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CORRELATION OF DENGUE VIRUS VIRULENCE WITH THE ABILITY TO STIMULATE HELPER T LYMPHOCYTES

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Executive Summary

Variation in the ability to stimulate helper T lymphocytes to secrete lymphokines in vitro together with differences of envelope glycoprotein sequences were determined in a group of dengue type 2 viruses which were recovered during the 1987 epidemic in Bangkok from dengue hemorrhagic fever patients with different clinical severity. Following the stimulation of lymphocytes with dengue-infected monocytes for a period of 8 days, IFN-γ, but not IL-2 and IL-4, was readily detectable. Variation of IFN-γ level did not correlate with the clinical severity of patients from whom viruses were isolated. Analysis of envelope protein sequence revealed two groups of dengue type 2 viruses co-circulating in Bangkok in 1987. The ratio of major group to minor-gruop was approximately 17:1. Members of the major group were very similar in their envelope protein sequences. There was no association between any group of viruses nor between specific amino acid changes with clinical severity of the patients. These findings will be important for public health care providers in the implementation of dengue type 2 vaccine candidate which is currently undergoing phase I/II testing in Thailand and also for subunit vaccine design. The skills that are related to in vitro cultivation and molecular characterization of human viral pathogen will be helpful to the study and management of other viral epidemic disease, such as AIDS, in the near future.

Research Objectives

Infection by any of the four serotypes of dengue viruses generally results in mild, self-limited illnesses, including asymptomatic infection, undifferentiated fever and dengue fever (DF). However, in several countries of Southeast Asia, severe and potentially-fatal disease (dengue hemorrhagic fever, DHF) can occur in a small fraction of all infected persons (Halstead, 1980; 1988). The pathogenesis of DHF is not known, but children who are secondarily infected have a greater risk of developing severe illness than those infected for the first time (Sangkawibha et al., 1984; Burke et al., 1988). Beause of the lack of appropriate animal model, the hypothetical difference in acquired host propensity to develop DHF has never been tested. On the other hand, it is possible that variation in viral virulence may serve as the determining factor of the outcome of dengue infection.

Variations in biological properties and antigenicity among dengue viruses are well documented. The intratypic differences of dengue viruses have been defined by: a) analysis of RNA genome using RNase T1 oligonucleotide fingerprinting (Repik et al., 1983; Trent et al., 1983), b) analysis of viral antigens with monoclonal antibodies (Monath et al., 1986; Morens and Halstead, 1987; Morens et al., 1987), c) sequence analysis of selected portions of the genome (Chu et al., 1989; Rico-Hesse, 1990; Blok et al., 1989; Blok et al., 1991), and d) comparison of the replicative ability in monocytic cells (Kliks, 1990; Morens et al., 1991). Structural analyses of dengue viruses isolated from various geographical areas indicate that viruses derived from a particular area are more similar to each other than viruses isolated from other locations (Repik et al., 1983; Trent et al., 1983; Chu et al., 1989; Rico-Hesse, 1990). These findings allow the classification of dengue viruses into structural topotypes (Repik et al., 1983; Trent et al., 1983; Monath et al., 1986; Rico-Hesse, 1990).

Two efforts were undertaken in order to delineate the variation and evolution of dengue viruses in a limited geographical area. In a longitudinal study of 54 type 2 viruses isolated from mosquitoes and patients with DF and DHF in Thailand over a span of 25 years (1962-1986), oligonucleotide fingerprints generated by RNase T1 digestion were compared in a pairwise fashion (Trent et al., 1989). Fingerprints of viruses isolated from within a decade are more similar to each others than to those from other decades. From this analysis, the RNA genome of dengue viruses type 2 in Thailand appears to undergo slow and continual changes over time. The rate of change is quite constant at about 1.4% of the long oligonucleotides per year. At any given year, circulating viruses have similar, but not identical, fingerprints; this quasispecies nature of dengue viruses is analogous to many other RNA viruses (Steinhauer and Holland, 1987). Trent et al. (1989) consider dengue type 2 viruses in Thailand as a single topotype that is gradually and constantly evolving.

In a cross-sectional study of 10 dengue type 2 viruses isolated during the 1980 epidemic in Bangkok, Walker et al.(1988) compared oligonucleotide fingerprints, restriction enzyme maps and reactivities with anti-NS1 monoclonal antibodies. A strain, D80-141, is unusually different from the rest with regards to all three analyses. Subsequent determination of the nucleotide sequences of E and NS1 genes of some of these 1980 viruses supports the subdivision of dengue

viruses type 2 of the 1980 Bangkok epidemic into two sets (Blok et al., 1989; Blok et al., 1991). The first set consists of eight isolates that are very similar to each other in the fingerprints and sequences of E and NS1 proteins, whereas the second set is represented by D80-141 and an additional strain. Analysis of the distribution of a distinct NS1 epitope that is lacking from D80-141 indicates that the second set may include as many as 12% of all dengue viruses type 2 found in this epidemic (Walker et al., 1988). However, because the unusual fingerprint of D80-141, which is derived from the 1980 epidemic, is not evident in the larger database examined by Trent et al.(1989), questions arise as to whether information from a variant would justify the subdivision, and whether this variant can compete effectively in the long run.

Attempts have also been made to identify variation of biological properties that may correlate with severe disease. Dengue type 2 viruses isolated from patients with clinically more severe diseases in the Bangkok 1980 epidemic appear to possess more infection-enhancing epitopes than isolates from milder cases (Morens and Halstead, 1987). However, this is evident only when monoclonal antibodies raised against dengue type 4, but not type 2, viruses are employed (Morens and Halstead, 1987; Morens et al., 1987). More recently, Kliks (1990) and Morens et al. (1991) report that dengue type 2 viruses isolated from severe cases tend to replicate to higher titer in human monocyte or peripheral blood leukocyte cultures than isolates from milder cases. It is not yet possible to correlate differences in the replicative ability with known genetic marker or structural variation.

It is well documented that dengue viruses interact with specific T lymphocytes in vitro such that T lymphocytes from dengue immune persons produce high titer of IFN-γ after stimulation with dengue antigens (Kontny et al., 1988; Kurane et al., 1989). However, the role of cell-mediated immune response in eradicating infecting dengue virus and in the pathogenesis of DHF is not yet known. Because, within a given epidemic year, dengue viruses of a particular serotype are not identical, we postulate that variation of viral structural proteins may involve linear epitope(s) which can be recognized by T lymphocytes, resulting in differences among dengue viruses in their ability to stimulate T lymphocytes. These differences, if exists, may constitute one of the determining factors which are involved in the pathogenesis of diseases caused by dengue infection. If this is the case, one should find that dengue type 2 viruses, which are isolated from DF patients and DHF patients with different clinical severity, will differ both in their ability to stimulate helper T lymphocytes in vitro and in primary sequence of the structural proteins (such as the envelope glycoprotein) and, more importantly, the differences will correlate with the clinical severity of patients from whom viruses were isolated. Our specific objectives are: 1) to compare the ability of dengue type 2 viruses in the stimulation of IFN-γ, IL-2 and IL-4 production from T lymphocytes, and 2) to determine the variation of their envelope glycoprotein sequence. In making the comparison, it is necessary to minimize variations due to geographical and temporal segregation; we, thus, deliberately employed for comparison a set of low passage dengue type 2 viruses isolated from DHF patients of differing clinical severity during an epidemic year of 1987 in Bangkok.

The information gained from the study will be helpful to our understanding of the pathogenesis of DHF. Specifically, as the issue of dengue virus variation has been examined in

more detail, it may become possible to confirm or dispute the concept of variation in dengue virus virulence, at least in the context of DHF. Moreover, the finding of heterogeneity, or lack, of the envelope protein will be important to the design, generation and implementation of dengue virus vaccine in the future.

Our attempts have been greatly facilitated by the Department of Virology, US Component, Armed Forces Research Institute for the Medical Sciences (AFRIMS), Bangkok (Drs. Bruce Innis and Ananda Nisalak) in allowing access to the dengue virus repository and generous gifts of reagents; the Division of Vector-Borne Diseases, Centers for Disease Control, Fort Collins, Colorado (Dr. Dennis Trent) and the Virus Research Institute, Ministry of Public Health, Nontaburi (Ms. Suntharee Rojanasuphot) for helps during the initial laboratory set up and gifts of antibodies; the Department of Virology, University of the Ryukyus, Okinawa, Japan (Drs. Toshihiko Fukunaga and Yoshihiro Makino) for providing cell line and assay protocol and Siriraj Medical Molecular Biology Center, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok (Dr. Prida Malasit) for access to flow cytometer and gifts of viral stock.

Methods and Results

During the course of investigation, it was necessary to: 1) adopt and improve a rapid method for the enumeration of infectious dengue viruses, 2) characterize and determine the susceptibility of monocytes, the major host cell of dengue virus in man, to dengue virus infection, 3) delineate the time course of lymphokine production from helper T lymphocytes following the stimulation by dengue-infected monocytes, and 4) develop a rapid nucleotide sequence analysis method which is readily applicable to the study of low passage, RNA-containing dengue viruses. The methods and results sections are divided accordingly.

A. Improvements of peroxidase-anti-peroxidase staining method for the enumeration of dengue type 2 viruses.

Quantitation of dengue viruses is generally done by employing the plaque assay (Schulze and Schlesinger, 1963; Russell et al., 1967). Depending on the type of cells and the replicating abilitiy of individual viruses, the time required for the formation of plaque may take from 7-10 days. Although there are several micro-methods for performing plaque assay (Fujita et al., 1975; Ksiazek and Liu, 1980; Morens et al., 1985), these methods still require long incubation time. Igarashi and Mantani (1974) applied an indirect fluorescent antibody technique for the titration of dengue viruses and were able to count foci of dengue-infected cells 2 days after the initiation of culture. Okuno et al.(1977) later adapted a peroxidase-anti-peroxidase (PAP) staining for the identification and titration of dengue viruses. This method has the advantage of short incubation time, but needs only regular light microscope and is readily adapted for microtitre plate. During the adaptation of PAP method, Okuno et al.(1977) tested the sensitivity of this method against the indirect fluorescent antibody assay, but did not employ the standard plaque assay for comparison. In this section, we seek to improve the peroxidase-anti-peroxidase (PAP) staining method by: a) adding 4-chloro-1-naphthol as co-substrate of peroxidase, and b) omitting a

washing step following the addition of sheep anti-rabbit IgG, the bridging antibody. We then make a direct comparison of the PAP staining method against plaque assay for dengue virus quantitation.

