Lymphocyte Activation and Hepatic Cellular Infiltration in Immunocompetent Mice Infected by Dengue Virus

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Activation and expansion of dengue virus-specific T cells and abnormal liver functions in dengue patients have been documented. However, it remains to be determined whether T cells are involved in the pathogenic mechanism of dengue virus infection. In this study, immunocompetent C57BL/6 mice were employed to study dengue virus-induced T cell activation. Mice were inoculated with 10⁸ PFU dengue virus serotype 2 strain 16681 by the intravenous route. Dengue viral core RNA was detected by RT-PCR in mouse serum, liver, spleen, and brain at different time points after infection. Splenic T cells were activated as evidenced by their expression of CD69 and *O*-glycosylated CD43 at as early as day 3 after infection. Splenic T cell expression of *O*-glycosylated CD43 and IFN- γ production coordinately peaked at day 5. Coincided with the peak of splenic T cell activation was hepatic lymphocyte infiltration and elevation of liver enzymes. Flow cytometric analysis revealed the infiltrating CD8⁺ T cell to CD4⁺ T cell ratio was 5/3. After a second inoculation of dengue virus, hepatic T cell infiltration and liver enzyme levels increased sharply. The infiltrating hepatic CD8⁺ T cell to CD4⁺ T cell ratio increased to 5.8/1. A strong correlation was found between T cell activation and hepatic cellular infiltration in immunocompetent mice infected with dengue virus. The kinetics of liver enzyme elevation also correlated with that of T cell activation. These data suggest a relationship between T cell infiltration and elevation of liver enzymes. **J. Med. Virol. 73:419–431, 2004.**

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INTRODUCTION

Dengue virus is a single positive-stranded RNA virus that belongs to the family *Flaviviridae*. Four antigenically related dengue viral serotypes cause a spectrum of clinical illness and significant mortality and morbidity throughout the tropics and subtropics [Monath, 1994]. In the year 1998, dengue fever became the most important tropical infectious disease next to malaria. There are an estimated 50–100 million dengue infections, 250,000–500,000 cases of dengue hemorrhagic fever and 25,000 deaths annually [Gubler, 1998; Guzman and Kouri, 2002].

It has been reported that soluble CD4 (sCD4), soluble CD8 (sCD8), and soluble IL-2 receptor (sIL-2R) are found in the sera of children with dengue hemorrhagic fever [Kurane et al., 1991]. The acute sera of patients

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with dengue fever contained higher than normal levels of sCD4 and sIL-2R but not sCD8. The levels of sCD8, sCD4, and sIL-2R in dengue hemorrhagic fever patients are significantly higher than in dengue fever patients on days 3–4 after onset of fever. Recently, Zivna et al. [2002] compared the frequency of peptide-specific T cells in subjects who experienced dengue hemorrhagic fever as a result of secondary infection and those who had dengue fever as a secondary infection, and found that the frequency of specific T cells were higher in the former than the latter. These findings suggest that the activation and expansion of dengue virus-specific T cells, especially that of CD8⁺ T cells is greater in subjects with more severe clinical syndrome and support the hypothesis that activation of dengue virus-specific T cells plays an important role in the pathogenesis of dengue hemorrhagic fever [Zivna et al., 2002]. Therefore, it is critical to understand the kinetics of T cell activation in relation to the pathogenesis of dengue. Such a study is best carried out in experimental animals.

Several small animal models have been established to study dengue and to test potential dengue vaccines. Early attempts by Falgout et al. [1990] showed that intracerebral inoculation of LD₁₀₀ and LD₅₀ dengue virus into BALB/c mice produced encephalitis. SCID mice engrafted with human peripheral blood lymphocytes (hu-PBL-SCID), human K562 or HepG2 cells were also used to study dengue virus infection [Wu et al., 1995; Lin et al., 1998; An et al., 1999]. In the former, hu-PBL-SCID model dengue virus serotype-1 Western Pacific strain 74 was used and the virus was recovered at low rates from different organ systems. In the latter, HepG2-SCID model, animals showed neurological signs after injection with dengue virus serotype-2 strain Tr1751 and died at 2 weeks post-infection. A/J mice infected intravenously with dengue virus serotype-2 strain PL-046 developed transient thrombocytopenia and produced anti-platelet antibody after primary as well as secondary infection [Huang et al., 2000]. AG129 mice with defects in IFN- α , β , γ receptor genes infected with mouse-adapted dengue virus serotype-2 of New Guinea C strain experienced transient viremia and died within 12 days of infection [Johnson and Roehrig, 1999]. Each of these animal models has its merit, but none of them has been used to evaluate T cell response to dengue virus.

