INFECTION OF DENGUE 2 VIRUS STRAINS ISOLATED FROM PATIENTS EXHIBITING DIFFERENT DISEASE SEVERITIES TO HUMAN PERIPHERAL BLOOD LEUKOCYTES AND PRODUCTION OF CYTOKINES IN THE INFECTED CULTURE SUPERNATANT

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Abstract. Three strains of type 2 dengue virus (DV-2), which had been isolated from patients exhibiting different disease severity, were inoculated to primary culture of human peripheral blood leukocytes from 3 healthy donors. The percentage of dengue antigen positive cells was highest for the strain isolated from a case of dengue shock syndrome, followed by the strain isolated from a case of dengue hemorrhagic fever, and lowest for the strain isolated from a mild case of dengue fever (DF). Generally, similar trend was observed for the amount of some cytokines released into infected culture supernatant, such as interleukin 6, and tumor necrosis factor-alpha. However, such a trend was not observed for interleukin 1 beta.

INTRODUCTION

Dengue hemorrhagic fever (DHF) is a serious epidemic virus disease in tropical and subtropical regions, caused by 4 different serotypes of dengue viruses. Several hypotheses have been postulated to explain the occurrence of DHF but the pathogenic mechanisms leading to develop severe clinical manifestations of the disease are still poorly understood. It has been suggested that DHF results from antibody-dependent enhancement of virus replication in the secondary infection by heterotypic dengue virus which is different from the primary infection (Halstead, 1988; Mady *et al*, 1991). It has also been proposed that the severity of the disease could be related to the virulence of the infecting dengue virus strain (Rosen, 1977). Mangada and Igarashi (1998) showed potential genetic determinants of dengue virus virulence by sequencing the entire genomes of several DV-2 strains isolated from patients exhibiting different disease severity in the same epidemic area during the same epidemic season. They showed several strain-specific amino acid replacements scattered mostly in the nonstructural protein gene region.

The major pathophysiological hallmark that distinguishes DHF from dengue fever (DF) is plasma leakage due to increased vascular permeability, which could be related to the release of immune mediator such as cytokines. Recently, several reports have described elevated level of cytokines in dengue patients (Hober *et al*, 1993; 1996; Kurane *et al*, 1992; Iyngkaran *et al*, 1995).

Moruo *et al* (1992) found that IL-6 increased permeability of the bovine vascular endothelial cell (BVEC) *in vitro*, by treating with IL-6 at 100 ng/ml for 21 hours as measured by increased permeability of flourescein isothiocyanate (FITC)-labeled albumin across the cell monolayer. Anderson *et al* (1997) supported this result with their finding that human umbilical vein endothelial cells become activated when exposed to culture fluids from dengue virus-infected peripheral blood monocytes.

The objective of this study was to compare the phenotypic characteristics of DV-2 strains isolated from patient exhibiting different disease severity, and to support the findings of molecular differences among these strains. In this study, we compared the infection rate of these DV-2 strains to human peripheral blood leukocytes (PBL), and levels of some cytokines released into the infected culture supernatant.

MATERIALS AND METHODS

Virus preparation

DV-2 strains used in this study were ThNH-p11/93, ThNH-28/93 and ThNH-7/93, which had been isolated from the sera of patients in Nakhon Phanom Provincial Hospital, northeast Thailand in 1993 (Mangada and Igarashi, 1998). ThNH-p11/93 was isolated from a male DF outpatient. ThNH-28/93 was isolated from a hospitalized male patient with

clinical diagnosis of DHF grade II. ThNH-7/93 was isolated from a hospitalized female patient showing DSS.

