et al., 1993).

2. Materials and methods

2.1. Patient specimens

Blood specimens were obtained from pediatric patients with suspected dengue infection admitted to the Queen Sirikit National Institute of Child Health (The Bangkok Children's Hospital, Bangkok, Thailand) and the Kamphaeng Phet Provincial Hospital (Kamphaeng Phet, Thailand) during 1994 and 1995. Two blood specimens (acute and convalescent) were collected per each patient. Sera or plasma were kept as 120-μl aliquots and stored at −70 °C within 6 h of collection. Acute specimens collected at admission were examined by virus isolation in live mosquitoes and the four RT-PCR procedures described below. Dengue serology was carried out on both acute and convalescent specimens. All assays were performed at the Armed Forces Research Institute of Medical Sciences (AFRIMS) except for the procedures M and Y that were performed at the Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand, and Division of Medical Molecular Biology, Department of Research and Development, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand, respectively. Specimens were distributed to each laboratory under code.

2.2. Serology

IgM and IgG to dengue and Japanese encephalitis (JE) viruses were determined by antibody capture EIA as described by Innis et al. (1989). An acute dengue infection was defined as

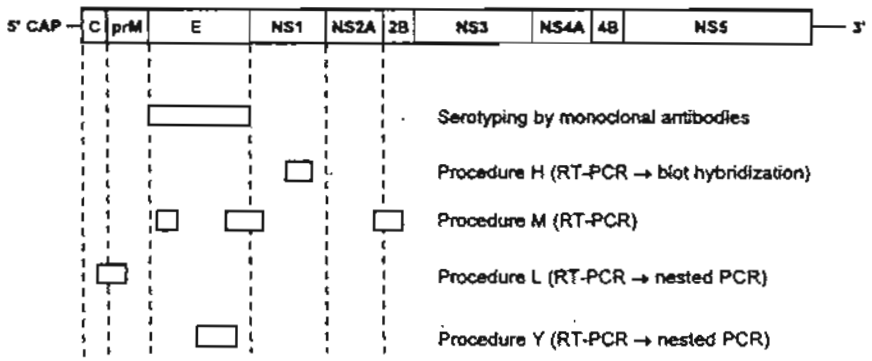

Fig. 1. Organization of dengue virus genome and gene regions used for serotyping by monoclonal antibodies and by RT-PCR. Dengue virus genome has a cap structure at 5' end, no poly(A) tail at the 3' end, and a single open reading frame, in which genes are arranged in the order: C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5. Serotyping of dengue virus utilizes monoclonal antibodies that recognize type-specific epitopes on the envelope (E) protein. The gene regions amplified by RT-PCR procedures H, M, L, and Y are mapped below. A brief format of each RT-PCR procedure is given in parenthesis (see text for details).

the presence of 40 units or more of dengue IgM in a single specimen (with dengue IgM greater than JE IgM) or a rise in dengue IgM from less than 15 units in the acute specimen to more than 30 units in the convalescent specimen. In the absence of dengue IgM of $\geqslant 40$ units, a twofold increase in dengue IgG with an absolute value of $\geqslant 100$ units indicated an acute secondary dengue infection. Specimens were considered negative for serologic evidence for a recent dengue virus infection if paired specimens were collected at least 7 days apart and lacked dengue-specific antibody as defined above. In certain cases, only acute specimens could be obtained or both acute and convalescent specimens could be collected but were not at least 7 days apart. If dengue-specific IgM and IgG titers did not reach the diagnostic criteria above, these cases were considered indeterminate by serology for acute dengue infection.

2.3. Virus isolation and serotyping

Dengue virus was isolated from sera or plasma by intrathoracic inoculation into live *Toxorhynchites splendens* mosquitoes (Rosen and Shroyer, 1985). Fifteen to twenty mosquitoes were used per specimen (0.34 μl of specimen per mosquito). Following a 14-day incubation, mosquito heads were examined for dengue virus using an indirect immunofluorescence assay (IFA). Mosquito bodies from those positive by IFA were triturated and placed in C6/36 cell culture. After 7 days of incubation, the cell culture supernatant was assayed for the presence of dengue virus by antigen-capture ELISA using dengue and JE virus type-specific monoclonal antibodies (Henchal et al., 1982).

2.4. Extraction of RNA

All RT-PCR procedures described below were set-up using RNA extracted by an acid guanidinium isothiocyanate phenol chloroform method of Chomczynski and Sacchi (1987). The amount of RNA used in the RT-PCR varied as indicated for each PCR procedure, depending on the set-up of each laboratory to obtain optimal results.

2.5. Procedure L

RNA equivalent to 10 μl of specimen was used for each RT-PCR reaction. RT-PCR was performed according to the protocol of Lanciotti et al. (1992) with the following modifications. Tris buffer pH was reduced from 8.5 to 8.3. Reverse transcriptase from avian myeloblastosis virus (AMV RT, Promega, Madison, WI) was used instead of rav-2 recombinant RT. In addition, to reduce the nonspecific background bands on the gel, concentrations of primers used in the RT-PCR and nested reactions were reduced by half and nested PCR amplification was set for 16 cycles rather than 20.

2.6. Procedure H

The same RNA preparations that were used in procedure L were subjected to RT-PCR as described by Henchal et al. (1991) with some modifications. Briefly, 5 μl of RNA (equivalent 10 μl of specimen) was mixed with 10 pmol of an AD3 antisense primer in a 10 μl reaction volume. The mixture was heated to 68 °C for 3 min and cooled on ice. The RT-PCR was set up by adjusting the RNA-primer mixture to 10 mM Tris (pH 8.3), 50 mM KCl, 3.5 mM $MgCl_2$, 25 pmol each of AD3 and AD4 primers, 40 units of RNAsin (Promega), 2 units of reverse transcriptase, and 2.5 units of AmpliTag (Perkin Elmer, Norwalk, CT) in a final volume of 100 μl. The RT was carried out at 42 °C for 45 min. Then the mixture was heated at 94 °C for 3 min followed by 40 cycles of PCR at 94 °C (1 min), 45 °C (1 min), and 72 °C (1 min). The last cycle was extended at 72 °C for 5 min.

Fifty microliters of the PCR product was extracted once with chloroform-isoamyl alcohol and treated with 4 μl of 3 M NaOH for 30 min at 70 °C. Samples were then neutralized with an equal volume of 2 M ammonium acetate. A one-fourth volume of each sample was blotted onto four separate sheets of Nytran membrane according to the manufacturer's instructions (Schleicher & Schuell, Keene, NH). Membranes were soaked at 42 °C for 10 min in a hybridization buffer (5 × SSC (1 × SSC = 0.15 M sodium chloride, 0.015 M sodium citrate), 0.5% hybridization

blocking reagent (Amersham, Arlington Heights, IL), 0.1% sarcosyl, 0.02% sodium dodecyl sulfate). Four separate hybridizations were carried out at 42 °C for 1 h in hybridization buffer containing 20 ng/ml of each dengue virus type-specific chemiluminescent probe (D1–D4, Henchal et al., 1991). The probes were synthesized from thiol modified oligonucleotides using a 5'thiol oligolabelling system (Amersham). Blots were washed and the hybridization was detected by an ECL gene detection kit (Amersham).