In this study, immunocompetent C57BL/6 mice were employed to investigate T cell response to dengue virus infection and to study the role of T cells in immunopathogenesis of dengue disease. Mice were infected with high titers of dengue virus serotype-2 strain 16681, originally isolated from a dengue hemorrhagic fever patient from Thailand [Russell et al., 1967]. RT-PCR and real-time RT-PCR revealed dengue viral RNA in the sera and various tissues of the infected mice. T cells in the infected mice were activated and functionally active, as evidenced by production of IFN- γ . CD8 T cells constituted the majority of activated T cells. Coincided with the kinetics of T cell activation was liver enzyme elevation and hepatic T cell infiltration. Taken together,

the results of this study point to the possibility of T cell-mediated liver damage in dengue disease.

MATERIALS AND METHODS

Virus

Dengue virus serotype-2 strain #16681 was used throughout this study. Dengue virus serotype-2 #16681 was originally isolated from a Thai patient who suffered dengue hemorrhagic fever [Russell et al., 1967]. The virus was propagated in insect cell line C6/36 cultured in DMEM (Gibco-BRL, Gaithersburg, MD) and M&M (Sigma-Aldrich, St. Louis, MO) (1:1 ratio) containing 2% heat-inactivated fetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel) at 28°C. The virus titer was at $\geq 2 \times 10^8$ PFU per ml. C6/36 culture supernatant (Mock) and UV-inactivated virus (UV-DV) were used as controls. Virus stocks were inactivated by UV treatment at 50 mJ/cm² in 30 sec by Spectrolinker XL-1000 crosslinker (Spectronics Corporation, Westbury, NY). Loss of infectivity was confirmed by plaque assay.

Infection

C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred at National Taiwan University College of Medicine Laboratory Animal Center. Mice at 4–5 weeks of age were injected intravenously with 1×10^8 PFU in 0.5 ml volume. Tissues were removed from mice at different time points after infection as indicated in the legend of each figure. Mice were bled from the orbital vein and sera were used for RT-PCR, real-time RT-PCR assays, and liver enzyme determinations. In some experiments, mice were given a second inoculation of virus (1×10^8 PFU) at day 7 after primary infection. Mice injected with 0.5 ml of UV-inactivated virus, mock C6/36 culture supernatant, or PBS were used as controls.

Reagents

Immunological reagents including: conjugated anti-CD4 (clone GK1.5), anti-CD8 (clone 53-6.7), anti-CD69 (clone H1.2F3), anti-CD44 (clone IM7), and IFN- γ (clone XMG 1.2) antibodies and IFN- γ ELISA kits were obtained from eBioscience (La Jolla, CA). Anti-O-glycosylated CD43 (clone 1B11) was obtained from BD PharMingen (San Diego, CA). RPMI 1640, HEPES buffer, L-glutamine, sodium pyruvate, non-essential amino acids, penicillin/streptomycin were purchased from Gibco-BRL. 2-Mercaptoethanol was purchased from Sigma-Aldrich.

Real-Time RT-PCR Assays

Real-time RT-PCR assay was employed to quantify dengue virus in sera [Wang et al., 2002]. Briefly, dengue viral RNA was isolated from serum using the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany). An aliquot (2.5 μ l) of purified RNA and known amounts of positive-sense RNA (2.5, 25, 250, 2,500, 25,000, and

2,500,000 copies) were subjected to real-time RT-PCR using the designed primers (d2C16A, 5'-GCTGAAACG-CGAGAGAAACC-3' and d2C46B, 3'-TCCCTGCTCCT-GGTIATTTTAC-5') and probe (VICd2C38B, 3'-TGT-CGACTGTTTCTCTAAGAGTGAACCTTACGA-5'), and the Taqman one-step RT-PCR master mix reagent kit (PE Biosystems, Foster City, CA). The amplification condition was 48°C for 30 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min, as recommended by the manufacturer. The ABI Prism 7700 sequence detector was used to analyze the emitted fluorescence during amplification. A positive result is defined by the cycle number (CT value) required to reach the threshold, which is ten times the standard deviation of the mean baseline emission calculated for PCR cycles 3–15. Since 2.5 µl of the 50-µl RNA elutes, which were derived from 100 µl of serum, was used in each reaction, the number of dengue virus RNA copies per reaction was divided by 5 µl (100 µl × 2.5 µl/50 µl) and multiplied by 1,000 to determine the number of RNA copies per ml of serum. The sensitivity of the assay is 2.5 copies of RNA per reaction, corresponding to 357 copies per ml of serum.