Virus infectivity assay

The virus strains were propagated in C6/36 mosquito cell line, and their infectivity was determined by focus formation in BHK-21 cells (Okuno *et al,* 1978). Approximately $1x10^4$ cells/ml of BHK-21 cells were inoculated into a 96 well flat bottom microplate (Nunc, Denmark) using 0.1 ml/well, and incubated at 37°C for 2 days in 5% CO_2 atmosphere using Eagle's medium supplemented with 10% fetal calf serum (FCS). The virus specimens were serially diluted in 10-fold steps in the virus diluent of 5% FCS in Eagle's medium. Growth medium was removed from confluent cell cultures and 50 µl of the diluted virus specimens were inoculated into each of the duplicate wells. Adsorption was carried out for 2 hours at 37°C in 5% CO_2 atmosphere with agitation at 30 minute intervals. The cultures were then covered with 100 µl/well of the overlay medium which contained 0.5 % methyl cellulose 4000 in the maintenance medium (2% FCS in Eagle's medium). The plate was incubated at 37°C for 3 days in 5% CO_2 atmosphere. Foci of the virus-infected cells were visualized by immunoperoxidase staining as follows. The overlay medium was removed, the plate was rinsed with PBS, and the cells were fixed with 5% formaldehyde in PBS for 20 minutes. After rinsing with PBS, the cells were permealized with 1% Nonidet P-40 in PBS by incubating at room temperature for 20 minutes, followed by blocking with Block Ace (Yukijirushi, Japan). After rinsing, the plate was successively reacted with DHF patient's serum at 1:500 dilution, and horseradish peroxidase (HRPO)-conjugated anti-human IgG (Cappel, USA) at 1:500 dilution for 60 minutes each. The HRPO reaction was carried out for 5 minutes using the substrate solution containing 0.5 mg/ml 3,3´-diaminobenzidine tetrahydrochloride (Sigma Chemicals, USA) and 0.02% H_2O_2. The plate was rinsed with tap water, and brown foci of virus infected cells were counted using an ordinary light microscope. Virus infectivity titer was expressed as focus forming units (FFU/ml), and in the following ranges in this study: 1.5 - 8.5 x 10^6 FFU/ml for ThNH-p11/93, 1.8 - 18 x 10^6 FFU/ml for ThNH-28/93, and 1.36 - 39 x 10^6 FFU/ml for ThNH-7/93, respectively.

Preparation of human PBL

Peripheral blood specimens were obtained from 3 healthy adult donors who had previously been infected with dengue virus type 1 (donor 1), type 3 (donor 2) or inapparent infection (donor 3). PBL were separated by Lymphoprep gradient centrifugation (Nycomed Pharma AS, Norway). Briefly, 10 ml of the heparinized venous blood from each donor was mixed with an equal volume of RPMI 1640 medium (GIBCO BRL, USA). The mixture was overlaid onto 15 ml of Lymphoprep, and centrifuged at 1,500 rpm at room temperature for 30 minutes. PBL were collected and washed once with Hanks' balanced salt solution (HBSS, Bio Whittaker, USA) to remove Lymphoprep and platelet. After counting the cell density, the PBL suspension was divided into 4 aliquots according the experimental design. Each aliquot of PBL was washed again with HBSS by centrifugation, and the pellet was resuspended with each of the 3 strains of DV-2 as infected C6/36 cell culture fluid or uninfected C6/36 cell culture fluid as negative control.

Infection of human PBL with dengue virus

Three different strains of DV-2 were incubated with 1:10,000 diluted each donor's serum, which is expected to contain anti-dengue virus antibodies, at 37°C for 1 hour (Kurane, 1987). PBL were infected with DV-2 strains at input multiplicity of infection of 10 FFU/cell by incubating at 37°C for 2 hours in 5% CO_2 atmosphere. Infected cells were washed twice with HBSS, and cultured at concentration of $1x10^6$ cells/ml in the medium which consisted of 6 parts of AIM-V medium (GIBCO BRL, USA), 3 parts of Iscove's modified Dulbecco's medium (GIBCO BRL, USA), and 1 part of heat-inactivated AB serum (Sigma Chemicals, USA). Controls were set up using uninfected C6/36 cell culture fluid instead of DV-2. At various time after infection, 0.2 ml aliquot of the PBL culture was harvested and centrifuged at 1,500 rpm for 5 minutes at 4°C. Supernatants were collected and assayed for cytokines levels and virus infectivity. The cell pellet was stained for DV-2 antigens by the indirect immunofluorescence. The first antibody was anti-DV-2 mouse hyperimmune ascitic fluid and the second antibody was FITC-conjugated sheep anti-mouse IgG (Cappel, USA).