Cells, viruses and antibody

LLC-MK2 cell line, dengue type 2 virus, strain 16681, and 4G2, an anti-flavivirus monoclonal antibody (Henchal et al., 1982), were provided by Drs. B. Innis and A. Nisalak, AFRIMS, Bangkok. The LLC-MK2 was maintained in M199 media + 20% fetal bovine serum (FBS). BHK-21 cell line was donated by Dr. T. Fukunaga, University of the Ryukyus, Okinawa, Japan. The BHK-21 cell was grown in MEM + 10% FBS and was passaged every 3-4 days.

Quantitation of dengue virus by PAP method

A 5-step PAP method was performed according to Okuno et al. (1977) and Ishimine et al.(1987). Briefly, a virus suspension was first diluted ten folds in MEM + 5% FBS, then a serial four-fold dilutions of the diluted suspension was made. Fifty µl of five serially diluted samples were inoculated in duplicate wells of 96-well plate (Costar) containing a one-day-old BHK-21 monolayer. After 2 h of incubation at 37°C, 100 µl of 1% gum tragacanth (Sigma) in MEM + 2% FBS was added as a top layer. The plate was incubated for two more days and then fixed with 3.7% formaldehyde and 1% Triton X for 10 min each. After washing with phosphate buffered saline (PBS), 50 µl of 4G2 at 1:1,000 dilution in PBS was added for 30 min and followed by another washing step. Fifty µl of the following reagents were then used successively: rabbit anti-mouse Ig antibody (at 1:1,000 dilution in PBS + 0.05% Tween 20, 2% FBS {PBSTF}; Dakopatts), sheep anti-rabbit IgG antibody (at 1:1,000 dilution in PBSTF), peroxidase-rabbit anti-peroxidase complex (at 1:5,000 dilution in PBSTF; Cappel) and 3,3'-diaminobenzidine/H_2O_2 mixture (Sigma) or 3,3'-diaminobenzidine/4-chloro-1-naphthol/H_2O_2 mixture. After the final washing, dry plates were observed under a light microscope and a group of >5 brown or bluish black cells was scored as a focus. All wells that contained <100 foci were used in the calculation of virus titer which was expressed as focus forming unit (ffu)/ml.

Quantitation of dengue virus by plaque assay

The same virus dilutions as above were simultaneously inoculated onto a one-day-old LLC-MK2 cell monolayer in 12-well tissue culture plate (Flow). After 2 h of incubation at 37°C, an agar overlayer medium containing M199, 5% FBS, 0.1% DEAE dextran (Pharmacia), 0.5% agar was added. The plate was incubated at 37°C in 5% CO_2 for seven days. On the last day, the cells were fixed with 10% formaldehyde and stained with 0.2% crystal violet. Plaques were counted in all wells that contained 40 plaques or less. The concentration of virus was calculated as plaque forming unit (pfu)/ml.

Results

Comparison of 3,3'-diaminobenzidine and 3,3'-diaminobenzidine + 4-chloro-1-naphthol as substrates for PAP staining

As a substrate for peroxidase, 3,3'-diaminobenzidine gave insoluble brown colour product with poor contrast when compared with a blue product of 4-chloro-1-naphthol, another substrate of peroxidase. Young (1989) advocated the combination of 3,3'-diaminobenzidine and 4-chloro-1-naphthol for immunoblot staining; the mixed substrates allowed high sensitivity of detection due to 3,3'-diaminobenzidine and at the same tine gave high contrast of 4-chloro-1-naphthol. We compared 3,3'-diaminobenzidine + 4-chloro-1-naphthol against 3,3'-diaminobenzidine alone as substrates for the PAP staining of dengue infected cells and found that the mixed substrates resulted in the formation of dark bluish black colour inside the cytoplasm of dengue infected cells. The foci observed with mixed substrates were easier to count than those bearing brown colour of 3,3'-diaminobenzidine. The former were stable for a few days and then slowly faded to brownish colour. Background staining of uninfected cells was minimal for both sets of substrate.

Reduction of staining time by omiting a washing step following sheep anti-rabbit IgG

In order to shorten the assay time and increase sensitivity of PAP method, we tried omitting a washing step following the addition of sheep anti-rabbit IgG. As introduced by Naser (1990), the omission should lead to better reaction among sheep anti-rabbit IgG, the bridging antibody, and rabbit anti-mouse Ig antibody and rabbit PAP complexes, the targets, resulting simultaneously in reduced assay time and enhanced sensitivity. We found that this omission did not affect the signal from infected cells as perceived visually. The omission, however, reduced assay time by about 20 min/plate.

Comparison of PAP method and plaque assay

To directly compare PAP method and plaque assay for the titration of dengue virus, five serially diluted samples of a suspension of strain 16681 were inoculated simultaneously onto BHK-21 and LLC-MK2 monolayers. Staining and counting of infected foci/plaques after appropriate incubation time revealed that the titers of dengue virus as determined by PAP and plaque methods were very similar (Fig. 1). In fact, the distribution of titers from three independent experiments was superimposable, indicating that, for practical purposes, these two methods are interchangeable.

Discussion

Plaque assay is currently accepted as the standard method for determining dengue virus titer. In practice, this method suffers from a long incubation time and poor adaptability to micro-culture. In order to avoid such inconvenience, we improve the PAP staining method and then compare it against plaque assay. For greater clarity of stained foci, we add

4-chloro-1-naphthol as co-substrate for peroxidase. This results in better contrast and easy counting of the foci. For reduction of the staining time, we try omiting a washing step following the addition of sheep anti-rabbit IgG, the bridging antibody, and are able to reduce the staining time by about 20 min. However, enhanced assay sensitivity is not obtained in the dengue system; this may be due to our use of direct visual observation of stained foci in contrast to photometric measurement performed by Naser (1990). We now routinely use the combination of diaminobenzidine and chloronaphthol as substrates, but seldomly omit the washing step. Reduction of the staining time can also be achieved by employing rabbit anti-dengue antiserum as the primary reagent (Okuno et al., 1977; Ishimine et al., 1987).

Direct comparison of the PAP staining method against plaque assay reveals comparable sensitivities. The PAP method, however, can be completed in a much shorter time than plaque assay, ie. 3 days vs. 7-10 days. In addition, if the monoclonal antibodies specific for each serotype are employed, the PAP method is also useful as a single-step identification of dengue virus serotype. Similar to the plaque assay, the optimal incubation time necessary for the development of foci of adequate size needs to be determined for each individual viruses. Low passage dengue type 2 viruses from Thailand require upto 3 or 4 days of incubation before appropriate focus size is obtained. Even when the low passage viruses are concerned, the PAP method still offers greater advantage in the reduction of total assay time. The disadvantage of the PAP method lies in the use of microscope for visual enumeration. This is especially true for the low passage viruses which produce small foci.

B. Characterization and testing of the effect of IFN-γ on dengue virus multiplication in human blood monocytes.

In this section, monocytes were prepared from peripheral venous blood by adhesion to and elution from gelatin/plasma-coated flask. The purity of monocytes was checked by staining for CD14 and CD64 expression. Their susceptibility to dengue virus infection in vitro was next determined in parallel to the testing of their responsiveness to anti-viral effect of interferon-γ.

Preparation of monocyte

Monocytes were prepared from peripheral blood of four Thai donors who maintained HI antibody titer against dengue type 2 of 40-160 throughout the study period (dengue-immune donors) and a rare donor who lacked detectable HI antibody against dengue type 2 virus. Heparinized venous blood was centrifuged at 150g for 30 min. After removal of platelet-rich plasma, mononuclear cells were separated by centrifugation through Histopaque 1077 (Sigma). According to Jones et al. (1989), mononuclear cells in RPMI 1640 and 10% FBS were added to plastic flasks (Costar) which were precoated with 30 mg/ml gelatin (May and Baker) and autologous plasma. Following an incubation at 37°C for 1 h, non-adherent cells were detached by several rinses of RPMI 1640; adherent cells were next eluted with RPMI 1640 + 10% FBS + 5 mM EDTA. Eluted cells were 85-90% monocytes by non-specific esterase staining. Recovery of monocytes ranged from 20-56%.

Determination of cell surface CD14 and CD64

Monocytes were incubated on ice with 10% human AB serum for 30 min to block FcR. Anti-CD64 or -CD14 was next added for another 30 min. After washing, cells were reacted with FITC-conjugated sheep $F(ab')_2$ anti-mouse Ig(G+M) (Grub, Scandic, Vienna, Austria) and fixed with 1% paraformaldehyde. As a negative control, an irrelevant antibody (CD1a) of identical isotype was employed. Analysis was done on a flow cytometer (FACSCAN, Becton Dickinson) (courtesy of Dr. P. Malasit, Siriraj Medical Molecular Biology Center, Bangkok). Monoclonal antibodies specific for human CD1a, CD14 and CD64 were gifts of Drs. W. Knapp and H. Stockinger, Institute of Immunology, University of Vienna, Austria.

Infection of monocyte

Monocytes were adjusted to $1x10^6$ cells/ml in RPMI 1640 containing 5% FBS, 2 mM glutamine, 1X non-essential amino acids, 0.1 mM sodium pyruvate, $5x10^{-5}$M 2-mercaptoethanol and 10 mM HEPES, pH 7.2 and $5x10^4$ cells/well were added to an 8-well glass chamber slide (Nunc). After 24 h at 37°C, strain 16681 was added with occasional shakings for 2 h. Unadsorbed viruses were washed off with three rinses of media. After a further incubation at 37°C in 5% CO_2 in air, culture supernatant was collected, centrifuged and stored at -70°C. In some experiments, dengue virus was mixed with optimal infection-enhancing dilutions of anti-dengue antibodies (10^{-5}, 4G2; 10^{-5} or $10^{-5.5}$, human antiserum) at 4°C for 1 h before infection. Serum from a Thai patient with the HI antibody titer against dengue type 2 of 10,240 was used as polyclonal anti-dengue antibody.