2.7. Procedure M

The RT-PCR procedure M was carried out as described originally by Morita et al. (1991) and modified by Maneekarn et al. (1993) except that the RT was done at 45 °C for 30 min and the RT-PCR was set up in four separate reactions using specific primer pairs for each dengue serotype instead of mixed primers. RNA equivalent to 25 μl of specimen was used in each virus type-specific RT-PCR. In certain RT-PCR runs, if contamination of carry-over PCR products was suspected, the testing was repeated. If extra aliquots of specimens were not available or results from repeat testing were still uncertain, the results were reported as indeterminate.

2.8. Procedure Y

Procedure Y was carried out as described previously (Yenchitsomanus et al., 1996). PCR primers used in this study were the sets used to amplify dengue sequences in the E region. RNA equivalent to 100 μl specimen was used in each RT-PCR. Indeterminate results were as described for procedure M.

2.9. Statistical analysis

Statistical analysis was performed using SPSS for Windows Version 9.01 (Chicago, IL). Differences in detection sensitivity in same group of specimens were determined by McNemar's test. Dependence of detection rate and time after onset of fever or levels of dengue-specific IgG were determined by χ^2-test. All P-values were two-tailed and values ≤ 0.05 were considered significant. Agreement between types of dengue virus identified using each typing method was determined by *Kappa* statistic.

3. Results

3.1. Study design

This study was conducted using two panels of coded specimens. The first panel comprised 98 acute serum specimens from suspected dengue patients. Use of serology as a reference for confirmation of acute dengue infection for this panel of specimens permitted a comparison of the relative sensitivity of virus detection between mosquito inoculation and the four RT-PCR procedures. The second panel included 143 acute specimens, 93 and 50 of which were from serologically confirmed dengue and non-dengue cases, respectively. It should be noted that dengue serotyping results by procedure L were used to select these 93 serologically positive specimens. The purpose of this design was to include an adequate number of each dengue serotype for study. Procedure L was chosen for the selection because it was the routine method in the reference laboratory at the time of study. Serology negative specimens included in panels 1 and 2 were used to assess the false positive rate for each detection method.

3.2. Sensitivity of dengue virus detection based on panel 1 specimens

Criterion testing (AFRIMS EIA) of specimens from 98 panel 1 cases revealed that 81 cases experienced acute dengue infections, 7 did not experience a recent flavivirus infection, and 10 had indeterminate serology results. Results of virus detection undertaken only on acute specimens of each case by mosquito inoculation, RT-PCR procedure H, M, L, and Y are summarized in Table 1. Analysis of 81 specimens obtained from serologically positive cases indicated that the sensitivity of virus detection by procedure L (79%) and Y (80%) were statistically higher than that of mosquito inoculation (54%), procedure H (52%),

Table 1
Detection of dengue virus in acute clinical specimens - panel 1 specimens ($n = 98$ cases)

Method of detection	Serologically positive cases ($n = 81$)			Serologically negative cases ($n = 7$)			Cases with indeterminate serology ($n = 10$)		
	No. of acute specimens with detection results			No. of acute specimens with detection results			No. of acute specimens with detection results		
	Positive[a]	Negative	Indeterminate	Positive	Negative	Indeterminate	Positive	Negative	Indeterminate
Mosquito inoculation	44 (54%, 43–65%)	37	0	0	7	0	7	3	0
Procedure H	42 (52%, 41–63%)	39	0	2[b]	5	0	6	4	0
Procedure M	49 (60%, 50–71%)	20	12	0	3	4	6	3	1
Procedure L	64 (79%, 70–88%)	17	0	1[c]	6	0	5	5	0
Procedure Y	65 (80%, 72–89%)	11	5	0	7	0	5	5	0

[a] Numbers in parenthesis are percentage of positive specimens per total numbers of serologically positive specimens tested. Lower and upper limits of 95% confidence interval are also shown.
[b] Dengue serotype 2 for both specimens.
[c] Dengue serotype 1.

Table 2
Detection of dengue virus in acute clinical specimens—panel 2 specimens ($n = 143$ cases)

Method of detection	Serologically positive cases ($n = 93$)			Serologically negative cases ($n = 50$)		
	No. of acute specimens with detection results			No. of acute specimens with detection results		
	Positive[a]	Negative	Indeterminate	Positive	Negative	Indeterminate
Mosquito inoculation	87 (94%, 89–99%)	6	0	0	50	0
Procedure H	78 (84%, 76–91%)	15	0	0	50	0
Procedure M	84 (90%, 84–96%)	9	0	2[b]	47	1
Procedure L	93 (100%)[c]	0	0	0	50	0
Procedure Y	90 (97%)	3	0	0	43	7

[a] Numbers in parenthesis are percentage of positive specimens per total numbers of serologically positive specimens tested. Lower and upper limits of 95% confidence interval, if applicable, are also shown.
[b] Dengue serotype 4 for both specimens.
[c] Typing results by procedure L were used to select panel 2 specimens; therefore, detection rate by this procedure was 100%.

and M (60%) (McNemar's test, $P < 0.05$). Some indeterminate results were reported by procedure M and Y. It should be noted that the 5 indeterminate specimens detected by procedure Y in the serologically positive group were also indeterminate by procedure M (Table 1). From 7 serologically negative cases, 2 and 1 false positive results were obtained by procedure H and L, respectively. For 10 cases with indeterminate serology results, most were positive for virus detection (i.e. 7 were virus isolation positive).

3.3. Sensitivity of dengue virus detection based on panel 2 specimens

This panel consisted of 93 and 50 acute specimens from dengue and non-dengue cases confirmed serologically. Results for testing of panel 2 specimens are shown in Table 2. As described earlier Section 3.1, results from procedure L were used to select the 93 specimens, thus yielding a detection rate of 100%. Sensitivity by other methods of virus detection were relatively high ranging from 84–97% and no statistical difference in sensitivity could be detected (McNemar's test, $P > 0.05$). For 50 acute specimens from non-dengue cases, 2 false positive results were reported by procedure M. For all 57 non-dengue specimens included in panel 1 and 2, the false positive rates by mosquito inoculation, procedure H, M, L, and Y were 0, 4, 4, 2, and 0%, respectively.

3.4. Sensitivity of detection by dengue virus type

The total numbers of each dengue virus serotype as identified by virus isolation and serotyping and by each of the RT-PCR procedures are presented in Table 3. To compare the sensitivity of the RT-PCR procedures in identifying dengue virus serotype, virus isolation by mosquito inoculation was used as the criterion standard. Results summarized in Table 4 suggest that some RT-PCR procedures were less sensitive for certain dengue serotypes than the others. For the first panel of specimens, the lowest sensitivity obtained by procedures H, M, L, and Y were for specimens containing dengue 4 (26%), dengue 3 (44%), dengue 1 (70%), and dengue 1 (60%), respectively. A similar rank of sensitivity was also seen in the second panel.