Assay of cytokine levels

Human TNF-α, human IL-1β and human IL-6 levels in the infected PBL culture supernatant were assayed by the ELISA kits, according to the manufacturer's instruction (Endogen, USA).

Statistical analysis

Statistical difference was examined by the Student's *t*-test and the chi-square test.

RESULTS

The percentage of PBL infected with DV-2 strains

Fig 1 shows percentages of PBL possessing DV-2 antigens as revealed by the indirect immunofluorescence from 1 to 4 days after infection (Halstead *et al*, 1983). DV-2 antigen-positive cells were detected as early as 3 hours after infection for all virus strains. The percentage was different from donor to donor even for the same DV-2 strain, however, the percentage was highest for the DSS strain (ThNH-7/93), followed by the DHF strain (ThNH-28/93), and lowest for the DF strain (ThNH-p11/93) for all donors. The observed difference among the DV-2 antigen positive cells depending on the infecting DV-2 strains was statistically significant. While, the infectivity assay of DV-2 in the infected PBL culture supernatant could not provide meaningful results, because BHK21 cells used for focus assay were apparently destroyed by incubation with the maintenance medium of the infected PBL.

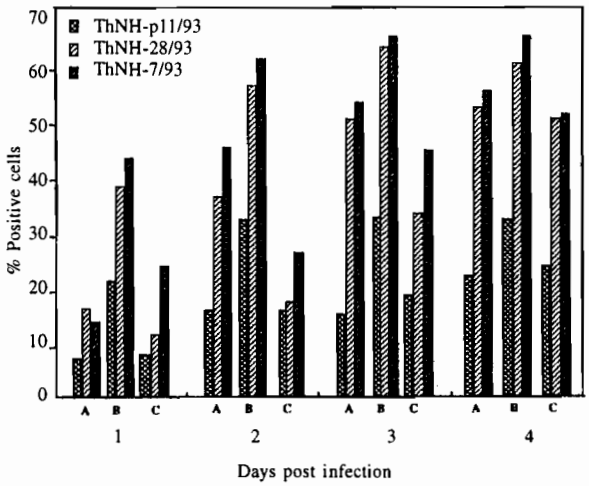

Fig 1–Percentages of PBL infected with DV-2 strains: ThNH-p11/93, ThNH-28/93, and ThNH-7/93, 1-3 days after infection, using the PBL from 3 donors (A, B, and C).

Amount of cytokines in the infected PBL culture supernatant

Fig 2 shows the amount of IL-6 in the culture supernatant of PBL infected with DV-2 strains or uninfected control. Significant amount of IL-6 was produced in the infected PBL cultures compared with the uninfected control, and the level at 12 hours after infection was highest for ThNH-7/93 strain, followed by ThNH-28/93, and lowest for ThNH-p11/93 strain. At 24 hours after infection, the levels of IL-6 were almost same for ThNH-7/93 and ThNH-28/93 strains, but the level by ThNH-p11/93 strain remained at the lowest. These IL-6 levels were maintained relatively stable until the end of experiment.

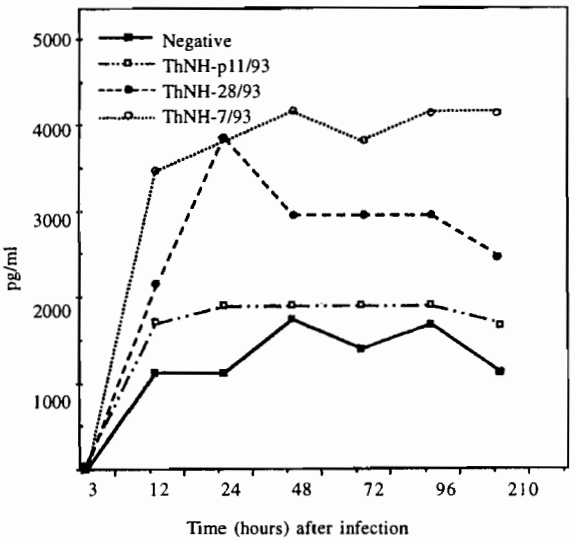

Fig 2–Amount of IL-6 secreted from PBL infected with DV-2 strains: ThNH-p11/93, ThNH-28/93, ThNH-7/93, at various time after infection.