Interferon

Recombinant human IFN-γ (specific activity $>2.0x10^7$ IU/mg) was from Boehringer Mannheim. Two other preparations of recombinant and leukocyte IFN-γ (activity 200 IU/ml and 500 IU/ml, respectively) were gifts of Dr. S. Yamazaki, National Institute of Health, Japan.

Quantitation of dengue virus-infected monocyte

To enumerate the number of dengue virus-infected monocytes, monocytes were fixed with acetone and then the 5-step PAP detection method described in Section A. was employed with the following modifications: a) monocytes were preincubated with 2% sheep serum for 30 min before the 4G2 reaction, b) rabbit anti-mouse Ig was diluted in PBS containing 0.05% tween 20, 2% FBS and 10% sheep serum, c) only 3,3'-diaminobenzidine was used, and d) cells were counterstained with 1% methyl green. Monocytes that contained yellow color in the cytoplasm against green nuclei were scored as positive. Five hundred - 1,200 cells were counted in each well.

Statistical analysis

At the level of 100 IU/ml of IFN-γ tested, the actual counts of stained foci from treated

wells were compared with those from untreated wells and tested for significant differences with Detre and White's method (Detre and White, 1970). For comparisons of the infected monocytes, Chi-square test was employed.

Results

Characteristics of monocyte and dengue virus infection of monocyte

Analysis of surface CD14 and CD64 (FcγRI) revealed that 85-92% of freshly eluted cells showed strong staining. Upon further culturing, enriched monocytes readily attached to glass surface and assumed a flat, spread-out morphology. After 24 h of culture, CD64 level decreased slightly. Stimulation with 100 IU/ml of IFN-γ for 24 h resulted in 7-11.5 folds increase of surface CD64.

Analysis of monocytes after direct infection with strain 16681 indicated that the percentage of infected cells increased proportionally with the multiplicity of infection (moi) reaching a plateau at the moi of 5-10 ffu/cell where about 10-30% of cells were infected at 24 h. At the moi of 5 and 0.05 ffu/cell, virus yields in the supernatant reached peak levels after 24-48 h and 72-96 h, respectively. When strain 16681 was mixed with human antiserum before infection, the highest level of enhancement of virus titer (mean=40 folds, range=24-71 folds, n=3) was observed when monocytes from a rare donor who lacked anti-dengue type 2 HI antibody were infected at the moi of 0.05 ffu/cell for 72 h. With monocytes from four dengue-immune donors, enhancement of virus yield by 4G2 or human antiserum was only 2.4 folds (range=1.6-3.4 folds, n=8). Varying the initial moi or preculturing cells for 3 and 6 days did not increase infection enhancement in monocytes from immune donors consistently.

Responsiveness of human blood monocyte to antiviral effect of IFN-γ

To assess the responsiveness to antiviral effect of IFN-γ, monocytes were treated with 100 IU/ml of IFN-γ for 6-24 h before infection. The inhibitory effect of IFN-γ was apparent as early as 6 h and becoming maximal at 24 h. When monocytes were treated with various amounts of IFN-γ for 24 h and then infected with strain 16681, a dose-related reduction of virus yields was observed (Fig. 2A). In six experiments, IFN-γ at the level of 100 IU/ml diminished virus yields by 12- to 97-folds ($p < 0.001$). Recombinant IFN-γ and leukocyte IFN-γ affected dengue infection similarly.

Dominant antiviral effect of IFN-γ in antibody-mediated enhanced infection

Having shown that enriched monocytes were responsive to the positive regulatory effect on FcγRI expression and the antiviral effect of IFN-γ, we next determined the effect of IFN-γ on dengue replication when infection was initiated by virus-antibody complex. In the first set of experiments, strain 16681 was incubated with 4G2 before infection of IFN-treated monocytes from dengue immune donors. Similar dose-related inhibitory effect was observed at various levels of IFN-γ tested (Fig. 2B). At 100 IU/ml of IFN-γ, the virus yields were reduced by 13- to 137-

folds ($p < 0.001$) and both IFN preparations were effective.

Because the antibody-mediated enhancement of dengue infection in immune monocytes was quite modest, another set of experiments was carried out with monocytes from a donor who lacked anti-dengue HI antibody. In three experiments where a human antiserum enhanced infection by a mean of 40 folds, IFN-γ still exerted a suppressive effect ($p < 0.001$).

The augmenting effect of IFN-γ on dengue infection of U937 cells was also evident when the number of infected cells was examined (Kontny et al., 1988). To assess this effect of IFN-γ on monocytes, IFN-treated monocytes were infected with dengue virus either directly or in the presence of 4G2 for 24 h and then stained for viral antigen. The antiviral effect of interferon was evident at 10-100 IU/ml of IFN-γ, and at 100 IU/ml the mean percentage of infected cells was reduced by 6.4 folds and 7.4 folds in the absence and presence of 4G2, respectively (17.3% vs. 2.7% and 28.8% vs. 3.9%, $n=3$, $p < 0.001$). Thus, IFN-γ treatment diminished the percentage of infected monocytes regardless of the mode of infection.

Discussion

By the criteria of CD14 and CD64 surface expression, monocytes that were prepared by adhesion to and elution from gelatin/plasma coated flask are highly enriched. They are readily infected with dengue viruses both in the absence and presence of enhancing antibodies. Both of the cytoplasmic antigen and infectious dengue virus in the culture supernatant can be detected. Furthermore, they are responsive to the anti-viral effect of IFN-γ both when the viral challenge is accomplished with or without enhancing antibodies.

Dengue virus infection of monocytes occurs via two major routes: a) uptake of virion through specific receptor and b) uptake of virus-antibody complexes through FcR (Halstead, 1988; Kontny et al., 1988; Littaua et al., 1990). IFN-γ can up-regulate FcγRI and increase the uptake of virus-antibody complexes into monocytic cells, but this positive effect is likely to be counteracted inside cells by concomitant induction of antiviral effect. Surprisingly, Kontny et al. (1988) showed augmenting effect of 1-10,000 units/ml of recombinant IFN-γ on dengue infection of U937 when infection was initiated in the presence of murine antiserum. However, induction of an anti-viral effect in U937 by IFN-γ, i.e. reduction of infection in the absence of added antiserum, was not demonstrated. Subsequently, Kurane et al. (1989) revealed, in two experiments, an enhanced dengue infection of human monocytes following pretreatment with stimulated leukocyte culture fluids that contained 3 units/ml of IFN-γ. In testing the generality of these findings, we checked the effect of IFN-γ on human monocytes under similar experimental conditions (dose of IFN-γ, duration of pretreatment, multiplicity of infection) and found that dengue infection of monocytes was always suppressed by IFN-γ.

The discrepancy between enhancing effect on U937 and suppressive effect on monocytes may be explained by a peculiar responsiveness of U937 to interferons. While it is well established that U937 is responsive to the antiviral effect of IFN-α and -β, induction of this effect by IFN-γ remains controversial. Hartshorn et al.(1987) find definite anti-HIV activity in U937

treated with IFN-γ. In contrast, IFN-γ induces the expression of HIV-1 in U1, a persistently infected subline of U937 (Biswas et al., 1992). Employing vesicular stomatitis virus, Improta et al.(1992) cannot detect any antiviral activity after treating U937 with IFN-γ, eventhough IFN-γ potentiates protection by other interferons. Segregation of intracellular responses to IFN-α and IFN-γ in U937 is also documented by studying of protein kinase C translocation and activation of interferon-stimulated proteins (Improta et al., 1992; Csermly et al., 1990).

Direct infection of U937 with dengue virus generally results in a very low yield (Kontny et al., 1988). The apparent lack of specific dengue virus receptor makes this cell line an attractive system for studying antibody-mediated infection enhancement. On the other hand, it is impossible to evaluate responsiveness of U937 to the anti-dengue effect of IFN-γ without added antibody. It is likely that the U937 cells that were studied by Kontny et al. (1988) are not protected by IFN-γ. Therefore, augmentation of dengue infection following IFN-γ treatment of such U937 cells can be explained by the up-regulation of surface FcγRI in the absence of anti-viral effect. Whether this is a general property of all lines of U937 or a peculiar characteristic of the line employed by Kontny et al. (1988) remains to be investigated.

Besides U937, infection enhancement has been studied extensively with monocytes from non-immune persons. Because DHF occurs more readily in children who possess anti-dengue antibodies (Halstead, 1988; Burke et al., 1988; Sangkawibha et al., 1984), the relevance of high level of enhancement observed with the cell line is unclear. Due to higher yields of virus following direct infection, enhancement by added antibodies is minimal with monocytes from immune donors. Similarly, infection enhancement in such monocytes by IFN-γ may be as inefficient. Nevertheless, our testing of IFN-γ with monocytes from a rare local non-immune individual, which allow comparatively higher level of antibody-mediated enhancement, still reveals anti-viral effect. Taken together, our results strongly suggest that once anti-viral response is established in monocytes, enhancement of virus uptake by added antibody and by up-regulating FcγRI at the levels seen in cells taken from donors in an endemic area does not abolish the anti-viral effect of IFN-γ. These results also underscore the need to employ host cells that are physiologically meaningful for evaluation of cytokines on virus replication

C. Stimulation of helper T lymphocytes by dengue type 2 viruses.

In this section, we employed dengue-infected monocytes as antigen presenting cells for the stimulation of lymphocytes from dengue immune donors. Production of IL-2, IL-4 and IFN-γ from stimulated lymphocytes was then measured.

Dengue viruses

All dengue virus type 2 strains employed were isolated during the epidemic season of 1987 from DHF patients who were cared for at the Children's Hospital, Bangkok. The clinical diagnosis and grading of disease severity were assessed by Dr. Siripen Kalayanarooj of the Department of Pediatrics, Children's Hospital according to the criteria established by WHO

(1986). The isolates were originally inoculated into *Toxorhynchites amboinensis* and then passed at most 4-5 times in C6/36 cell clone of *Aedes albopictus* mosquito cells (Igarashi, 1978) until the titer of 10^4-10^6 ffu/ml as measured by PAP method was reached. Before being employed in the stimulation experiments, the isolates were titrated again. The characteristics of all dengue virus type 2 isolates were summarized in Table 1. The laboratory-adapted dengue virus type 2 strain 16681 was isolated from a fatal case of DHF in Thailand in 1964 (Halstead and Simasthien, 1970) and was serially passaged in C6/36 cells.