3.5. Typing discrepancy

Statistical analysis of the typing results shown in panel 1 and 2 specimens (rating: dengue 1, 2, 3, 4, or negative) revealed a 0.63–0.77 range of agreement (*Kappa* statistics, 70–82% concordance). Seven specimens yielded discrepancy in the type of dengue virus identified therefore repeat testing of these specimens was performed (Table 5). Results either remained the same or became negative upon repeat testing of three specimens 01125/94, 01199/94, and 01327/95. Specimen

00938/94 yielded two PCR product bands (dengue 1 and 2) on the second run by procedure Y while only dengue 1 virus could be isolated on the second attempt. Repeat testing of three specimens 00846/94, 01517/94, and 30191.95 was not done due to unavailability of additional specimen for testing.

3.6. Sensitivity of virus detection by days of illness

Specimens from the 81 confirmed dengue cases (panel 1) were grouped by days after the onset of fever. The percentage of specimens with detectable dengue virus by each method is shown graphically in Fig. 2A. During the first 4 days of illness, the sensitivity of virus detection by mosquito inoculation and by procedure H was above 50%; subsequently, the sensitivity dropped to 50% or below. In contrast, sensitivity of detection by procedures M, L, and Y remained above 50% though declining over time.

Statistical analysis revealed significant dependence of sensitivity of virus detection by mosquito inoculation on days after onset of fever (χ^2-test, $P < 0.05$). For specimens collected on day 4 after onset of fever (Fig. 2A, $n = 38$), sensitivity of virus detection by procedure L was significantly higher than those by mosquito inoculation, procedure H, and M (McNemar's test, $P < 0.05$) but was not different from that of procedure Y. For day 5 specimens ($n = 19$), significant higher sensitivity was detected for procedure Y as compared to mosquito inoculation and procedure H (McNemar's test, $P < 0.05$)

3.7. Effect of antibody on the sensitivity of detection

Of the 81 panel 1 specimens, 69 had secondary antibody responses and 12 had primary antibody responses. Dengue-specific IgG titers of these panel 1 specimens were stratified into three groups: 0–50, 51–100, and more than 100 EIA units (37, 22, and 22 specimens, respectively). The dengue IgG level of 100 units was chosen since it was a cut-off value used in some cases when dengue IgM was not at diagnostic level (< 40 units, defined in Section 2.2 above). The 50-unit value (half of 100 units) was chosen arbitrarily to spread out the group. In the presence of dengue IgG titers of 100 units or less, sensitivity of virus detection by all methods were relatively high (> 50%, Fig. 2B). For specimens with dengue IgG titers greater than 100 units, sensitivity of virus detection by mosquito inoculation and procedure H appeared to drop sharply to 32 and 27%, respectively or slightly to 45% by procedure M. In contrast, virus detection rates by procedures L and Y remained relatively high (68–94%) for all three groups of specimens.

Statistical analysis revealed significant dependence of sensitivity of virus detection by mosquito inoculation and procedure H on levels of dengue

Table 3
Detection of dengue virus in acute clinical specimens—details of dengue serotypes identified by each method of detection

Method of detection	Panel 1—serologically positive cases ($n = 81$)				Panel 2—serologically positive cases ($n = 93$)			
	No. specimens yielding typing results[a]				No. specimens yielding typing results			
	Dengue 1	Dengue 2	Dengue 3	Dengue 4	Dengue 1	Dengue 2	Dengue 3	Dengue 4
Mosquito inoculation	10	6	9	19	24	26	24	13
Procedure H	17	8	12	5	27	27	21	3
Procedure M	21	7	4	17	25	24	20	15
Procedure L	12	9	20	23	27	27	25	14
Procedure Y	20	13	9	23	25	27	24	14

[a] Only specimens with positive typing results are shown. Specimens that yield negative or indeterminate results are as in Tables 1 and 2.

Table 4
Comparison of four PCR procedures for detecting and typing of dengue viruses using mosquito inoculation and serotyping as a gold standard

Methods	Panel 1—serologically positive cases				Panel 2—serologically positive cases			
	No. of specimens yielding following isolates				No. of specimens yielding following isolates			
	Dengue 1	Dengue 2	Dengue 3	Dengue 4	Dengue 1	Dengue 2	Dengue 3	Dengue 4
Mosquito inoculation	10	6	9	19	24	26	24	13
Procedure H	9 (90%)[a]	5 (83%)	9 (100%)	5 (26%)	24 (100%)	26 (100%)	21 (88%)	3 (23%)
Procedure M	9 (90%)	4 (67%)	4 (44%)	14 (74%)	22 (92%)	24 (92%)	20 (83%)	13 (100%)
Procedure L	7 (70%)	6 (100%)	9 (100%)	17 (89%)	N/A[b]	N/A	N/A	N.A
Procedure Y	6 (60%)	6 (100%)	9 (100%)	16 (84%)	21 (88%)	26 (100%)	24 (100%)	13 (100%)

[a] No. of PCR positive specimens. Number in parenthesis was the percentage of PCR positive specimens per total number of each dengue virus type isolated by mosquito inoculation.
[b] Not applicable since typing results by procedure L was used to select panel 2 specimens (see text).

IgG titers (χ^2-test, $P < 0.05$). For specimens with dengue IgG titers less than 50 units, detection sensitivity by procedure L and Y were significantly higher than those by mosquito inoculation, procedure H, and M (McNemar's test, $P < 0.05$). For specimens with dengue IgG titer greater than 100 units, a significantly higher sensitivity was detected for procedure L and Y as compared to those of mosquito inoculation and procedure H ($P < 0.05$).

4. Discussion

A number of RT-PCR methods for the detection and typing of dengue viruses in clinical specimens have been reported. In the present study, four of these RT-PCR procedures (Henchal et al., 1991; Morita et al., 1991; Lanciotti et al., 1992; Yenchitsomanus et al., 1996) were evaluated along with mosquito inoculation using the same set of serologically confirmed specimens. Our analysis utilized serologically confirmed dengue cases rather than the known viremic specimens used in the previous reports in order to allow the determination of sensitivity of mosquito inoculation to be included in the comparison. In addition, the relatively larger number of specimens used in the present study has made a detailed evaluation of comparative performance of the methods possible.

Analysis of results using specimens from the first panel indicated that procedure L and Y had the relatively higher sensitivity of detection (79–80%) followed by procedure M, mosquito inoculation, and procedure H, respectively. A similar ranking of sensitivity was also observed when data from the first panel was analyzed by days after onset of fever and by dengue IgG titers (Fig. 2). Our study reconfirmed the higher sensitivity of RT-PCR over virus isolation reported in some earlier studies (Deubel et al., 1990; Henchal et al., 1991).

When data from the second panel, which were selected based on serology and positive RT-PCR result employing procedure L, were compared, procedure Y again gave the highest sensitivity (97%), followed by mosquito inoculation, procedures M and H, respectively. There was no statistically difference in detection sensitivity among the three RT-PCR procedures as well as mosquito inoculation (McNemar's test, $P > 0.05$). This result suggests that once virus positive specimens are used as references for the analysis, it is difficult to detect the difference in the performance of the three RT-PCR procedures and mosquito inoculation since all of them performed well (sensitivity 84–97%).