Detection of Dengue Virus in Tissues by RT-PCR

Spleen, liver, brain, and serum were collected from control as well as infected mice at days 1, 3, 5, and 7 after infection. RNeasy mini kit (Qiagen) was used to extract RNA from 30 mg of tissue. Tissue was homogenized in 600 µl of RLT buffer with 0.1% of β-ME (2-mercaptoethanol) and RNA was extracted with 70% ethanol. RNA extract was filtered through RNeasy mini column (Qiagen) into collection tube by centrifugation for 15 sec at 10,000 rpm. The RNA was washed with buffer RPE and DEPC water and stored at -80°C. The RNA was reverse transcribed and cDNA amplified in one reaction tube with primer set C14A and C69B and RT/Tag mix (Gibco-BRL). The sequences of C14A and C69B are 5'-AATATGCTGAAACGCGAGAGAAACCGCG-3' (corresponding to genome positions 136–163 of the DEN-2 Jamaica strain) and 5'-CCCATCTCTTCAGTATCCC-TGCTGTTGG-3' (corresponding to genome positions 278–305 of the dengue virus serotype-2 Jamaica strain), respectively [Deubel et al., 1986; Wang et al., 2000]. They were designed to amplify a 170-bp product in the C region of dengue viral RNA. The RT-PCR reaction took place in the following condition: 40 min at 55°C, 2 min at 94°C followed by 35 cycles of 45 sec at 94°C, 45 sec at 62°C, and 45 sec of 68°C, then 5 min at 68°C. The PCR products were diluted 1:500 and amplified by 40 cycles of PCR reaction (45 sec at 94°C, 45 sec at 62°C, and 45 sec at 68°C, followed by 5 min at 68°C) with C22A (5'-GCGTGTCTGACTGTACAACAG-3') and C63B (5'-GAG-TGTTAGGGTGGTTGTCGT-3') primer set and Taq DNA polymerase (Klen Tag, St. Louis, MO). The nested RT-PCR products were loaded onto 2% agarose gel for separation. The size of nested RT-PCR products was 125 bp. *HPRT* gene was amplified as a control. The

primer set for *HPRT* amplification was 5'-GTTGGATA-CAGGCCAGACTTTGTTG-3' and 5'-GAGGGTAGGCT-GGCCTATGGCT-3'. The molecular size for PCR product of *HPRT* was 352 bp.

Immunostaining and Flow Cytometric Analysis of Cell Surface Marker

One million of spleen cells were placed in the wells of V-bottomed 96-well plate. Cells were spun down at 4°C, supernatants removed, and washed two times in staining buffer. Phycoerythrin (PE)-conjugated anti-CD4 or PE-conjugated anti-CD8 and fluorescein isothiocyanate (FITC)-conjugated anti-CD69, FITC-conjugated anti-CD43 activation-associated glycoform, or FITC-conjugated anti-CD44 antibodies at final concentration of 1 µg/ml were added to the wells. Thirty minutes after incubation at 4°C, the cells were spun down and washed. The cell pellets were resuspended in staining buffer containing 1% paraformaldehyde. Cells were acquired by FACSCalibur (BD Biosciences, San Jose, CA). Data were analyzed by CellQuest.

IFN-γ Enzyme-Linked Immunoabsorbent Assay

Spleen cells were harvested from uninfected control mice and mice inoculated with mock supernatant, UV-inactivated virus or live dengue virus at different time points after inoculation. Single cell suspension was prepared and the red cells were lysed by treatment in 0.85% Tris-NH₄Cl buffer. Splenocytes at 5×10^5 in 100 µl of RPMI medium containing 20 mM of HEPES, L-glutamine, sodium pyruvate, non-essential amino acids, 2-mercaptoethanol (5×10^{-2} mM), streptomycin/penicillin, 10% heat-inactivated fetal calf serum, and rhIL-2 (0.04 ng/ml) were added to round-bottomed wells in the 96-well plates. Virus at 1.5×10^8 PFU/ml was inactivated by UV as described above. One tenth of 1 ml of inactivated virus was added to each well. The culture plate was left in 37°C incubator for 48 hr before culture supernatants were harvested for determination of IFN-γ concentration by ELISA assay. Coating and blocking antibody set for IFN-γ was purchased from eBioscience. The avidin-horseradish peroxidase and TMB substrate were used for color development. Known concentrations of recombinant murine IFN-γ were used as standards.