Although, IL-1β was under detectable level at 3 hours after infection, it reached to the maximal level at 12 hours after infection, and then gradually decreased (Fig 3). The maximal level at 12 hours after infection was 700 pg/ml for both ThNH-7/93 and ThNH-p11/93 strains, 370 pg/ml for ThNH-28/93 strain, and 180 pg/ml for uninfected control, respectively. Throughout the observation period, the IL-1b level produced by ThNH-28/93 strain was always lower than those by ThNH-p11/93 or ThNH-7/93 strain, and the levels by ThNH-p11/93 were higher than those by ThNH-7/93 at 24 and 48 hours after infection. Therefore, no significant correlation was found between IL-1β level produced by a particular DV-2 strain and disease severity of the patient from whom that strain was isolated.

The time course of TNF-α production in the culture supernatant of PBL infected with DV-2 strains was shown in Fig 4. The amount of TNF-α from PBL infected with DV-2 was higher than the negative control, and was detected at its maximum level after 12 hours of infection followed by decrease to undetectable level at 72 hours after infection. At 12 hours after infection, the amount of TNF-α was almost

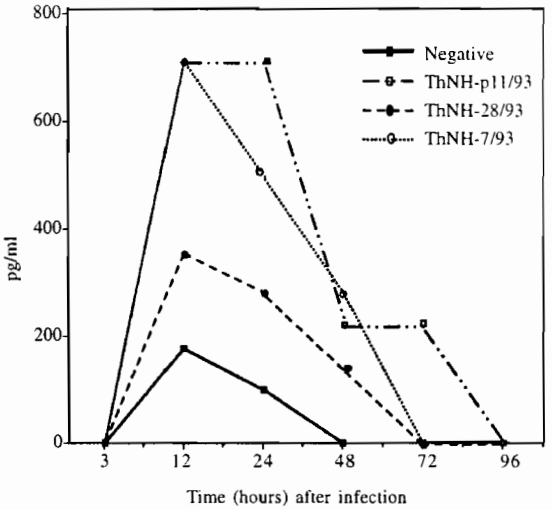

Fig 3–Amount of IL-1β secreted from PBL infected with DV-2 strains: ThNH-p11/93, ThNH-28/93, ThNH-7/93, at various time after infection.

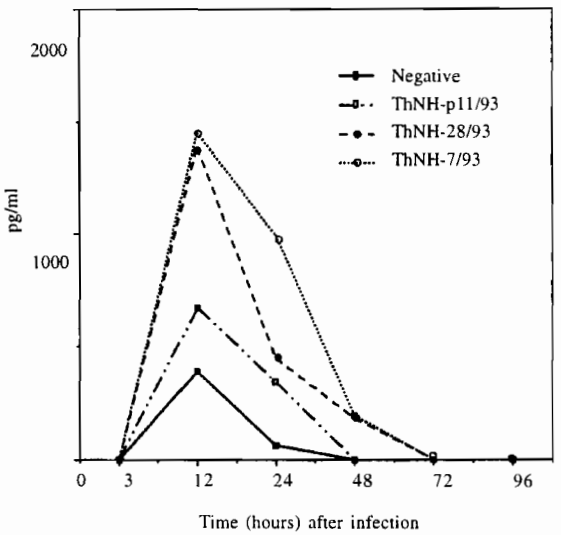

Fig 4–Amount of TNF-α secreted from PBL infected with DV-2 strains; ThNH-p11/93, ThNH-28/93, ThNH-7/93, at various time after infection.

same for the cells infected with ThNH-28/93 and ThNH-7/93, but lowest for ThNH-p11/93 strain.

Our results indicated that the amount of IL-6 and TNF-α secreted by PBL infected with different DV-2 strains roughly paralleled with the clinical severity of the patient from whom each DV-2 strain was isolated.