Serologic confirmation with indirect immunofluorescence assay

Serotypic classification of all dengue viruses employed was confirmed by an indirect immunofluorescence assay (Henchal et al., 1982). C6/36 cells were infected with each isolates at 28°C for seven days. At the end of incubation, infected cells were spotted onto glass slide, fixed with cold acetone and reacted sequentially with 1:2,000 dilution of a mouse anti-dengue virus type 2 (3H5) antibody (Henchal et al., 1982) and fluorescein-conjugated goat anti-mouse Ig (Dakopatt). Murine monoclonal antibodies specific for flaviviruses (4G2), dengue viruses type 1 (1F1), type 3 (10C10), and type 4 (1H10) were also used. C6/36 cells infected with known dengue viruses type 1, 2, 3, and 4 were employed as positive controls. All of the 1987 Bangkok isolates reacted with only 4G2 and 3H5, but not with others, indicating that they were actually dengue virus type 2.

Stimulation of lymphocytes

To determine the capacity of dengue virus to stimulate T lymphocytes, enriched monocytes were infected with dengue viruses at the moi of 0.5 in 24-well plate. In each well, $3x10^4$ monocytes were added with $1.5x10^4$ ffu in 0.5 ml of media; they were then incubated with intermittent shakings for 2 hr at 37°C. Lymphocytes (the non-adherent fraction from gelatin/plasma-coated flask) were next introduced at the ratio of lymphocyte/monocyte of 16:1 and the suspension was incubated for 8 days. On each of days 3-8 following the initiation of culture, supernatants were collected, pooled, spun down to remove cells/debris, aliquoted and stored at -70°C until assayed for lymphokine content. For each isolate, infection and stimulation were performed in quintuplicate. As a positive control for stimulation, PHA at 2 μg/ml was employed to activate $6x10^5$ lymphocytes in the presence of $1.5x10^5$ monocytes/well.

Depletion of T lymphocyte subset

In some experiments, subset of T cells were depleted from non-adherent population by negative selection. Twenty and thirty million lymphocytes were reacted with 10 μg/ml of murine anti-CD4 and anti-CD8 monoclonal antibodies (VIT4 or VIT8b, courtesy of Drs. W. Knapp and H. Stockinger, Institute of Immunology, University of Vienna, Austria), respectively, at 4°C for 30 min. Following two washes with media, corresponding subset of T cells were removed with the addition of anti-mouse IgG-coated beads and the application of magnet according to the manufacturer's instruction. Remaining cells were more than 98% viable by trypan blue exclusion.

Lymphokine assay

Human IL-2, IL-4 and IFN-γ were measured by 1-step or 2-step ELISA according to the manufacturer's instruction (human IL-2 and IL-4 test kits from Amersham, human IFN-γ kit for medium from GIBCO/BRL). The assays were performed in duplicates on precoated, flat-bottom 8-well strip and the O.D. measured with an ELISA reader. Lymphokine content of supernatants was derived by comparing the mean O.D. of samples with those of standard amounts of lymphokine provided by the manufacturer.

Results

Different kinetics of stimulation of IFN-γ production between strain 16681 and low passage 1987 isolates

Monocytes were infected separately with strain 16681 and two low passage 1987 isolates and were employed as antigen presenting cells in coculture with lymphocytes from dengue-immune person. During a 7-day period, only IFN-γ, but not IL-2 nor IL-4, was detected. Strain 16681 stimulated a more rapid IFN-γ response than two low passage strains. In 16681-stimulated culture, the level of IFN-γ reached the plateau level on day 4-5 and declined slowly thereafter. Under the same conditions, the plateau was delayed to day 7 with two low passage isolates.

IFN-γ production from lymphocytes was dependent on virus replication because viruses that were inactivated with UV light treatment before being added to monocytes did not stimulate IFN-γ production. Lack of stimulatory effect of UV-inactivated viruses was concurrent with an absence of focus forming ability when inactivated viruses were tested simultaneously on BHK cell monolayer. Comparing the input virus at the moi of 0.1, 0.5, 1 and 5 revealed that maximal stimulation was achieved with moi of 0.5-1.

Depletion of $CD4^+$ cells from non-adherent lymphocytes consistently reduced IFN-γ production to the background level, indicating that $CD4^+$ T lymphocyte subset was the main producer of IFN-γ in this in vitro system.

Variation of stimulation of IFN-γ production by 1987 isolates

Monocytes were infected with 7 low passage isolates from 1987 that had attained high enough titer. Following co-culture of infected monocytes with lymphocytes for 7 days, supernatants were assayed for IFN-γ content. In two experiments, there were consistent variations of the levels of IFN-γ following the stimulation by individual viruses. However, there was no linear correlation between the clinical severity of patients from whom viruses were isolated with the level of IFN-γ (Table 2). A grade II DHF isolate (D87-1441) stimulated the highest level of IFN-γ while the lowest level was observed with a grade I isolate and a grade III isolate.

To investigate whether variation in the stimulation of IFN-γ from lymphocytes was secondary to the changes in kinetics of IFN-γ production, the time course of IFN-γ production

was determined for two isolates. Although the levels of IFN-γ following the stimulation by D87-1441 and D87-1421 differed by almost 4 folds on day 7, it was apparent that such difference was detected since day 3 and was maintained during the 5-day period (Table 3). Thus, variation of stimulation of IFN-γ production was independent of the changes in the kinetics of stimulated IFN-γ production and may be due to intrinsic differences of these virus strains.

Discussion

Based on a comparison of the kinetics of stimulation of IFN-γ production between low passage viruses and a laboratory adapted strain, 16681, it is evident that low passage viruses induce a lower level and a slower kinetics of IFN-γ production than 16681. The difference is likely to be due to an enhanced in vitro replicative ability of this laboratory adapted strain. Although 16681 has been serially passaged in C6/36 mosquito cells, data from previous section indicate that it replicate quite well in human monocytes. The finding implies that in the comparison of the 1987 isolates, the effect of passage history of each isolate on the stimulation of IFN-γ production must be taken into account. Thus, we have employed only those isolates that grow well (to the titer of 10^4-10^6 ffu/ml) in the first 4-5 passages in mosquito cells for the comparison. I n order to confirm the similarity of stimulation kinetics of these low passage viruses, we follow the time course of stimulation of two low passage isolates that are highly discordant with regard to the level of IFN-γ stimulated and, as expected, we detect no difference in the stimulation kinetics.

While IFN-γ is readily detectable in the co-culture system, little or no IL-2 or IL-4 can be found under the same conditions. The disparity may be caused by one or more of the following possibilities: 1) the ELISA for IL-2 and IL-4 are not as sensitive as the one for IFN-γ, 2) secreted IL-2 and IL-4 are consumed by activated lymphocytes to the greater extent than IFN-γ, 3) dengue-specific T cell subset that is responsible for IL-2 and/or IL-4 secretion does not circulate in peripheral blood of dengue-immune persons, 4) dengue-infected monocytes preferentially stimulate T cell subset (or intracellular pathway) that is responsible for IFN-γ production, or 5) there is suppressive influence of IFN-γ-producing T cell (or IFN-γ itself) on the production of IL-2 and IL-4. These possibilities are not mutually exclusive; it is likely that more than one mechanisms are responsible for the lack of IL-2/IL-4 response. It now becomes clear that multiple factors are involved in the differential regulation of Th1 (IFN-γ producer) and Th2 (IL-4 producer) activation, proliferation and lymphokine secretion (Fitch et al., 1993). In this regard, it will be interesting to further investigate the effect of IFN-α that is secreted by dengue-infected monocytes on the differentiation of dengue-specific T cells.

Comparison of the stimulatory capacity of seven dengue isolates from the 1987 Bangkok epidemic reveals differences in IFN-γ response which is not correlated with clinical severity of patients from whom viruses were isolated. This finding may reflect the low number of isolates being tested in this experiment; increasing the number of viruses for comparison may give a different picture. However, low passage dengue viruses generally do not grow to high titer in vitro. It will be necessary to modify the method of monocyte infection and the co-culture system so that low titered viruses can be studied. Alternatively, it is more desirable to be able to

concentrate viruses before employing them for monocyte infection. The lack of an ultracentrifuge has severely hampered us in this matter.

Differences in IFN-γ response to this group of 1987 Bangkok isolates do not correspond with relative homogeneity of their envelope glycoprotein sequences. As presented in the next section, all of the 1987 isolates employed in this section, except D87-1421, are very similar in the amino acid sequence of envelope protein. The difference may be due to high level of amino acid substitution in other structural/non-structural proteins as there are other proteins (such as NS4 proteins) that accumulate higher amino acid substitution than the envelope protein (Blok et al., 1992). It is also possible that low level of nucleotide substitution in the non-coding regions may greatly affect the rate of replication, resulting in higher T lymphocyte response.

D. Sequence variation of envelope protein gene of dengue viruses type 2.

In this section, the variation of envelope (E) protein sequence of dengue type 2 viruses derived from the 1987 epidemic in Bangkok was examined by employing polymerase chain reaction (PCR) and cycle sequencing of the amplified product.

Oligonucleotide primers

Oligonucleotides suitable for use as primer pairs in the PCR reaction were selected by using a computer program described by Lowe et al. (1990). The primer sequence was based on the nucleotide sequence of dengue type 2 Jamaica prototypic strain (Deubel et al., 1986); they were purchased from Synthetic Genetics, San Diego, California. The expected size of amplified products is 1,700 bp. Twelve oligonucleotide primers used for the sequence analysis of E gene were selected to allow reading of about 200-300 base apart (Table 4). Two sets of six primers were employed for sequencing of each coding and non-coding strands, respectively.

Preparation of genomic RNA

Genomic RNA was prepared from culture fluids of infected C6/36 cell by phenol-chloroform extraction. The culture fluid, containing 8×10^4 ffu, was added with an equal volume of 0.1 M Tris-HCl, pH 8.0, 0.1 M EDTA, 0.2 M NaCl, 1% SDS and 100 μg/ml proteinase K at 56°C for 1.5 h. The RNA was recovered by precipitating with ethanol and collected by centrifugation. After washing once with 75% ethanol, RNA pellet was allowed to dry and then resuspended in diethyl pyrocarbonate-treated water. After adding three volumes of absolute ethanol, genomic RNA was stored at -70°C.