Intracytoplasmic IFN-γ Staining

Single cell suspension was prepared from freshly harvested spleens at different time points after infection. Spleen cells at 5×10^5 cells were suspended in 100 µl of RPMI 1640 medium containing rhIL-2 (0.04 ng/ml). Cells were added to flat-bottomed 96-well plates that were coated with 100 µl of anti-CD28 antibody 10 µg/ml (clone 37.51, BD PharMingen). Dengue virus of 1.5×10^7 PFU was added to the wells. Six hours before harvest, monesin (2 µM, Sigma-Aldrich) was added to the culture. Cells were stained with allophycocyanin (APC)-conjugated anti-CD4 or anti-CD8 antibody, and FITC-conjugated 1B11 antibody at 1 µg/ml final concentration for 30 min. Cells were then washed, fixed,

and permabilized by Cytofix (4% paraformaldehyde in dPBS) for 20 min. After two washes, PE-conjugated anti-IFN- γ antibody in the Perm/Wash buffer was added for 30 min. After incubation on ice, cell were washed and fixed in FACS staining buffer/2% paraformaldehyde. FACSCalibur flow cytometer was used for cell acquisition. Data were analyzed by CellQuest.

Immunohistochemical Staining

Liver tissues removed from control as well as dengue virus-infected mice were embedded in O.C.T. in dry ice bath. Frozen tissues were then cryosectioned (Shandon, CRYOTOME[®] SME, Pittsburgh, PA) at 5 μ m thickness, fixed in acetone for 5 min and air-dried. The sections were washed with PBS and blocked in medium containing 5% goat serum for 20 min. Rat anti mouse-CD4 (clone GK 1.5 at 1:100 dilution) or anti-CD8 (clone 53-6.7, at 1:100 dilution) antibody was added to the sections and left in wet chamber at 4°C overnight. Sections were washed twice in PBS before peroxidase-conjugated goat anti-rat IgG antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was added. After 5 hr incubation at 37°C, the sections were washed and DAB substrate (Vector Lab., Burlingame, CA) was applied. One to three minute(s) was allowed for color development. Hematoxylin was used as a counterstain.

H & E Staining

Liver tissues were fixed in 4% neutral formalin solution for at least 24 hr and then embedded in paraffin. The tissues were sectioned at 3 μ m in thickness. After deparaffinization and rehydration, the sections were stained in hematoxylin for 15 min, washed, and stained in eosin for 3 min. The sections were then dehydrated before mounting.

Isolation and Identification of Lymphocytes in the Liver

Liver tissues were collected from mice with one or two inoculations of virus or from mice receiving intravenously injection of Con A (20 mg/kg, Sigma-Aldrich). The tissues were pressed between two ground glass slides. Cell debris was removed by passing the suspension through the cotton column packed in Pasteur pipette. The single cell suspension was centrifuged and resuspended in HBSS. The cell suspension in 4 ml was overlaid on 3 ml of Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden). After centrifugation at 400g at room temperature for 30 min, the cells at the interface were collected and washed. Cells were spun down at 4°C, and washed two times in staining buffer. The cells were stained by PE-conjugated anti-CD4 or anti-CD8 and FITC-conjugated anti-CD44 antibodies at a final concentration of 1 μ g/ml.

Determination of Liver Enzyme Levels

Mouse blood was collected from the orbital vein. Serum obtained from clotted blood was frozen at -70°C

before assay. ALT and AST levels were determined in separate vials by a colometrical method. Coenzyme pyridoxal-5-phosphate was added to facilitate the determination of ALT activity. ALT catalyzes and produces pyruvate and glutamate. The pyruvate produced is then reduced to lactate by lactate dehydrogenase (LD) with the concomitant oxidation of NADH to NAD⁺. The rate of NADH oxidation is proportional to the ALT activity of the sample. AST catalyzes and produces oxaloacetate and is reduced to malate dehydrogenase (MDH) with the concomitant oxidation of NADH to NAD⁺. Likewise, the rate of NADH oxidation is proportional to the AST activity of the sample. NADH oxidation was measured at 340 nm and read by automated Abbott Alcyon 300i (Abbott Park, IL).