DISCUSSION

Although most reports on dengue virus infection have used the adherent fraction of PBL (Hotta *et al*, 1984; Wiharta *et al*, 1994; Anderson *et al*, 1997), we used PBL containing both non-adherent and adherent fractions in this study. Kurane *et al* (1990) demonstrated that DV-2 readily establishes persistent infection in human T, B and myelomonocytic cell lines *in vitro*. It has been reported that human B cells and some B cell lines possess FcγRII similar to human monocytes.

To increase the number of infected cell by DV-2, PBL were infected with the virus which was preincubated with each respective donor's serum at final dilution of 10^{-4}, which presumed to contain anti-dengue antibodies, at 4°C for 1 hour. It was expected that such pre-incubation enhanced dengue virus infection through antibody-dependent enhancement (ADE) by subneutralizing levels of antibodies (Halstead 1988; Mady *et al*, 1991).

In this study, the percentage of DV-2 antigen positive PBL appeared to correlate with the disease severity of the patients from whom each strain was isolated. The percentage of DV-2 positive PBL infected with the isolate associated with mild disease (ThNH-p11/93) was consistently less than those infected with the isolates from DHF grade II (ThNH-28/93) or DSS (ThNH-7/93). When we observed the number of DV-2 antigen-positive cells after 3 hours of infection to see the early stage of virus infection, the number of PBL infected with DF strain (ThNH-p11/93) was less than those infected with the strains isolated from DHF (ThNH-28/93) and DSS (ThNH-7/93). The results indicated significant difference among DV-2 strains in term of their intrinsic infecting as well as replication capability.

TNF-α, IL-6 and IL-1β are cytokines produced by monocytes and macrophages which are involved in various pathological phenomena such as septic shock and inflammation (Tracey *et al*, 1988; Hack *et al*, 1989). Recently, Hober *et al* (1993) reported elevated value of TNF-α, IL-6 and IL-1β in sera of dengue patients.

Our results showed that the amount of IL-6, TNF-α, and IL-1β from PBL infected with DV-2 was higher than negative control, indicating that DV-2 induced PBL to release these cytokines. The correlation was observed between the amount of IL-6 and TNF-α with the disease severity of the patients from whom each strain was isolated, but such correlation was not clearly observed for IL-1β. The

amount of IL-6 remained at relatively stable level until the end of the experiment, but the levels of TNF-α and IL-1β decreased relatively rapidly after 12 hours of infection. Schindler *et al* (1990) reported that IL-6 suppressed TNF-α, and IL-1β from PBMC induced by lipopolysaccharide or phytohemagglutinin. This suppression was also observed on the level of transcription. Suppression factor by IL-6 might be responsible for the decreased amounts of TNF-α and IL-1β in this study. IL-6 could play a major role in the pathogenesis of hemorrhage and shock, through mediating increased permeability of endothelial cells (Mauro *et al,* 1992). Relatively stable level of IL-6 until the end of experiment in this study may be responsible for above mention phenomena.

The pathogenesis of DHF may be complex, involving virus and host factors. Our preliminary observations suggest that viral factor including infectivity for entry into PBL and intrinsic replication properties in PBL might be responsible for the disease severity. These data should be supported by further studies using more numbers of virus strains and PBL from various donors in order to correlate *in vitro* growth capability with disease severity of dengue. Following two interesting questions remain to be elucidated in order to understand the pathogenesis of dengue hemorrhagic fever:

1. Are amino acids differences among the DV-2 strains (ThNH-p11/93, ThNH-28/93 and ThNH-7/93) responsible for their infectivity and intrinsic replication properties in PBL?

2. Does IL-6 produced by PBL infected with ThNH-28/93 (DHF grade II) or ThNH-7/93 (DSS) causes increased endothelial permeability?

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REFERENCES

Anderson R, Wang S, Osiowy C, Issekutz AC. Activation of endothelial cells via antibody-enhanced dengue virus infection on peripheral blood monocytes. *J Virol* 1997; 71: 226-32.

Hack CE, Degroot ER, Feltbersma RJF, *et al.* Increased plasma levels of interleukin-6 in sepsis. *Blood* 1989; 74: 1704-10.