Synthesis of complementary DNA

Genomic RNA derived from 4×10^4 ffu was reverse transcribed into cDNA as follows. Genomic RNA was first added with 20 units of ribonuclease inhibitor (Promega), then heated to 70°C for 10 min and quickly chilled on ice. Reverse transcription was carried out in 10 mM Tris-

HCl, pH 8.3 at 25°C, 1.5 mM $MgCl_2$, 50 mM KCl, 0.001% gelatin, 10 mM dithiothreitol, 0.5 mM each of the four dNTPs, 20 pmol of antisense primer, and 150 units of recombinant MMLV RNaseH⁻ Reverse Transcriptase (Superscript, GIBCO/BRL) at 45°C for 1 h and then stopped by heating at 95°C for 5 min. The cDNA products were used directly in the amplification reactions without any purification.

Amplification of dengue envelope gene

The cDNA was amplified in 100 µl of the mixture containing 10 mM Tris-HCl, pH 8.3 at 25°C, 1.5 mM $MgCl_2$, 50 mM KCl, 0.001% gelatin, 0.1 mM each of the four dNTPs, 10 pmol of sense primer, and 2.5 units of Taq DNA polymerase. After overlayering the mixture with mineral oil, the amplification was allowed to proceed for 35 cycles according to the following parameters: denaturation, 94°C for 1 min; annealing and extension, 72°C for 5 min. The last extension step was at 72°C for 7 min. Five µl of the products were analyzed by electrophoresis through 1% agarose gel in Tris/acetate/ EDTA buffer; this revealed an expected 1,700 bp DNA band together with a few other smaller bands. The 1,700 bp DNA band was confirmed as a specifically amplified product by digestion with each of the following enzymes: BamHI, EcoRI, HindIII, KpnI and PstI and electrophoresis either in 1% agarose gel or 4-5% polyacrylamide gel. Purification of amplified DNA fragment from agarose gel was accomplished by using GlassMax DNA isolation system (GIBCO/BRL). Purified DNA was eluted with preheated (65°C) 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA. The amount of DNA in eluates was estimated by comparing fluorescence of the DNA band in ethidium bromide-stained gel with that of a known amount of HindIII-digested λDNA fragments.

Nucleotide sequence analysis

Nucleotide sequence analysis of the PCR products was performed by using the dsDNA cycle sequencing system (GIBCO/BRL). Sequencing primers were first labeled with [γ-^{32}P] ATP in a kinasing reaction. One picomol of each of the sequencing primers was 5'end-labeled with T_4 polynucleotide kinase and 1 pmol of [γ-^{32}P] ATP (>5,000 Ci/ mmol; Amersham) in 5 µl of 60 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, and 0.2 M KCl. Labeled primers were used in the sequencing reaction directly without removing of unincorporated [γ-^{32}P] ATP.

The prereaction mixture was next prepared by combining 5 µl of the end-labeled primer with approximately 50 fmol of the PCR product, 2.5 units of Taq DNA polymerase, 6.2 mM $MgCl_2$, 37.5 mM KCl, 37.5 mM Tris-HCl, pH 9.0. Four micro tubes labeled A, C, G, and T were added with 2 µl of the termination Mix-A, -C, -G, and -T, respectively. After adding equal portions of prereaction mixture to each tubes, the mixture was overlayered with mineral oil. The reaction tubes were then placed in a thermal cycler (Perkin Elmer Cetus) and were preheated at 95°C for 5 min. Depending on the melting temperature and efficiency of each sequencing primers, either or both of the quick cycle or normal cycle of the cycling temperature parameters were employed. The products of cycle sequencing reaction were analyzed within 24 h by electrophoresis on a 33x39.4x0.04 cm polyacrylamide gel in the S2 sequencing apparatus (GIBCO/BRL). The 6% or 8% gel was run at a constant power of 45 watts in TBE buffer. After

fixation and drying of the gels, exposure to X-ray film was done without the use of enhancing screen.

Optimization of the parameters for cycle sequencing analysis of the PCR products

Oligonucleotide primers selected for sequence analysis of the envelope gene vary in size, G+C content, Tm and conservation of their target sequences. It was then necessary to determine for each primer the optimal sequencing parameters. Amplified envelope gene from strain 16681 and one of the 1987 isolates were employed for testing individual primer.

With the quick PCR protocol (denaturation, 95°C for 10 sec; primer annealing and strand extension, 65°C for 10 sec; 20 cycles) and strain D87-499 or D87-1441 template, nice sequencing ladders were obtained with the following primers: S838, S1151, S1699, S2260, C1204, C1763, C1976, C2260 and C2434. Due to a lower melting temperature, sequencing with primer S1380 at the annealing temperature of 55°C for 25 cycles was found to be optimal. Both of the reduction of the annealing temperature to 50-55°C and the addition of the second set of reactions (denaturation of 95°C for 10 sec; and primer annealing and strand extension of 65°C for 60 sec; 10 cycles) was necessary for primer S1974 for some templates. The primer C1518 was successful with the normal cycle program: the first reaction set of denaturation, 95°C for 10 sec; primer annealing, 55°C for 30 sec; and strand extension, 65°C for 60 sec; 20 cycles and the second set of denaturation of 95°C for 10 sec, and primer annealing and strand extension of 65°C for 60 sec; 10 cycles.

Results

Nucleotide and deduced amino acid sequences of envelope gene of strain 16681

Because of the lower fidelity of Taq DNA polymerase, it was important to determine the reliability of dengue envelope sequence obtained by using Taq DNA polymerase. The nucleotide sequence of envelope gene of dengue type 2 virus strain 16681 was determined by cycle sequencing of the PCR products with Taq DNA polymerase and then compared with a published sequence of strain 16681 which has been obtained by direct sequencing of genomic RNA (Blok et al., 1989; 1992). Our 16681 sequence differed by only one nucleotide: at the base position 509, a C base was found instead of U, leading to an amino acid substitution at position 170 (Thr vs. Ile). This comparison revealed a low level of base difference (1 out of 1485) between two sequencing methods and indicated that nucleotide sequences generated by cycle sequencing of PCR products employing Taq DNA polymerase at both amplification and sequence analysis were quite reliable.

Nucleotide and deduced amino acid sequences of the envelope gene of dengue type 2 virus isolated from the 1987 Bangkok epidemic season

The envelope gene of the first set of nine dengue type 2 strains were amplified and sequenced by employing six non-coding strand primers. Regions that yielded ambiguous

sequencing ladders were subjected to reanalysis with corresponding coding strand primers. Due to a high degree of sequence variation between D87-1421 strain and the other eight strains, both strands of strain D87-1421 were sequenced. The envelope gene sequences and translated amino acid sequences of all nine strain were then compared; only the amino acid sequence of selected strains is shown in Fig. 3.

Examination of the envelope protein sequences revealed no deletion nor insertion. There were 12 conserved Cys residues and two potential N-glycosylation sites: Asn-Thr-Thr and Asn-Asp-Thr at the positions 67-69 and 153-155, respectively. Two long hydrophobic stretches, which were separated by a hydrophilic residue and probably served as transmembrane anchor, were present at the carboxy-terminal. The general structure of the envelope protein of these 1987 Bangkok isolates was, thus, identical to those previously observed in dengue type 2 prototypic strains and five 1980 Bangkok strains (Deubel et al., 1986; Hahn et al., 1988; Irie et al., 1989; Blok et al., 1989; Blok et al., 1992).

Comparison of nucleotide sequences revealed that single base substitutions were scattered throughout the entire length of the envelope gene. The actual number of nucleotide differences between each pairs of the nine dengue strains is shown in Table 5. Eight dengue type 2 isolates derived from four grades I and II DHF patients (D87-642, D87-1121, D87-1372, and D87-1441) and four grade III DHF patients (D87-279, D87-499, D87-704, and D87-1040) differed from each others by only 16/1485 bases or less (sequence identity of over 98.9%). Strains D87-642 and D87-1121 were almost identical throughout the gene. In contrast, strain D87-1421 which was isolated from a grade IV DHF patient varied by as many as 90 to 99 bases from any of the other eight strains. The identity of E gene sequence between strain D87-1421 and others ranged from 93.3 to 93.9%. The strain D87-1421 appeared to be quite distinct from the other dengue type 2 viruses found in the 1987 epidemic year.

At the protein level (Table 5), six strains (D87-642, D87-1121, D87-1372, D87-1441, D87-279, and D87-704) were identical in the amino acid sequence of envelope protein. Two other strains (D87-499 and D87-1040) differed from the first six isolates by only one residue (position 162, Ile vs. Val). D87-1421 differed from the rest by 10-11 amino acid residues (97.7-97.9% sequence identity). Amino acid substitutions were located at the following amino acid positions: 64, 96, 141, 162, 164, 181, 203, 308, 346, 484 and 491. Except for two changes that result in acquisition of charged residues (position 203, Asn vs. Asp; position 346, Tyr vs. His), all others substitutions are conservative changes of uncharged residues (7 positions) or negatively-charged residue (position 64, Lys vs. Arg).

Based on the differences detected by oligonucleotide fingerprinting, E and NS1 gene sequence analyses and distribution of antigenic determinants on NS1 protein, dengue type 2 viruses isolated during the 1980 epidemic season in Bangkok have been separated into two groups: the major group, represented by D80-100, PUO-218 and PUO-280, and the minor group, D80-141 and D80-038 (Walker et al., 1988; Blok et al., 1989; Blok et al., 1991). The relative prevalence has been estimated to be about 88 : 12 (Walker et al., 1988). Comparing the variations among the 1987 isolates with those of 1980 (Table 5) showed that members of the

major group of both years were about 97.9-98.5% identical to each others, but they differ from the minor group by up to 4.1-5.7% (Blok et al., 1989). The major group of 1980 was more similar to the major group of 1987 (97.4-98.5% base identity) than to the minor group of 1987 (93.7-94.3% base identity). Similarly, the minor group of 1980 was only 93.5-95.8% identical to the major group of 1987 while they were from 96.4-98.6 identical to the minor group of 1987. Thus, there appears to be a closer genetic relatedness between the major groups of 1980 and 1987, and between the minor groups of 1980 and 1987, respectively.