Some indeterminate results were reported by procedures M and Y. Due to the number of different methods that each specimen was sub-

jected to, repeat testing was not possible for some specimens. It should be noted that 5 specimens with indeterminate results by procedure Y were also reported as indeterminate by procedure M (in 12 specimens, panel 1 serological positive specimens, Table 1), suggesting that some indeterminate results might be associated with certain specimens. The indeterminate results reported by procedure M and Y in serologically negative groups (Tables 1 and 2) also led to an incomplete comparison of specificity of all detection methods. If all specimens with indeterminate results were excluded (5 by procedure M and 7 by procedure Y), mosquito inoculation, procedure H, M, L, and Y would have a specificity of 100% (57/57), 96% (55/57), 96% (50/52), 98% (56/57), and 100% (50/50), respectively.

Detailed typing results of 81 specimens in the first panel (Table 3) suggested that most RT-PCR procedures were more sensitive than the mosquito inoculation in detection of each of the 4 dengue serotypes. It was also noted that procedure H was not as sensitive as the others in detection of serotype 4 viruses. This was evident for both panels. A similarly decreased sensitivity was also noted for procedure M in detecting dengue serotype 3. When the data were analyzed by using mosquito inoculation as a reference, lower sensitivity by procedure H was also observed for serotype 4 viruses (Table 4). Similar observations were noted for dengue serotype 3 by procedure M. In addition, data in Table 4 also suggests that procedures L and Y might have decreased sensitivity in the detection of serotype 1.

Our observed low sensitivity for dengue 4 detection by procedure H was in accord with a previous report (Henchal et al., 1991) in which the lowest sensitivity among the four dengue serotypes was documented for dengue 4 virus (60%, 6/10 specimens). This finding may be due to insufficient sequence homology of the dengue 4 probe for the dengue serotype 4 viruses circulating in our study locations. The low sensitivity which we detected for procedure M for dengue 3 and procedure L for dengue 1, to our knowledge, has never been reported. It was previously reported that the inner nested primers failed to amplify the PCR product of the outer primers in one of eleven dengue 1 virus samples tested (Yenchitsomanus et al., 1996). A similar situation might have occurred in our study, thus revealing low detection sensitivity for some serotype 1 viruses by procedure Y. A failure of nested primers to amplify products from the first round PCR was also observed with pro-

Table 5
Discrepancy in dengue typing

	Type of dengue virus identified by				
	Virus isolation and serotyping	Procedure H	Procedure M	Procedure L	Procedure Y
01125/94	Negative	Dengue 1	Dengue 1	Dengue 3 Dengue 3[a]	Dengue 1 Dengue 1[a]
01199/94	Negative	Negative	Negative	Dengue 3 Dengue 3[a] Negative[a]	Dengue 4 Negative[a] Negative[a]
01327/95	Negative	Negative	Negative	Dengue 3 Dengue 3[a] Negative[a]	Dengue 1 Negative[a] Negative[a]
00938/94	Dengue 1 Dengue 1[a]	Negative	Dengue 2	Negative Negative[a]	Dengue 2 Dengue 1 and 2[a]
00846/94	Dengue 4	Dengue 2	Indeterminate	Dengue 4	Dengue 2
01517/94	Negative	Dengue 1	Dengue 4	Dengue 1	Dengue 1
30191/95	Dengue 2	Dengue 2	Dengue 1	Dengue 2	Dengue 2

[a] Indicates results of repeat testing of serum specimens done at the AFRIMS.

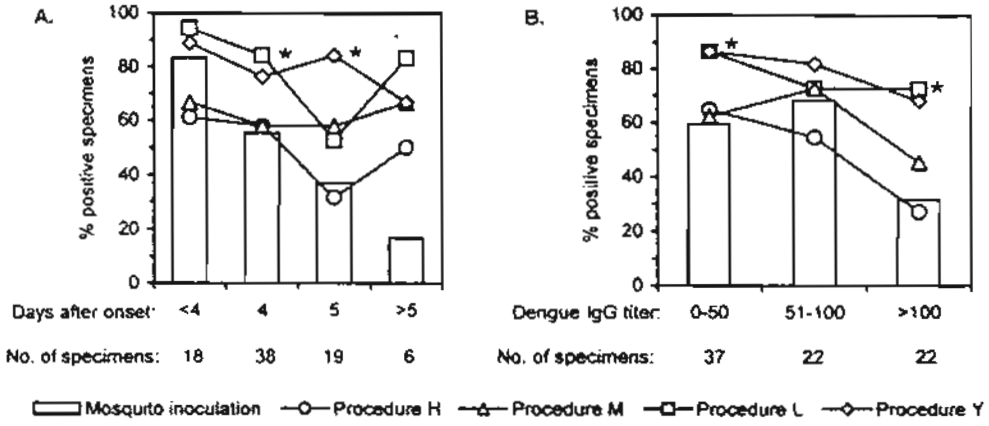

Fig. 2. (A) Sensitivity of dengue virus detection by day after onset of fever. The percentage of dengue positive specimens by each method of virus detection are plotted against the number of days after the onset of fever. Numbers of specimens for each study day are shown. (B) Effect of the presence of dengue-specific IgG in serum specimens on sensitivity of virus detection. Specimens were grouped by level of dengue-specific IgG titer (0–50, 51–100, and more than 100 units). Percentages of dengue positive specimens by each method are shown. The symbol * indicates that a statistically significant difference was noted ($P < 0.05$, see text for details).

cedure L for two dengue serotype 1 specimens in our study. Similarly, dengue 4 probes used for detection of PCR products by procedure H failed to hybridize to PCR products in two of our dengue serotype 4 specimens (data not shown). Taken together, lower detection sensitivity for particular virus types observed in each PCR procedure may lie on the inadequate sequence homology of the nested primers or probes to those of the actual viruses circulating in the area of study.

Varying detection sensitivity among the four PCR procedures may be due to various factors, including the method of RNA extraction, amount of RNA used for the RT step, PCR parameters, primer design, and performance of enzymes. For example, detection of PCR products by probe hybridization as used in procedure H may not be as sensitive and specific as nested PCR and visual inspection of the correct product sizes as used in procedures L and Y. Procedure M, which involved only one set of amplification reaction and visual inspection of PCR products in stained agarose gel, is clearly less sensitive than the other three procedures.

A number of possibilities may be considered for the discrepancy in typing results by serology (using monoclonal antibodies) and by PCR. First, the patient could have been concurrently infected with multiple dengue types. This phenomenon has been documented in endemic areas where multiple serotypes co-circulated and has been thought of as not uncommon (Gubler et al., 1985; Laille et al., 1991; Maneekarn et al., 1993; Lorono-Pino et al., 1999). In a case of concurrent infection, the RT-PCR would have an advantage over virus isolation in that, if properly designed and of equal sensitivity, the RT-PCR should be able to simultaneously detect multiple serotypes in the specimens without interference with one another (Lorono-Pino et al., 1999). Another possibility for the discrepancy was that there might be only one infecting virus with a recombinant genome (Worobey et al., 1999). This was possible since primers or probes used in these four RT-PCR procedures were selected from different regions of the dengue genome (Fig. 1). A possible example may be specimen 01125/94 (Table 5) in which procedure L amplified a dengue 3 fragment in C-PrM region while other PCR procedures gave dengue 1 results (procedure H probe in NS1 region, procedure M primers in E-NS1 region, and procedure Y primers in E region). Specimens shown in Table 4 could fall in either or both of these categories (one or multiple viruses). Additional investigations such as careful virus isolation/characterization and/or quantitative and

highly sensitive RT-PCR are required to clarify the issues.