Detection and Isotyping Dengue Virus Specific Antibodies

Mice were bled from the orbital vein. Sera were collected after the clotted blood was centrifuged. Sera were kept in -70°C before assay. EIA plates (Nalge Nunc, Denmark) were used for antibody detection. Dengue virus at 5×10^7 PFU/ml was sonicated for total of 15 min on ice and diluted tenfold in coating buffer. Supernatants from uninfected C6/36 culture were diluted tenfold and used as mock control. The wells were coated with 50 μ l of the virus preparation and the mock supernatant overnight at 4°C. The wells were then washed with PBS/0.5% Tween-20 for three times and blocked with blocking buffer at 4°C overnight and then washed. Duplicate samples of sera and control supernatants were diluted 1:200 before addition to wells. The plates were left at room temperature for 1 hr. After washing, detection antibody biotin-conjugated goat anti-mouse IgG in 1:5,000 or biotin-conjugated goat anti-mouse IgM in 1:5,000 was added. The plates were again left at room temperature for 1 hr before wash with buffer. After the addition of avidin-conjugated horseradish peroxidase (1:250) the plates were left at room temperature for 30 min. The wells were then washed five times with buffer and let stand for 1–2 min before addition of TMB substrate (eBioscience). The plates were left at room temperature and protected from light for 15 min. The color development was stopped by 2 N H₂SO₄. The absorbance was read at 450 nm.

In Situ Detection of DNA Fragmentation

Liver tissues were fixed in 4% paraformaldehyde. Fixed sections were treated with 0.1% Triton[®] X-100 in 0.1% sodium citrate for 2 min on ice. The sections were then blocked with 0.1 M Tris-HCl (pH 7.5) containing 20% FCS. DNA fragmentation was detected on sections by using the In Situ Cell Death Detection Kit AP (Boehringer Mannheim, Mannheim, Germany). The staining was performed according to manufacturer's instructions. In brief, TUNEL reaction mixture (TdT-mediated dUTP nick end labeling) at enzyme solution to label solution 1:9 was added. Converter-POD (peroxidase-conjugated anti-fluorescein antibody) as added

after wash. DAB was used as substrate for color development and methyl green as a counterstain.

RESULTS

Dengue Virus Disseminates in Immunocompetent Mice

C57BL/6 mice were inoculated intravenously with 10⁸ PFU of dengue virus serotype-2, strain 16681. RT-PCR results showed that dengue viral RNA was detectable in mouse spleen, liver, brain, and serum at days 1, 3, 5, and 7 after infection (Fig. 1A,B), demonstrating that dengue virus was disseminated in the mouse. Real-time RT-PCR revealed that viral RNA peaked at day 3 when there was 2.7 × 10⁶–3.6 × 10⁶ copies of viral RNA per ml of serum in the mouse. The level fell to 5.1 × 10⁴–4.6 × 10⁵ copies per ml of serum at day 5. Together, these results show that dengue virus serotype-2 strain 16681 inoculated intravenously established viremia and disseminated to various tissues in immunocompetent mice.

T Cells Are Activated After Dengue Virus Infection

The expression of CD69 is commonly used as a marker to assess early lymphocyte activation [Testi et al., 1989]. In the course of dengue virus infection, the numbers of CD4⁺ and CD8⁺ T cells expressing CD69 at days 3, 5, and 7 after infection were significantly higher than the

control mice (Table I). During this time, the number of T cells in infected mice that expressed CD69 was 2.0–3.5 times of that in the control mice. These data show that dengue virus infection induced T cell activation.

To understand further the kinetics of T cell activation and function, another activation marker was studied. It has been reported in the LCMV model that increased expression of O-glycosylated CD43, as recognized by 1B11 monoclonal antibody, on CD8⁺ T cells, correlates with their effector function [Harrington et al., 2000]. The expression of O-glycan by CD4⁺ T cells and its correlation with activation state has also been reported in mice after infection with Moloney murine sarcoma and leukemia virus complex (MoMSV) [Schepers et al., 2002]. Therefore, 1B11 monoclonal antibody was used to identify and enumerate activated and functional T cells. Data in Figure 2 show that at day 3 of infection, the numbers of CD8⁺ and CD4⁺ T cells expressing O-glycosylated CD43 in dengue virus-infected mice became significantly higher than the control mice. The mean numbers of O-glycan-expressing CD8⁺ and CD4⁺ T cells peaked at day 5 of infection and declined thereafter. At the peak, they were 7.0- and 1.2-fold, respectively, higher than the control mice. In contrast, O-glycosylated CD43 T cells in mice inoculated with mock or UV-inactivated virus were not significantly different from control mice. The results suggest that T cells were functionally activated after dengue virus infection.