To examine more fully the possible association of the minor group of the 1987 season with grade IV DHF, we performed nucleotide sequence analysis of 6 and 3 additional isolates from grade I-III and grade IV DHF patients, respectively. All of the additional sequences were very similar (1-2 amino acids substitutions) to the major group of 1987 described above. Therefore, D87-1421 appears to represent a very minor (1 out of 18) subset of dengue type 2 virus population in this epidemic. Furthermore, there is no apparent association of this minor group with grade IV DHF. Analysis of 2 other isolates from Khanjanaburi province (the western region) and Buriram province (the northeastern region) from this epidemic season also revealed that their envelope protein sequences were almost identical to that of the major Bangkok group.

Discussion

The envelope glycoprotein of 18 dengue type 2 viruses isolated from the 1987 epidemic season in Bangkok maintains the structural features that had been observed previously with the prototypic and the 1980 Bangkok strains. Variations occur as amino acid substitutions scattered throughout the protein, sparing the conserved Cys residues and potential N-glycosylation sites. Among the major group, which are consisted of 17 Bangkok isolates and 2 provincial isolates, there are 5 isolates that contain 1 amino acid substitution and only 1 isolate with 2 substitutions when they are compared with the concensus sequence of the group. D87-1421 represents a minor group (1/18 Bangkok isolates; 1/20 total isolates) with 10-11 amino acid substitutions.

The functional significance of amino acid substitution seen in D87-1421 is not yet known. This level of sequence alteration is as high as those observed between 3 pairs of flavivirus virulent strains and their corresponding vaccine candidates. For example, there are 12, 5 and 3 amino acid differences between Asibi and 17D yellow fever viruses, a virulent and an attenuated JE virus, and dengue 2 16681 strain and 16681-PDK-53 attenuated dengue-2 viruses, respectively (Hahn et al., 1987; Nitayaphan et al., 1990; Blok et al., 1992). In the most extreme case, a neurovirulent revertant of yellow fever virus vaccine differs from its parental strain by only 2 amino acids of the envelope protein (Jennings et al., 1994). In D87-1421, two changes that occured in or distal to the carboxy terminal membrane anchor will most likely not affect its function. Of 8-9 changes proximal to the membrane anchor, the majority represents conservative change of mostly uncharged residues and only one negatively charged residue. The substitution at position 181 (Val vs. Ile) has been reported earlier between 16681 and 16681-PDK153 vaccine candidate (Blok et al., 1992). It will be interesting to determine the effect of substitutions at the positions 203 and 346 that result in the acquisition of one positively charged residue and one negatively charged residue, respectively, as it is the loss of two charged residues that has been

implicated in the acquisition of neuroinvasiveness of the yellow fever revertant (Jennings et al., 1994). Recently, a full-length cDNA clone of dengue type 4 virus has been described (Lai et al., 1991). This plasmid-based cDNA clone can be transcribed in vitro, yielding infectious RNA that is capable of initiating replication in host cells. With this vector, it is now possible to introduce single or multiple changes into dengue genome in order to determine the effect of specific mutations on viral behaviours in vitro and in vivo (Bray and Lai, 1991; Pletnev et al., 1992).

Based on the comparison of of 21 dengue type 2 isolates from the 1987 epidemic season, there appears to be no association between amino acid changes with severity of DHF in patients from whom viruses are derived. This has been observed previously in the study of 1980 epidemic in the same city (Blok et al., 1989; Blok et al., 1991). However, it should be noted that these viruses are derived by intrathoracic injection of mosquitoes and subsequent propagation in C6/36 cells; these procedures may have selected against variants which are not capable of high level of replication in the mosquito system. This issue can be studied further by examining sequence variation of PCR-amplified gene derived directly from clinical samples.

There has been examples for dengue virus variants which can be detected only once in the period of several years. TH-SMAN and TH-36 represent dengue type 1 and 2 variants of this type (Shiu et al., 1992). D80-141 and D87-1421 are, however, different in that they are clearly related viruses that are persistent at least during the 7 year period. It will be necessary to continue monitoring the population dynamics of these viruses further as selective forces may in the future favour the expansion of the variant such that they become the predominant species. Meanwhile, it is clear that even in a major epidemic as large as the 1987 Bangkok season, the majority of circulating type 2 viruses are quite homogeneous in their envelope protein sequence. This major group has persisted for a long period of time and they are related to strain 16681 from which a vaccine candidate 16681-PDK-153 has been developed. If the major group continues to predominate, 16681-PDK-153, if proved effective, can be safely implemented at least in Thailand and possibly in adjacent countries.

Impact, Relevance and Technology Transfer

Impact and Relevance: As considerations in the development and implementation of any viral vaccine will include variability of the virus in the community. The finding that dengue viruses that are causing a major epidemic in a given locality at a given point of time are not very different in the envelope pretein will be important to the implementation of the vaccination program by the public health providers in the future. Currently, a dengue type 2 vaccine candidate (16681-PDK-153) is undergoing a phase I/II testing in Thailand together with other three belonging to type 1, 3, and 4. The dengue vaccine candidates has been prepared by a group of investigator at Mahidol University. The type 2 component of this vaccine candidate was derived from strain 16681 which correlates to the major group of dengue type 2 virus in this study. Our finding will also be important to the design of envelope subunit vaccine in the future.

Technology Transfer: The capability to grow, enumerate and characterize viruses of medical

importance by serological method and molecular biological methods constitutes the majority of technology transfered. The molecular biological techniques, including PCR amplification, molecular cloning and nucleotide sequence analysis, are now firmly established in the Department of Microbiology. Among the equipments that are left and functioning are: a thermocycler, an incubator appropriate for insect cell culture, a set of nucleotide sequencing apparatus, a horizontal electrophoresis system and a gradient forming apparatus. Most significantly, the expertise gained in isolating and characterising viral pathogens will be very helpful to our study of other viral disease, such as HIV which is now rampaging Thailand and other developing countries.

Project Activities/Output

1. Meetings attended:

1.1 International Symposium on Dengue and Dengue Hemorrhagic Fever. October 1-3, 1990. ASEAN Institute for Health Development, Mahidol University at Salaya, Thailand (A. Kanjanahaluethai).

1.2 Second Asia-Pacific Congress of Medical Virology. November 17-22, 1991, Ambassador Hotel, Bangkok, Thailand. Paper presented: Lack of an augmented effect of interferon gamma on dengue virus replication in human peripheral blood monocytes (N. Sittisombut); Detection and typing of dengue viruses in the culture fluid by reverse transcriptase polymerase chain reaction (N. Maneekarn).

1.3 Seminar for Asia and the Pacific on "Immunoassay Methods and Labelled DNA Probes in the Diagnosis of Communicable Diseases". Organizer: International Atomic Energy Agency. November 23-27, 1992, the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. Paper presented: Rapid identification of sequence variation in dengue virus type 2 envelope type 2 envelope gene (N. Sittisombut).

1.4 Workshop on the Pathogenesis of Dengue and Dengue Hemorrhagic fever. Organizer: Armed Forces Research Institute of Medical Sciences. December 5, 1992, the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. Paper presented: Effect of IFN- on dengue virus replication in human monocytes (N. Sittisombut).

1.5 XIIIth International Congress for Tropical Medicine and Malaria. November 29-December 4, 1992; Pataya, Thailand. Paper presented: Applications of polymerase chain reaction for the detection and typing of dengue viruses in patients sera (N. Maneekarn).

2. Publication:

2.1 Sittisombut, N., Maneekarn, N., Kanjanahaluethai, A., Kasinrerk, W., Viputtikul, K.,

Supawadee, J. Lack of augmenting effect of interferon-γ on dengue virus multiplication in human peripheral blood monocytes. Submitted.

2.2 Viputtikul, K., Kanjanahaluethai, A., Maneekarn, N., Sittisombut, N. (1993) Improvements and comparison of peroxidase-anti-peroxidase staining method with plaque assay for the titration of dengue type 2 virus. Chiang Mai Medical Bulletin. In press.

2.3 Sittisombut, N. (1994) Molecular biology of dengue viruses. In Current Status of the Knowledge on dengue fever/dengue hemorrhagic fever/ dengue shock syndrome in Thailand. Chapter 3. Etiology and Diagnosis. Faculty of Tropical Medicine, Mahidol University. In press.

Project Productivity

We have not accomplished all of the proposed goals. One of the most interesting part of the project is to compare the viruses derived from DF patients with those from DHF patients. Our problems in recovering the former type of viruses has severely limited the scope and importance of this work. Instead, we have to resort to comparing viruses derived from different severity of DHF patients.

Future Work

From the molecular epidemiological point of view, it will be necessary to continually monitor the changes in the population dynamics of the dengue viruses in Thailand and other parts of Southeast Asia. In later years the dengue type 2 minor variant of 1987 may become predominate or die out. If it becomes predominant, the effect of 16681-PDK-153 vaccine candidate may have to be retested.

Monitoring will be made easier by using PCR amplification of the selected region of the dengue virus genome directly from the viruses present in the patient blood samples, such as envelope gene as in this study, and then determine the sequence variation either by direct nucleotide sequence analysis of the amplified products or hybridization with sequence-specific oligonucleotide probes. In order to maximize the chances of finding variation, it will be desirable to perform limitind dilution of blood sample or cDNA before PCR amplification step. The amplified product can then be subcloned prior to nucleotide sequence analysis.

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Fig. 1. Comparison of PAP method and plaque assay for titration of dengue type 2 virus. Five serially diluted samples of strain 16681 were assayed simultaneously by PAP method and plaque assay on BHK-21 cells and LLC-MK2 cells, respectively. Virus titers from three independent experiments are expressed as ffu/ml (PAP method, filled symbols) or pfu/ml (plaque assay, open symbols).