Results shown in Fig. 2 suggested that dengue virus was detectable by RT-PCR for more days of illness and in the presence of dengue-specific antibody when compared to virus isolation by mosquito inoculation. These findings confirmed earlier findings that virus isolation rates correlate inversely with the levels of dengue-specific antibody (Gubler et al., 1981; Innis et al., 1989; Chan et al., 1994; Vaughn et al., 2000). In one study, dengue RT-PCR was found to be more sensitive than virus isolation for both acute and convalescent specimens and in the presence of dengue-specific antibody (Chan et al., 1994).

In conclusion, we have compared four RT-PCR procedures that have potential to be used in a routine laboratory to detect and identify the dengue types. For the two panels of specimens employed, the highest sensitivity of detection was procedure Y, followed by procedures L, M, mosquito inoculation, and procedure H. Our data also suggest that each of the four PCR methods might have lower sensitivity in detecting a particular dengue type when compared with the others. We speculate that the problem may be due to low sequence homology between primers or probes used for typing and the target sequences of some virus strains circulating in the area of study. To improve further the performance of these RT-PCR procedures, certain primers or probes should be re-designed to accommodate sequence variations of local dengue strains.

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Comparison of penaeid shrimp and insect parvoviruses suggests that viral transfers may occur between two distantly related arthropod groups

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Abstract

The DNA and putative amino acid sequences of representative insect and shrimp parvoviruses (subfamily *Densovirinae*) were analyzed using computer programs. Shrimp viruses included hepatopancreatic parvovirus (HPV) of *Penaeus monodon* (HPVmon) and *P. chinensis* (HPVchin), spawner-isolated mortality virus from *P. monodon* (SMVmon) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) from *P. vannamei*. Insect viruses included *Aedes aegypti* densovirus (*Aae*DNV), *Aedes albopictus* densovirus (*Aal*DNV), *Junonia coenia* densovirus (*Jc*DNV), *Galleria mellonella* densovirus (*Gm*DNV), *Bombyx mori* densovirus 5 (*Bm*DNV), *Diatraea saccharalis* densovirus (*Ds*DNV) and *Periplaneta fuliginosa* densovirus (*Pf*DNV). Virion size for all these viruses ranged between 18 and 30 nm diameter and ssDNA genome length was between 4 and 6 kb. Using BLAST or Clustal W with the sequence fragments available, no significant DNA homology was found except for 77% DNA identity between HPVmon and HPVchin. However, phylogenetic trees constructed by comparing DNA genome sequences for putative viral polypeptides, capsid proteins and nonstructural proteins placed the parvoviruses into two Clades: Clade 1 with SMVmon, *Pf*DNV, *Ds*DNV, *Gm*DNV, *Jc*DNV, and *Bm*DNV; and Clade 2 with HPVmon, HPVchin, IHHNV, *Aal*DNV and *Aae*DNV. The four shrimp parvoviruses fell into two different clades that grouped with different insect parvoviruses.

Keywords: Insect; Crustacean; Shrimp; Parvovirus; *Densovirinae*; Phylogeny

1. Introduction

Viruses in the Family *Parvoviridae* consist of small icosahedral, non-enveloped particles 18–26 diameter (Afanasiev et al., 1991). The genome

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(*Parvovirinae* or *Densovirinae*) is single stranded, linear DNA with only two overlapping genes that generate two sets of structurally related but biologically distinct polypeptides called nonstructural (NS) protein and the viral capsid polypeptide (VP) (Shike et al., 2000). The family *Parvoviridae* includes two subfamilies, *Parvovirinae* with viruses that infect vertebrates, and *Densovirinae* with viruses that infect invertebrates, mainly insects. Boublik et al. (1994) suggested that the overall organization of three densoviruses, *Bombyx mori* densovirus (*Bm*DNV), *Aedes* DNV and the *Junonia coenia* densovirus (*Jc*DNV), revealed striking differences.

Of the parvoviruses in crustaceans, only those of shrimp have been studied in any detail. These include hepatopancreatic parvovirus (HPV) in *P. chinensis* (HPVchin) (Lightner and Redman, 1985) and *Penaeus monodon* (HPVmon) (Sukhumsirichart et al., 1999; Phromjai et al., 2001), spawner-isolated mortality virus from *P. monodon* (SMVmon) (Fraser and Owens, 1996; Owens et al., 1998, 2000; Owens and McElnea, 2000) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Lightner et al., 1983; Bonami et al., 1990; Shike et al., 2000). These viruses have caused disease in cultivated shrimp in many countries all over the world (Flegel, 1997). Shike et al. (2000) recently reported that IHHNV of shrimp and the mosquito densoviruses *Aae*DNV and *Aal*DNV shared similar overall genomic organization. Specifically, the left ORF of these viruses most likely encoded the major NS protein (NS1) since it contained conserved replication initiator motifs and NTP-binding and helicase domains similar to those in NS-1 from all other parvoviruses, and the putative NS1 of IHHNV shared amino acid sequence homology with NS1 of the mosquito densoviruses.

Spawner-isolated mortality virus (SMV) of *P. monodon* was described as a parvovirus based on its morphological characteristics by transmission electron microscopy (TEM) and its specific gravity (Fraser and Owens, 1996). It has also been recently reported from freshwater crayfish in Australia (Owens and McElnea, 2000). It was of interest to know the relationship amongst the three types of shrimp parvovirus and also their relationship to known insect parvoviruses. To this end, recent sequence information from these three shrimp virus types was compared with that published for the insect parvoviruses.

2. Materials and methods

2.1. DNA sequences

The shrimp DNA and putative protein sequences used for alignment and comparison were SMVmon (GenBank AF499102), HPVmon (GenBank AF456476), HPVchin (GenBank AY008257) and IHHNV (GenBank AF218266). Insect viral sequences from GenBank were *Aal*DNV (X74945), *Aae*DNV (M37899), *Bm*DNV (AB042597), *Ds*-DNV (NC001899), *Gm*DNV (L32896), *Jc*DNV (S47266) and *Pf*DNV (NC000936). A key to the numbers and sources is shown in Table 1.

2.2. Computer analysis

BIOEDIT software (version 5.0.6) was used to manipulate the retrieved sequences (Hall, 1999). The alignment of sequences was performed by using the CLUSTAL W software contained in the BIOEDIT program (Thompson et al., 1994). For full-length genomes as well as noncoding regions, nucleotide sequences were aligned. For coding regions, the alignment was performed for amino acid sequences. Phylogenetic analysis was performed by using several methods. For all methods, positions containing an alignment gap were excluded from pairwise sequence comparisons. Bootstrap re-sampling was performed for each analysis (100 replications). Nucleotide distances were analyzed by using the neighbor-joining algorithm (http://bioweb.pasteur.fr/seqanal/phylogeny phylip-uk.html) as implemented in the PHYLIP package (NEIGHBOR), based on the Kimura two-parameter distance estimation method or the proportion of differences (*p* distance). The program TreeView was also used to view the phylograms (Page, 1996).