Halstead SB, Larsen K, Kliks S, *et al.* Comparison of P388D1 mouse macrophage cell line and human monocyte for assay of dengue-2 infection enhancing antibodies. *Am J Trop Med Hyg* 1983; 32: 157-63.

Halstead SB. Pathogenesis of dengue:challenges to molecular biology. *Science* 1988; 288: 476-81.

Hober D, Poli L, Roblin B, *et al.* Serum levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin 1b (IL-1b) in dengue-infected patients. *Am J Trop Med Hyg* 1993; 48: 324-31.

Hober D, Delonnoy AS, Benyoucef S, *et al.* High levels of sTNFRp75 and TNF-α in dengue infected patients. *Microbiol Immunol* 1996; 40: 569-73.

Hotta H, Wiharta AS, Hotta S , *et al.* Dengue type 2 virus infection in human peripheral blood monocyte cultures. *Microbiol Immunol* 1984; 28: 1099-109.

Iyngkaran N, Yadav M, Sinniah M. Augmented inflammantory cytokines in primary dengue infection progressing to shock. *Singapore Med J* 1995; 36: 218-21.

Kurane I, Innis FA. Induction of interferon α from human lymphocytes by autologous, dengue virus infected monocytes. *J Exp Med* 1987; 166: 999-1010.

Kurane I, Kontny U, Janus J, *et al.* Dengue-2 virus infection of human mononuclear cell lines and establishment of persistent infections. *Arch Virol* 1990; 110: 91-101.

Kurane I, Innis, FA, Immunity and immunopathology in dengue virus infection. *Semin Immunol* 1992; 4: 121-7.

Mady BJ, Erbe DV, Kurane I, *et al.* Antibody-dependent enhancement of dengue virus infection mediated by bispecific antibodies against cell surface molecules other than Fc g receptors. *J Immunol* 1991; 147: 3139-44.

Mangada MNM, Igarashi, A. Sequences of terminal noncoding regions from four dengue-2 viruses isolated from patients exhibiting different disease severities. *Virus Gene* 1997; 14: 5-12.

Mangada MNM, Igarashi A. Molecular and *in vitro* analysis of eight dengue type 2 viruses isolated from patients exhibiting different disease severities. *Virology* 1998; 244: 458-66.

Maruo N, Morita I, Shirao M, *et al.* IL-6 increases endothelial permeability *in vitro*. *Endocrinology* 1992; 131: 710-4.

Okuno Y, Igarashi A, Fukai K. Neutralization tests for dengue and Japanese encephalitis viruses by the focus reduction method using peroxidase-anti-peroxidase staining. *Biken J* 1978; 21: 137-47.

Okuno Y, Fukunaga T, Srisupaluck S, *et al.* A modified PAP (peroxidase-anti-peroxidase) staining technique using sera from patients with dengue hemorrhagic fever (DHF):4 step PAP staining technique. *Biken J* 1979; 22: 131-5.

Pang T. Pathogenesis of dengue haemorrhagic fever: towards a more balanced view. *Southeast Asian J Trop Med Public Health* 1987; 18: 321-5.

Rosen L. The emperor's new clothes revisited, or reflections on the pathogenesis of dengue hemorrhagic fever. *Am J Trop Med Hyg* 1977; 26: 337-43.

Schindler R, Mancilla J, Endres S, *et al.* Correlations and interactions in the production of interleukin-6 (IL-6), IL-1, and tumor necrosis factor (TNF) in human blood mononuclear cells:IL-6 suppresses IL-1 and TNF. *Blood* 1990; 75: 40-7.

Tracey KJ, Lowry SF, Cerami A, *et al.* Cachectin:a hormone that triggers acute shock and chronic cachexia. *J Infect Dis* 1988; 157: 413-20.

Wiharta AG, Hotta H, Sujudi, *et al.* Dengue virus multiplication in cultures of peripheral blood monocytes from Indonesian and Japanese residents, in relation to effects of parasitic and bacterial components and anti-dengue antibodies. *ICMR Ann Kobe Univ* 1994; 4: 105-15.