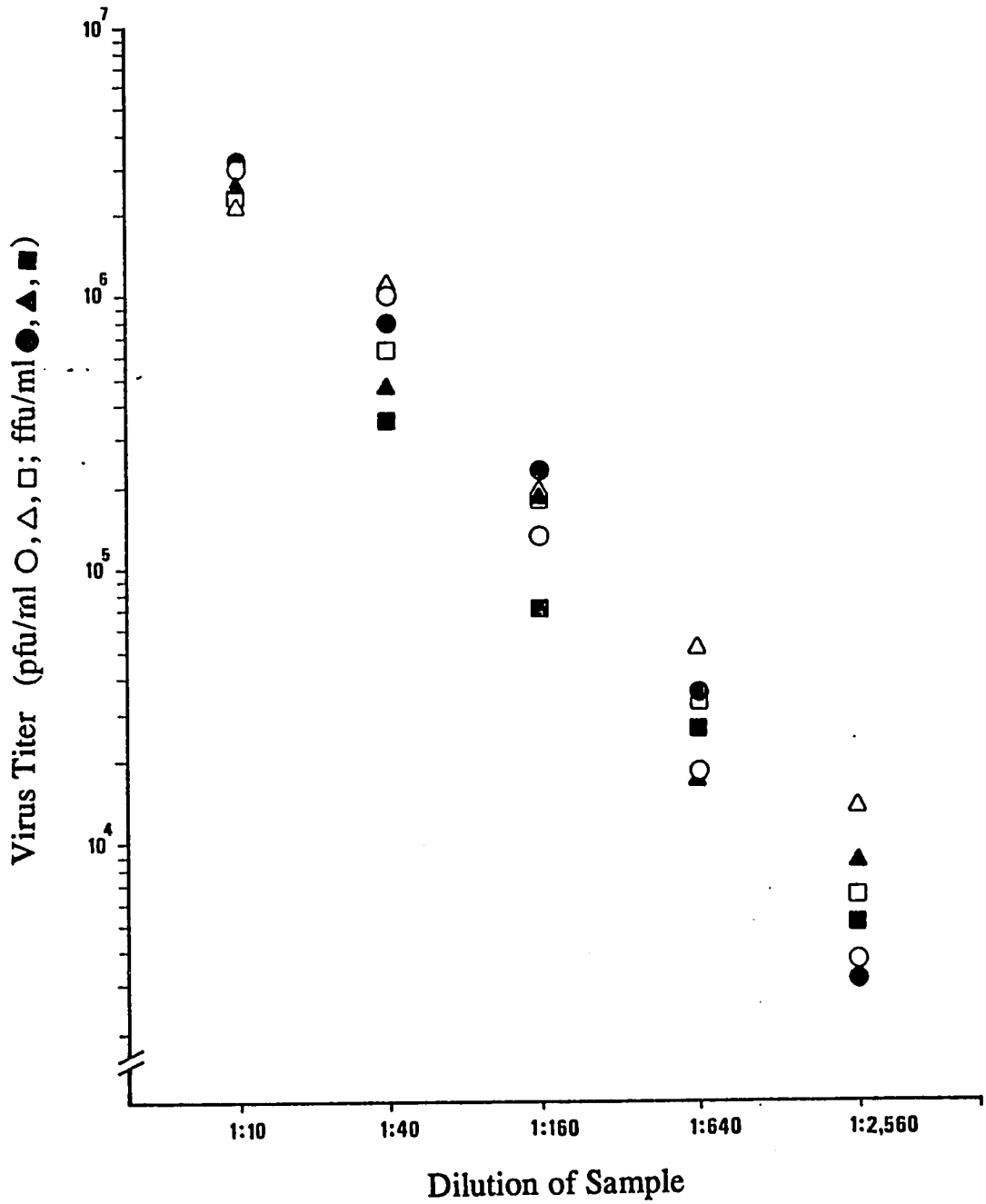

Fig. 2. Effect of IFN-γ on dengue virus replication in monocytes. Peripheral blood monocytes from dengue immune donors were treated with various concentrations of recombinant IFN-γ (□, ◊, △) or leukocyte IFN-γ (■, ♦, ▲) for 24 h and then infected with strain 16681 at the moi of 5 ffu/cell in the absence (A) or presence (B) of 10^{-5} dilution of 4G2. Virus yields in the culture supernatants were determined at 24 h after infection.