The phylogenetic tree for DNA was constructed based on 696 bp of aligned DNA sequences. We used this 696 bp as the basis for the comparisons with all the other parvovirus sequences because it

was the only sequence available from HPVmon. Proteins that were analyzed by using BLAST program from NCBI (Tatusova and Madden, 1999) and the most similar sequences were used for the phylogenetic analysis. For the analysis of protein similarities, the LALIGN program version 2.0 (http://www.ch.embnet.org/software/LALIGN -form.html) was used to calculate global alignment of sequences (Myers and Miller, 1988).

3. Results and discussion

3.1. Characteristics of insect and penaeid shrimp parvoviruses

From a literature review (Afanasiev et al., 1991; Bando et al., 1987a,b, 1990; Bonami et al., 1990; Boublik et al., 1994; Dumas et al., 1992; Jousset et al., 2000; Lightner et al., 1983, 1994; Lightner and Redman, 1985; Owens et al., 1991; Sukhumsirichart et al., 1999), virion size for insect and penaeid shrimp viruses ranged between 18 and 30 nm in diameter and all had genomes of ssDNA that ranged between 4 and 6 kb (Table 2). These characteristics would place them in the Family *Parvoviridae*. The smallest virion size (18 nm) and shortest length genome (4009 bp) belonged to the densovirus from *Aedes aegypti* (*Aae*DNV) and the longest (about 6 kb) to the densovirus from *Culex pipiens* (*Cp*DNV). The fact that these viruses are in the same family and all occur in arthropods opened the possibility that they might be ancestrally related in some way.

3.2. Sequences comparisons of insect and penaeid shrimp parvoviruses

Using BLAST program to compare all of the tested nucleic acid sequences, the highest homology (77% DNA identity) was found between HPVmon and HPVchin. However, using the global alignment method from LALIGN program to detect homology of putative proteins from the two showed only 15.3% similarity. No other significant DNA homology was found. Results using the CLUSTAL W program were the same. However, when CLUSTAL W was used to compare isolated pairs of DNA sequences, some homology was found between IHHNV and *Aae*DNV or *Aal*DNV.

Table 1
Source and origin of parvoviruses sequences used

Abbreviation	Full name	Source animal	Common name	GenBank accession number
SMVmon	Spawner-isolated mortality virus	*P. monodon*	Black tiger shrimp	AF499102
HPVmon	Hepatopancreatic parvovirus	*P. monodon*	Black tiger shrimp	AF456476
HPVchin	Hepatopancreatic parvovirus	*P. chinensis*	Chinese white shrimp	AY008257
IHHNV	Infectious hypodermal and hematopoeitic necrosis virus	*P. vannamei*	American white shrimp	AF218266
*Aal*DNV	*Aedes albopictus* densovirus	*Aedes albopictus*	Mosquito	X74945
*Aae*DNV	*Aedes aegypti* densovirus	*Aedes aegypti*	Mosquito	M37899
*Bm*DNV	*Bombyx mori* densovirus	*Bombyx mori*	Silkworm	AB042597
*Ds*DNV	*Diatraea saccharalis* densovirus	*Diatraea saccharalis*		NC001899
*Gm*DNV	*Galleria mellonella* densovirus	*Galleria mellonella*		L32896
*Jc*DNV	*Junonia coenia* densovirus	*Junonia coenia*		S47266
*Pf*DNV	*Periplaneta fuliginosa* densovirus	*Periplaneta fuliginosa*		NC000936

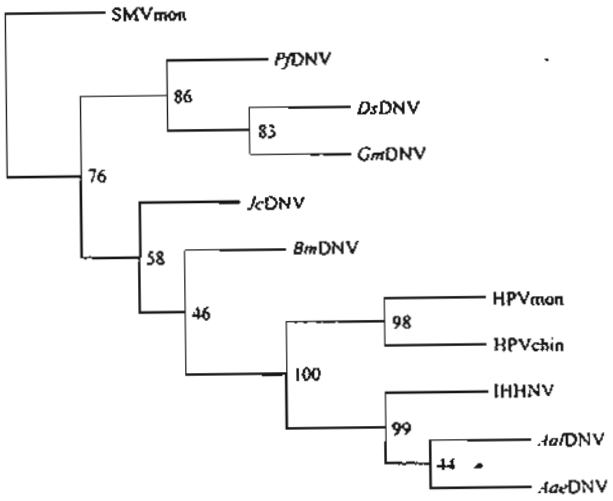

Fig. 1. The neighbor-joining phylogenetic tree generated from a heuristic search of aligned insect and shrimp parvoviruses DNA sequences. The phylogenetic tree was constructed based on 696 bp of aligned DNA sequences. The branch lengths are scaled according to the number of base substitutions. The scale shown in the left-hand corner of the figure indicates the proportion of distance differences. Bootstrap values are indicated as number at each branch (100 replications). For the sources of DNA sequences data are shown in Table 1.

A phylogenetic tree based on genomic DNA of shrimp and insect parvoviruses (Fig. 1) gave two main clades (clusters) that included: Clade 1 with SMVmon, *Pf*DNV, *Ds*DNV, *Gm*DNV, *Jc*DNV, and *Bm*DNV; and Clade 2 with HPVmon, HPVchin, IHHNV, *Aal*DNV and *Aae*DNV. The grouping of IHHNV with the mosquito brevidensoviruses *Aae*DNV and *Aal*DNV was in agreement with the study of Shike et al. (2000) who found the same relationship in a phylogenetic tree based on a 199-aa conserved region characteristic of the NTP-binding and helicase domains of the nonstructural protein (NS1) of IHHNV, of insect parvoviruses and vertebrate parvoviruses. However, the clustering of SMV with a different group of insect densoviruses and the separation of HPV into yet another group suggests that the shrimp parvoviruses studied are of diverse origin and that they are not closely related. Shike et al. (2000) reported that IHHNV was the first example of a densovirus with a host range outside the class Insecta that diverged from the Crustacea over 500 million years ago. Our analysis suggests that SMVmon and HPVmon/HPVchin may represent two other groups of distantly related densoviruses in the class Crustacea.

Comparisons were also made using amino acid sequences for nonstructural protein (NS1), structural protein, capsid/coat proteins (VP) and putative proteins of unknown function that showed similarity using global alignment from LALIGN program. There was some homology between the shrimp viral proteins and known capsid proteins of insect viruses (Fig. 2A). Other proteins that showed homology were capsid protein of HPVchin and unknown protein of HPVmon (15.3%) and proteins of insect parvoviruses (Fig. 3A). It was interesting that the capsid protein of IHHNV showed very low similarity to capsid proteins of *Aae*DNA and *Aal*DNV, even though its NS1 showed similarity to their NS1 (i.e. IHHNV and *Aae*DNV, 18.2%; IHHNV and *Aal*DNV, 20.4%). The results were used to construct a phylogenetic tree (Fig. 2B).

NS1 of IHHNV showed some similarity to the protein translated from orf2 of *Jc*DNV (13.7%), to NS1 from *Ds*DNV (14.3%) and to NS1 or putative proteins of other viruses (Fig. 3A). The results of the comparison were used to generate another phylogenetic tree (Fig. 3B). A putative protein of HPVmon showed high homology to the capsid protein of HPVchin but both showed very low homology to the capsid protein of IHHNV.