Fig. 1. Dengue virus disseminated in immunocompetent mice. (A) Viral core genes were detected by RT-PCR in the spleen (Sp), liver (L), brain (Br), and (B) serum at different time points after infection. RT-PCR product of viral core gene is 125 bp. Control mice were injected with PBS. +, Culture supernatant from dengue virus-infected C6/36 cells; Live-DV, tissues or serum RNA from mice inoculated with live dengue virus mice; UV-DV, tissues or serum RNA from mice inoculated with UV-inactivated dengue virus; HPRT, house-keeping gene. The molecular size for HPRT is 352 bp.

TABLE I. T Cells Express CD69 After Infection

Cell type ^a	Cell number ($\times 10^6$)			
	Control	Days after infection		
		3	5	7
CD8 ⁺ T cells	0.4 $\pm$ 0.1	1.4 $\pm$ 0.3** (3.5) ^b	1.1 $\pm$ 0.8** (2.8)	1.0 $\pm$ 0.4** (2.5)
CD4 ⁺ T cells	0.8 $\pm$ 0.4	2.7 $\pm$ 0.7** (3.4)	2.8 $\pm$ 1.7** (3.4)	1.6 $\pm$ 0.9* (2.0)

^aSpleen cells were collected from control mice and mice infected with dengue virus serotype-2 virus at days 3, 5, and 7 after infection. The number of lymphocytes expressing CD69 ($\times 10^6$) was calculated from the total number of CD4⁺ or CD8⁺ T cells and the percent of cells expressing CD69. Six to nine mice were included for each time point.

^bThe mean number of cells expressing CD69 in infected mice divided by that in control mice.

* $P < 0.05$, comparing the number of CD69⁺ cells in infected mice with that in uninfected mice.

** $P < 0.005$, comparing the number of CD69⁺ cells in infected mice with that in uninfected mice.

Activated T Cells Produce IFN- γ

To investigate the effector function of activated T cells, spleen cells collected at different time points of infection were cultured and the supernatants were assayed for IFN- γ . Not only spleen cells from infected mice produced IFN- γ (Fig. 3) but the kinetics of IFN- γ production also followed that of T cell activation (Fig. 2). IFN- γ production (19.3 ± 6.9 ng/ml) peaked at day 5 of infection, when 18.3% of splenic CD8⁺ T cells and 4.6% of CD4⁺ T cells were IFN- γ producers (Fig. 4A). In contrast, spleen cells from mice inoculated with UV-inactivated dengue virus and those with mock supernatants produced $<1/10$ levels of IFN- γ at 5 days after inoculation (Fig. 3). Interestingly, of the total IFN- γ producing cells, about 76% were CD8⁺ T cells and 24% CD4⁺ T cells, demonstrating that the majority of functionally active cells were CD8⁺ T cells. Phenotyping showed 82.8% of CD8⁺-IFN- γ ⁺ T cells and 61.6% of CD4⁺-IFN- γ ⁺ T cells expressed O-glycosylated CD43 and $>97\%$ of all IFN- γ ⁺ T cells were CD44^{hi} (Fig. 4B). These results demonstrate that most activated CD8⁺ T cells expressing O-glycan and CD44^{hi} were functionally active in mice infected by dengue virus.

Hepatic Infiltration in Mice Infected With Dengue Virus

In examining liver sections, cellular infiltration was observed in mice at day 5 after infection (Fig. 5B). H & E stain revealed that the infiltrating cells were mostly lymphoid cells. Immunohistochemical staining revealed that the infiltrating cells constituted of both CD8⁺ and CD4⁺ T cells (Fig. 6B,E). The distribution of CD4⁺ and CD8⁺ T cells followed different patterns. While most of the CD4⁺ T cells clustered around the portal vein with some scattered in the parenchyma, most of CD8⁺ T cells were scattered in the hepatic acinus (personal observation). Flow cytometric analysis of hepatic infiltrating T cells showed that of total infiltrating T cells isolated, about 62.5% were CD8⁺ T cells and 37.5% CD4⁺ T cells (Fig. 7B), contrast to acute hepatitis induced by Con A where the majority of infiltrating cells were CD4⁺ T cells (71.7%) (Fig. 7A). Moreover, 69.9% of infiltrating CD8⁺ T cells were of CD44^{hi} phenotype (Fig. 7D). These results are consistent with the observation in Figure 4A that

most of the functionally active T cells in the spleen were CD8⁺ T cells.