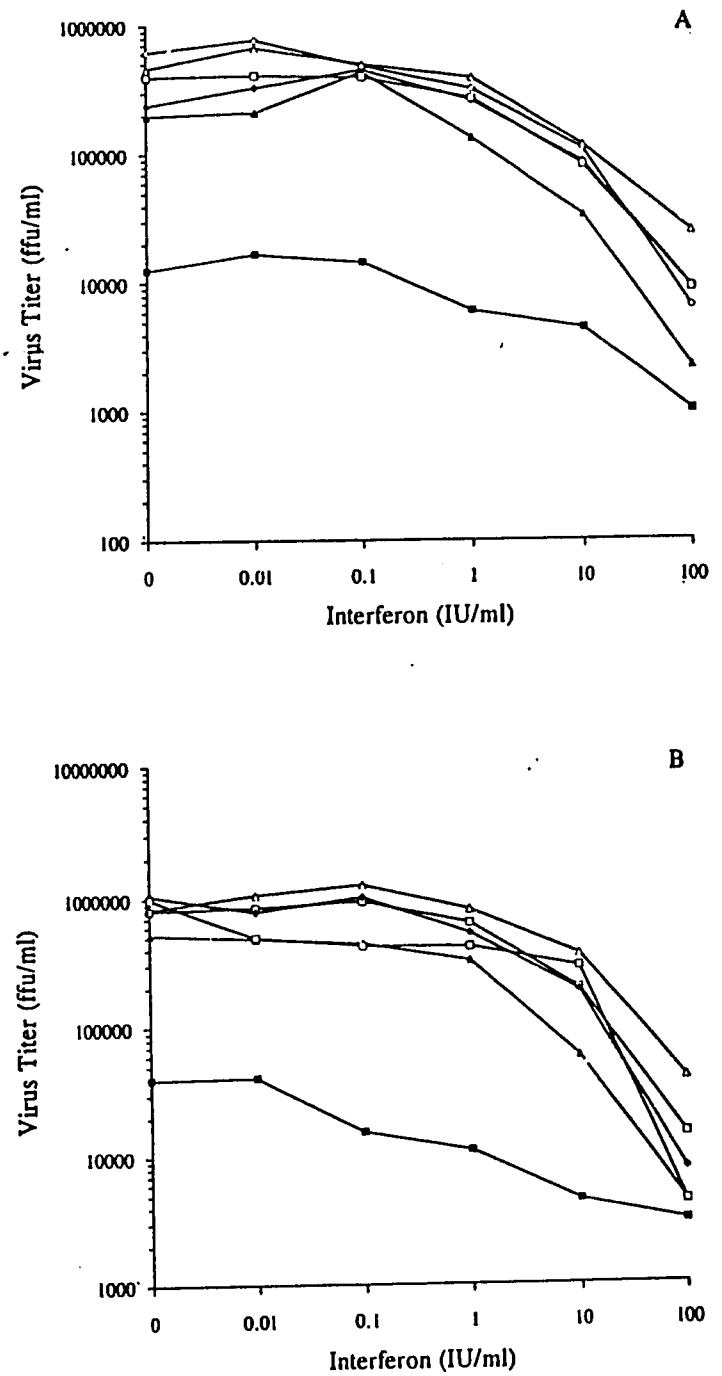

Fig. 3. Deduced amino acid sequence of envelope protein of thirteen dengue type 2 viruses. Only the amino acids that differ from D87-642 sequence have been indicated for the other viruses. The two potential glycosylation sites are underlined. The sequences of D80-141, D80-038, PUO-280, and D80-100 are from Blok et al. (1989).

```
D87-642     MRCIGISNRD FVEGVSGGSW VDIVLEHGSC VTTMAKNKPT LDFELIKTEA 50
D87-1121    .......... .......... .......... .......... ..........
D87-1372    .......... .......... .......... .......... ..........
D87-1441    .......... .......... .......... .......... ..........
D87-279     .......... .......... .......... .......... ..........
D87-499     .......... .......... .......... .......... ..........
D87-704     .......... .......... .......... .......... ..........
D87-1040    .......... .......... .......... .......... ..........
D87-1421    .......... .......... .......... .......... ..........
D80-141     .......... .......... .......... .......... ..........
D80-038     .......... .......... .......... .......... ..........
PUO-280     .......... .......... .......... .......... ..........
D80-100     .......... .......... .......... .......... ..........
16681       .....M.... .......... .......... .......... ..........

D87-642     KQPATLRKYC IEAKLTNTTT ESRCPTQGEP SLKEEQDKRF VCKHSMVDRG 100
D87-1121    .......... .......... .......... .......... ..........
D87-1372    .......... .......... .......... .......... ..........
D87-1441    .......... .......... .......... .......... ..........
D87-279     .......... .......... .......... .......... ..........
D87-499     .......... .......... .......... .......... ..........
D87-704     .......... .......... .......... .......... ..........
D87-1040    .......... .......... .......... .......... ..........
D87-1421    .......... ...R...... .......... .......... .....I....
D80-141     .......... .......... .......... ..N....... ..........
D80-038     .......... .......... .......... ..N......L ..........
PUO-280     .......... M......... .......... ..N....... ..........
D80-100     .......... .......... .......... ..N......L ..........
16681       .......... .......... .......... ..N....... ..........

D87-642     WGNGCGLFGK GGIVTCAMFT CKKNMEGKIV QPENLEYTIV VTPHSGEEHA 150
D87-1121    .......... .......... .......... .......... ..........
D87-1372    .......... .......... .......... .......... ..........
D87-1441    .......... .......... .......... .......... ..........
D87-279     .......... .......... .......... .......... ..........
D87-499     .......... .......... .......... .......... ..........
D87-704     .......... .......... .......... .......... ..........
D87-1040    .......... .......... .......... .......... ..........
D87-1421    .......... .......... .......... .......... I.........
D80-141     .......... .......... .......... .......... I.........
D80-038     .......... .......... .......... .......... I.........
PUO-280     ......F... .......... .......... .......... ..........
D80-100     .......... .......... .......... .......... ..........
16681       .......... .........R ........V. .......... I.........
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D87-642     VGNDTGKHGK EIKVTPQSSI TEAELTGYGT VTMECSPRTG LDFNEMVLLQ 200
D87-1121    .......... .......... .......... .......... ..........
D87-1372    .......... .......... .......... .......... ..........
D87-1441    .......... .......... .......... .......... ..........
D87-279     .......... .......... .......... .......... ..........
D87-499     .......... .V........ .......... .......... ..........
D87-704     .......... .......... .......... .......... ..........
D87-1040    .......... .V........ .......... .......... ..........
D87-1421    .......... ...I...... .......... I......... ..........
D80-141     .......... ...I...... .......... .......... ..........
D80-038     .......... .......... .......... .......... ..........
PUO-280     .......... .......... A......... .......... ..........
D80-100     .......... .......... .......... .........V ..........
16681       .......... ...I...... .......... .......... ..........

D87-642     MENKAWLVHR QWFLDLPLPW LPGADTQGSN WIQKETLVTF KNPHAKKQDV 250
D87-1121    .......... .......... .......... .......... ..........
D87-1372    .......... .......... .......... .......... ..........
D87-1441    .......... .......... .......... .......... ..........
D87-279     .......... .......... .......... .......... ..........
D87-499     .......... .......... .......... .......... ..........
D87-704     .......... .......... .......... .......... ..........
D87-1040    .......... .......... .......... .......... ..........
D87-1421    ..D....... .......... .......... .......... ..........
D80-141     ..D....... .......... .......... .......... ..........
D80-038     ..D....... .......... .......... .......... ..........
PUO-280     .......... .......... .......... .......... ..........
D80-100     .......... .......... .......... .......... ..........
16681       .......... .......... .......... .......... ..........

D87-642     VVLGSQEGAM HTALTGATEI QMSSGNLLFT GHLKCRLRMD KLQLKGMSYS 300
D87-1121    .......... .......... .......... .......... ..........
D87-1372    .......... .......... .......... .......... ..........
D87-1441    .......... .......... .......... .......... ..........
D87-279     .......... .......... .......... .......... ..........
D87-499     .......... .......... .......... .......... ..........
D87-704     .......... .......... .......... .......... ..........
D87-1040    .......... .......... .......... .......... ..........
D87-1421    .......... .......... .......... .......... ..........
D80-141     .......... .......... .......... .......... ..........
D80-038     .......... .......... .......... .......... ..........
PUO-280     .......... .......... .......... .......... ..........
D80-100     .......... .......... .......... .......... ..........
16681       .......... .......... .......... .......... ..........

D87-642     MCTGKFKVVK EIAETQHGTI VIRVQYEGDG SPCKIPFEIM DLEKRYVLGR 350
D87-1121    .......... .......... .......... .......... ..........
D87-1372    .......... .......... .......... .......... ..........
D87-1441    .......... .......... .......... .......... ..........
D87-279     .......... .......... .......... .......... ..........
D87-499     .......... .......... .......... .......... ..........
D87-704     .......... .......... .......... .......... ..........
D87-1040    .......... .......... .......... .......... ..........
D87-1421    .......I.. .......... .......... .......... .....H....
D80-141     .......I.. .......... .......... .......... .....H....
D80-038     .......I.. .......... .......... .......... .....H....
PUO-280     .......... .......... .......... .......... .....H....
D80-100     .......... .......... .......... .......... .....H....
16681       .......... .......... .......... .......... .....H....
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D87-642     LITVNPIVTE KDSPVNIEAE PPFGDSYIII GVEPGQLKLN WFKKGSSIGQ 400
D87-1121    .......... .......... .......... .......... ..........
D87-1372    .......... .......... .......... .......... ..........
D87-1441    .......... .......... .......... .......... ..........
D87-279     .......... .......... .......... .......... ..........
D87-499     .......... .......... .......... .......... ..........
D87-704     .......... .......... .......... .......... ..........
D87-1040    .......... .......... .......... .......... ..........
D87-1421    .......... .......... .......... .......... ..........
D80-141     .......... .......... .......... .......... ..........
D80-038     .......... .......... .......... .......... ..........
PUO-280     .......... .......... .......... .......... ..........
D80-100     .......... .....K.... .......... .......... ..........
16681       .......... .......... .......... .......... ..........

D87-642     MFETTMRGAK RMAILGDTAW DFGSLGGVFT SIGKALHQVF GAIYGAAFSG 450
D87-1121    .......... .......... .......... .......... ..........
D87-1372    .......... .......... .......... .......... ..........
D87-1441    .......... .......... .......... .......... ..........
D87-279     .......... .......... .......... .......... ..........
D87-499     .......... .......... .......... .......... ..........
D87-704     .......... .......... .......... .......... ..........
D87-1040    .......... .......... .......... .......... ..........
D87-1421    .......... .......... .......... .......... ..........
D80-141     .......... .......... .......... .......... ......T...
D80-038     .......... .......... .......... .......... ..........
PUO-280     .......... .......... .......... .......... ..........
D80-100     .......... .......... .......... .......... ..........
16681       .......... .......... .......... .......... ..........

D87-642     VSWTMKILIG VIITWIGMNS RSTSLSVSLV LVGIVTLYLG VMVQA      495
D87-1121    .......... .......... .......... .......... .....
D87-1372    .......... .......... .......... .......... .....
D87-1441    .......... .......... .......... .......... .....
D87-279     .......... .......... .......... .......... .....
D87-499     .......... .......... .......... .......... .....
D87-704     .......... .......... .......... .......... .....
D87-1040    .......... .......... .......... .......... .....
D87-1421    .......... .......... .......... ...V...... A....
D80-141     .......... .......I.. .......... ...V...... A....
D80-038     .......... .......... .......... ...V...... A..L.
PUO-280     .......... .......... .......P.. .......... .....
D80-100     .......... .......I.. .......... .......... .....
16681       .......... .......... .......T.. .......... .....
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Table 1. Characteristic of the dengue type 2 virus isolated from Bangkok in 1987.

Virus strain	Grade of DHF	Sex*	Address	Date of virus isolation
D87-642	I	F	1875 Praram 4 Rd., Lumpinee, Pratoomwan	Jun. 15
D87-1121	I	M	1805/109 Soi Vudruak-bamru, Bangkoknoi	Sep. 4
D87-1372	I	M	13 Soi Charoennivas, Romgmaung, Patumvan	Nov. 15
D87-1441	II	M	65/6 Soi Charoennivas, Praram 1 Rd.	Dec. 6
D87-1283	II	F	35/1 Klongluang. Pratoomtani	Oct. 26
D87-279	III	F	666 Soi Payanark, Prayatai	Mar. 24
D87-499	III	F	186 Soi Ratpan, Rachaparot Rd., Prayatai	May. 19
D87-704	III	M	1875 Lumpini, Patumvan	Jun. 24
D87-1040	III	M	426 Tepsirin, Promprab	Aug. 21
D87-1421	IV	M	990/6 Soi Vudpaingean, Bangklo	Nov. 28
D87-738	IV	M	5/5 M 6 Klongluang, Pratoomtani	Jun. 29

* Sex : F, Female ; M, Male.

Table 2. Stimulation of IFN-γ production by 7 low passage dengue type 2 viruses from the 1987 Bangkok epidemic.

Strain	DHF severity	IFN-γ level (pg/ml)	
	(Grade)	Experiment 1	Experiment 2
D87-1121	I	60	160
D87-1441	II	2,150	Not done
D87-1283	II	140	140
D87-279	III	980	625
D87-1040	III	200	120
D87-1421	IV	430	190
D87-738	IV	750	680
16681	IV	2,240	3,800

Table 3. Kenetics of IFN-γ production following stimulation of lymphocyte with 2 low passage dengue isolates from the 1987 Bangkok epidemic.

Day	IFN-γ level (pg/ml)	
	D87-1441	D87-1421
3	430	120
4	750	160
5	1,000	200
6	1,200	230
7	2,150	430
8	1,750	430

Table 4. Nucleotide sequences of synthetic primers for sequence analysis.

Primer	Sequence	Position	Tm
S838	GGG TAC CAT AAT GGC AGC AAT CCT GG	838-859	66
S1380	GCA TGC GGT AGG TAA TGA CAC A	1380-1401	62
S1151	CGC GCT GCC CAA CAC AAG GG	1151-1170	66
S1699	TCT CAA GAA GGG GCC ATG CA	1699-1718	61
S1974	TGT CTT AGG TCG CCT GAT TA	1974-1993	58
S2260	GCA ATC TAT GGA GCT GCC TTT	2260-2280	60
C1204	GGA GTG TTT GCA GAC GAA	1221-1204	57
C1518	CAT CTG CAG CAA CAC CAT CTC	1539-1519	64
C1518	CAT CTG CAG CAG CAC CAT CTC	1539-1519	63
C1530	GCT TTG TCT TTC ATT TGC AG	1550-1531	56
C1763	GAG ATG TCC TGT GAA GAG TAA GT	1785-1763	60
C1976	TGT AAT CAG GCG ACC TAA GA	1995-1976	58
C2260	AAA GGC AGC TCC ATA GAT TGC	2280-2260	60
C2434	TTT TCC AGC TCA CAA CGC AAC C	2449-2428	62

Table 5. Number of nucleotide differences (above diagonal line) and amino acid differences (below diagonal line) of envelope gene/protein among dengue type 2 viruses isolated from Bangkok in 1987

	642	1121	1372	1441	279	499	704	1040	1421	16681	141	038	280	100	218
D87-642	-	4	3	2	5	10	0	14	93	45	90	65	26	30	32
D87-1121	0	-	5	4	7	12	4	16	95	47	92	67	28	32	34
D87-1372	0	0	-	1	6	11	3	13	92	44	89	64	27	31	33
D87-1441	0	0	0	-	5	10	2	12	93	45	90	65	26	30	32
D87-279	0	0	0	0	-	7	5	11	90	42	87	62	23	27	29
D87-499	1	1	1	1	1	-	10	10	95	47	92	67	28	32	34
D87-704	0	0	0	0	0	1	-	14	93	45	90	65	26	30	32
D87-1040	1	1	1	1	1	0	1	-	99	51	96	71	32	36	38
D87-1421	10	10	10	10	10	11	10	11	-	81	21	53	94	94	85
DV-16681	8	8	8	8	8	9	8	9	12	-	76	61	44	49	33
D80-141	9	9	9	9	9	10	9	10	6	10	-	48	84	82	82
PUO-038	9	9	9	9	9	10	9	10	7	11	5	-	61	61	61
PUO-280	5	5	5	5	5	6	5	6	13	9	12	11	-	22	32
D80-100	7	7	7	7	7	8	7	8	14	10	10	9	8	-	34
PUO-218	3	3	3	3	3	4	3	4	11	5	9	8	5	5	-