The phylograms based on capsid or related protein sequences (Fig. 2B) and NS1 or related sequences (Fig. 3B) were somewhat different. The phylogenetic tree based on NS1 was similar to that described by Shike et al. (2000) with the closest relationship found between IHHNV and *Aae*DNV or *Aal*DNV. However, when capsid proteins were used, IHHNV fell closer to *Bm*DNV and *Pf*DNV than to *Aae*DNV or *Aal*DNV. The phylogram based on NS1 sequences corresponded most closely to the phylogram constructed based on DNA sequences and it suggested that NS1 might be more conserved than the capsid protein.

Jousset et al. (2000) reported that there was a lack of sequence homology between *Cp*DNV and *Aal*DNV genomes, and that lack of antigenic

(A)

HPVmon Unk	HPVmon Unk						
IHHNV NS1	1.6	IHHNV NS1					
*Aae*DNV NS	1.3	18.2	*Aae*DNV NS				
*Aal*DNV NS1	1.5	20.4	75.8	*Aal*DNV NS1			
*Jc*DNV Orf2	0.3	13.7	11.7	12.1	*Jc*DNV orf2		
*Bm*DNV NS_orf1	0.5	0.3	0.2	0.4	17.7	*Bm*DNV NS_orf1	
*Pj*DNV NS	1.1	0.2	0.4	11.2	28.0	18.0	*Pj*DNV NS
*Ds*DNV NS1	0.3	14.3	29.7	11.5	92.8	17.8	29.7	*Ds*DNV NS1

(B)

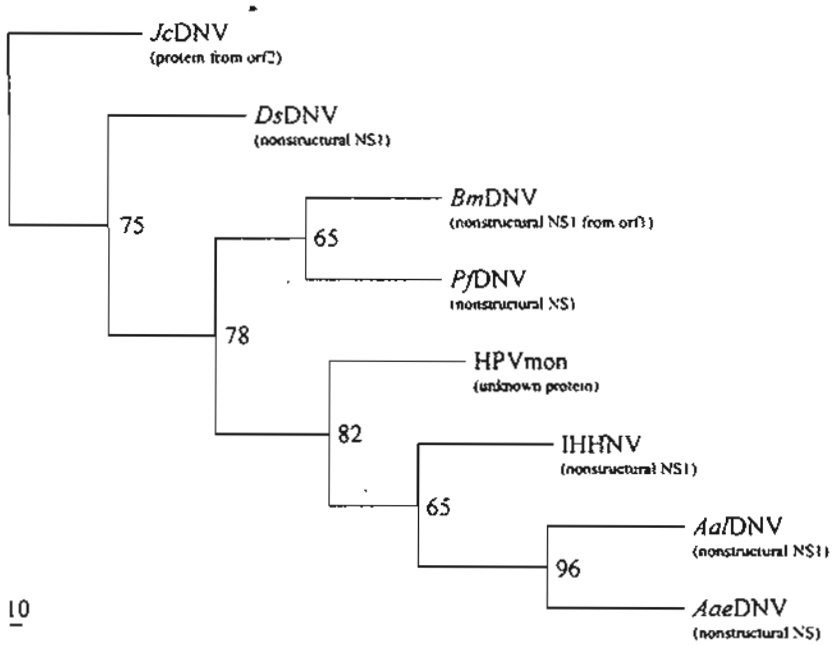

Fig. 3. Comparisons based on putative protein sequences of insect virus nonstructural protein (NS) and most similar proteins using global alignment method (LALIGN). (A) Overall parvoviral protein similarities. (B) The neighbor-joining phylogenetic tree generated from a heuristic search of aligned insect and shrimp parvoviruses deduced amino acid sequences. The phylogenetic tree was constructed based on deduced amino acid sequences of structural proteins. The scale shown in the left-hand corner of the figure indicates the proportion of distance differences. Bootstrap values are indicated as number at each branch (100 replications).

Based on DNA sequence comparisons, we were surprised to find that the shrimp parvoviruses did not form one cluster and the insect parvoviruses another. The fact that the three shrimp viruses fell into two different parvovirus clades, including two different insect virus groups suggests that there may have been and may still be viral transfers between these distantly related arthropods. Indeed, Lo et al. (1996) found that a PCR probe for white spot syndrome virus of penaeid shrimp gave positive results with aquatic insect larvae and suggested that they were possible reservoir hosts. Since this work was not followed up with more detailed tests such as in situ DNA hybridization

assays, it may have been that the insect larvae were simply mechanical carriers of the shrimp virus. On the other hand, cultivated penaeid shrimp larvae and some insect larvae are carnivorous and known by shrimp farmers to eat each other when sizes are appropriate and opportunity arises. It would be worthwhile looking in the shrimp environment to determine whether any aquatic insect larvae there can be infected with shrimp parvoviruses and act as reservoirs for them.

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ORIGINAL PAPER

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Construction and characterization of phage-displayed leukocyte surface molecule CD99

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Abstract The phage display technique has been described for the production of various recombinant molecules. In the present report, we used this technique to display a leukocyte surface molecule, CD99. PCR subcloning of CD99 cDNA from the mammalian expression vector pCDM8 to the phagemid expression vector pComb3HSS was performed. The resulting phagemid, pComb3H-CD99, was transformed into *Escherichia coli* XL-1 Blue. CD99 was displayed on the phage particles following infection of the transformed *E. coli* with the filamentous phage VCSM13. Using sandwich ELISA, the filamentous phage-displayed CD99 was captured by a CD99 monoclonal antibody (mAb) then detected with anti-M13 conjugated to horseradish peroxidase, confirming that the CD99 molecule was displayed on the phage particles. The CD99-phages inhibited induction of Jurkat cell aggregation by CD99 mAb MT99/1. Proper folding of the displayed CD99 bioactive domain was inferred from this finding. Our results demonstrate that the phage display technique can be applied to the generation of full-length CD99 molecules. The phage carrying this cell surface protein will be useful for identification of its counter receptor or ligand.

Introduction

The filamentous phage display technique, first described by Smith (1985), has been widely used for expression of polypeptides and proteins. Currently, several phagemid vectors are available for different purposes (McCafferty et al. 1994; Crameri and Blaser 1996; Persic et al. 1997). Basically, the recombinant molecules are fused with phage coat proteins, gp3 or gp8, and displayed on the surface of phage particles. The phage display technique delivers the recombinant molecule to the periplasm of *Escherichia coli* with the assistance of signal peptides. Due to the higher oxidizing conditions in comparison to the cytoplasm, the periplasmic environment effectively promotes disulfide bond formation (Becker and Hsiung 1986; Dracheva et al. 1995). Therefore, using phage display technique, the correct conformation of a recombinant protein is obtained. In 1995, Barbas and Wagner constructed the phagemid vector pComb3HSS for delivering a Fab fragment to the periplasmic space of *E. coli* (Barbas and Wagner 1995). Phage-displayed Fab libraries were produced and used to select Fabs specifically interacting with the epitope of interest. Recently, this vector was applied to the production of a tissue plasminogen activator deletion mutant (K2S) that contains nine disulfide bridges (Manosroi et al. 2001). Other complex molecules have also been displayed using this vector (Lasters et al. 1997; Appenzeller et al. 2001; Kurokawa et al. 2002). Production of recombinant protein by phage display technique has the major advantage that the displayed recombinant molecules can be directly and easily harvested from the *E. coli* culture supernatant by PEG precipitation.

Leukocytes express a large number of molecules on their surfaces. These leukocyte surface molecules are important for cell function. Antibodies raised against these molecules have become a major tool in characterizing the structure and function of these surface molecules. In addition to specific antibodies, the isolated cell surface molecules themselves have been broadly used for identification and functional characterization of their counter receptors or specific ligands (Bowen et al. 1996; Vilardell et al. 1998; Martinez-Pomares et al. 1999). However, to prepare these molecules from cell membranes, cumbersome steps of specific cell isolation are

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required. In addition, contamination with undesired proteins is difficult to avoid. To overcome this problem, molecular techniques in mammalian cell expression systems have been employed. The Fc of immunoglobulin was used as a fusion partner of certain CD molecules, e.g., CD31 (Prager et al. 1996) and CD147 (Koch et al. 1999). The fusion protein was then secreted into the culture medium and purified using a protein A column. Although the recombinant proteins obtained have an almost native conformation, mammalian cell expression systems are more expensive and time-consuming in comparison to prokaryotic expression systems. Moreover, the hydrophobic nature of the transmembrane region of the CD molecule makes it impossible to produce the entire length version as a secreted protein. An influence of the transmembrane domain on the conformational structure of the external domain has already been shown (Gaudin et al. 1999). In contrast, lipocalin-1 interacting membrane receptor, a molecule with nine putative transmembrane domains, was successfully expressed using phage display (Wojnar et al. 2001).

In an attempt to produce recombinant leukocyte surface molecules using a prokaryotic expression system, the potential of the phage display technique was evaluated. We demonstrated that the phage display technique could be used to generate phage displayed CD99 molecules. The constructed phages were able to inhibit Jurkat cell aggregation induced by monoclonal antibodies (mAb) against CD99, indicating the presence of a bioactive domain. Our findings suggest that the phage display technique is useful for displaying cell surface molecules when the corresponding cDNA is available.

Materials and methods

Primer design

A pair of primers, CD99MatF 5'-GAGGAGGAGGTGGCCCAGGCGGCCGATGGTGGTTTCGATTTA-3' and CD99MatR 5'-GAGGAGGAGCTGGCCGGCCTGGCCTTTCTCTAAAAGAGTACG-3' (synthesized at the Bioservice Unit, National Center for Genetic Engineering and Biotechnology, Thailand) were designed to amplify the mature CD99-encoding gene carried by the mammalian expression vector pCDM 8 (Kasinrerk et al. 2000). The primers were designed with *Sfi*I end cloning sites (underlined) to maintain the correct reading frame of the inserted sequence from the ATG to the gpIII gene in the phagemid vector pComb3HSS.

PCR amplification of the CD99 gene

Primers CD99MatF and CD99MatR (1 µg each) together with 50 ng CD99-encoding cDNA (pCDM 8-CD99) template were suspended in a 100 µl PCR mixture. *Taq* polymerase (2.5 U; Roche, Indianapolis, Ind.) was added last to the solution. The titrated amplification condition was initiated with a jump start at 85°C for 4 min, then denaturation at 95°C for 50 s, annealing at 42°C for 50 s, then extension at 72°C for 1.5 min for 35 cycles. The mixture was further incubated at 72°C for 10 min. The amplified product of 537 bp was subsequently purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The correct identity of the purified product was confirmed by restriction enzyme fragment analysis.

Construction of a phagemid expressing CD99

The purified CD99 PCR product and the phagemid pComb3HSS (kindly provided by C.F. Barbas, Scripps Institute, La Jolla, Calif.) (Barbas and Wagner 1995), were digested with *Sfi*I (Roche) to prepare specific cohesive cloning sites. Purified PCR product (4 µg) was digested with 60 U *Sfi*I at 50°C for 18 h; for pComb3HSS, 20 µg phagemid was treated with 100 U *Sfi*I. Digested fragments of the purified PCR products and pComb3HSS (~3,300 bp) were subsequently gel-purified using a QIAquick Gel Extraction Kit (Qiagen). A ligation reaction was performed by introducing 5 U T4 DNA ligase (Roche) to a mixture of 0.7 µg purified *Sfi*I-digested pComb3HSS and 0.9 µg purified *Sfi*I-digested PCR product. Ligation was performed at 30°C for 18 h. The newly constructed phagemid was named pComb3H-CD99.

Transformation of XL-1 Blue

$CaCl_2$ competent *E. coli* XL-1 Blue (200 µl) (Stratagene, La Jolla, Calif.) were transformed with 70 ng ligated product. The transformed cells were propagated by spreading on LB agar containing 100 µg/ml ampicillin and 10 µg/ml tetracycline (Sigma, St. Louis, Mo.). After cultivation at 37°C for 18 h, several antibiotic-resistant colonies were selected for plasmid minipreps using the alkaline lysis method. Each purified plasmid was subjected to *Sfi*I restriction site analysis. A transformant harboring a plasmid with the correct *Sfi*I restriction pattern was subsequently propagated for 18 h at 37°C in 100 ml LB broth with antibiotics as above. A plasmid maxiprep was performed using the Qiagen Plasmid Maxi Kit. The purified plasmid was re-examined by *Sfi*I digestion.

Preparation of CD99-ϕ

After transforming XL-1 Blue with pComb3H-CD99, the phage display technique was performed. A clone of pComb3H-CD99-transformed XL-1 Blue was propagated in 10 ml super broth containing 100 µg/ml ampicillin and 10 µg/ml tetracycline at 37°C until an OD at 600 nm of 1.5 was reached. The bacterial culture was subsequently propagated in 100 ml of the same medium and cultured for another 2 h. The transformed XL-1 Blue were infected with 10^{12} pfu VCSM13 helper phage (Stratagene). After 3 h incubation, kanamycin (final concentration 70 µg/ml) was added to the culture, which was then left shaking (200 rpm) for a further 18 h at 37°C. Bacteriophages harboring CD99 on gpIII (CD99-ϕ) were then precipitated using 4% (w/v) PEG MW 8000 (Sigma) and 3% (w/v) NaCl. Finally, the harvested phage was resuspended in 2 ml phosphate-buffered saline (PBS), pH 7.4.

Immunoassay for CD99-ϕ

CD99-specific mAbs, MT99/1 (IgM isotype) and MT99/3 (Ig2a isotype), were generated in our laboratory (unpublished data, and Kasinrerk et al. 2000). Solid phase was separately coated with 1 µg MT99/1 and MT99/3. The same amount of a CD54 mAb, MT54 (Moonsom et al. 2001), was used as a control. Standard ELISA washing and blocking processes were performed. CD99-ϕ or VCSM13 phages (50 µl; 10^{11} pfu/ml) were added to each mAb-coated well. A suitable dilution of horseradish peroxidase (HRP)-conjugated sheep anti-M13 (Pharmacia, Uppsala, Sweden) was added to each reaction well after the washing step. The 3,3',5,5'-tetramethylbenzidine plus H_2O_2 substrate was added to every well and the reaction was finally stopped with H_2SO_4 solution after a 30-min incubation.