Liver Injury

Serum ALT and AST levels were then assayed to determine whether T cell infiltration was associated with liver damage. The results in Table II show that liver enzyme levels in dengue virus-infected mice were significantly higher than control mice (days 3 and 5 for ALT and days 3, 5, 7 for AST), although UV-inactivated dengue virus also induced a transient elevation of ALT and AST at day 3 after inoculation. In situ detection of DNA fragmentation by TUNEL assay shows that liver cells are apoptotic (Fig. 8), similar to what is reported in infected humans [Couvelard et al., 1999]. Interestingly, the kinetics and peak of liver enzyme levels correlated with that of activated T cells (Fig. 2) suggesting that activated T cells were associated with liver damage. Moreover, the liver enzyme levels also correlated with the peak of IFN- γ production (Fig. 3). The degree of cellular infiltration also positively correlated with the level of IFN- γ produced (Table III).

Second Inoculation of Dengue Virus Induces Greater Liver Damage

In a separate experiment, mice were given a second inoculum of dengue virus at the time when activated T cell numbers and specific IgM levels declined and before specific IgG rose. The AST levels in these mice increased rapidly. At the third day after second inoculation, AST levels were 1.7 times that of mice given a single dose of the virus. Interestingly, a second virus inoculation resulted in more pronounced cellular infiltration (Figs. 5C and 6C,F). The infiltrating CD8⁺/CD4⁺ T cell ratio was greater in mice receiving a second dose of virus (5.8/1) than in those given one inoculation (3/2) (Fig. 7B,C). Moreover, although in mice receiving only one dengue virus inoculation only 69.9% of CD8⁺ T cells expressed CD44^{hi} phenotype, $>95\%$ of infiltrating CD8⁺ T cells in mice receiving second dengue virus inoculation expressed CD44^{hi} phenotype (Fig. 7E), illustrating further the association of activated CD8⁺ T cells and liver injury in dengue.

Fig. 2. T cell expression of O-glycosylated CD43 peaks at day 5 after dengue virus infection. Splenocytes were harvested from dengue virus-infected mice at days 1, 3, 5, and 7 after infection. Splenic cells from mice receiving PBC (control), C6/36 culture supernatant (mock), and UV-inactivated dengue virus (UV-DV) were harvested at day 5 after inoculation. Cells were stained with PE-conjugated anti-CD8 or PE-conjugated anti-CD4 and FITC-conjugated 1B11 antibodies. The number of CD8⁺ (A) and CD4⁺ (B) T cells expressing O-glycan in the course of infection was calculated from the total number of T cells and the percentage of cells stained positive by 1B11 monoclonal antibody. Each symbol represents one mouse. The horizontal lines indicate the mean value. *,** indicate $P < 0.05$ and $P < 0.005$, respectively, comparing the mean number of cells from infected mice at each time point after infection with that from control mice without infection.

DISCUSSION

Both immune enhancement and viral virulence hypotheses recognize the importance of achieving high viral titer in the cause of severe dengue disease [Halstead, 1970, 1980, 1988; Rosen, 1977a; Gubler et al., 1978; Gubler, 1988]. The antibody enhancement hypothesis attempts to explain the observation of individuals experiencing secondary infection with a heterologous dengue serotype had a significantly higher

Fig. 3. IFN- γ production peaks at day 5 of infection. Splenic cells were harvested from same groups of mice at the same time points as described in legend of Figure 2. Virus were UV-inactivated and added to the wells containing 5×10^5 splenic cells. Culture supernatants were collected after 48 hr of incubation. IFN- γ concentration in the supernatants was determined by ELISA. Six to ten mice were used at each time point. *, ** indicate $P < 0.05$ and $P < 0.005$, respectively. The P value was calculated from comparing the IFN- γ concentration in the splenic cell culture supernatants from mock supernatant, UV-inactivated virus, or live virus infected mice with that in supernatants from uninfected control mice.

risk of developing dengue hemorrhagic fever/dengue shock syndrome [Halstead, 1970, 1980, 1988; Sangkawibha et al., 1984; Burke et al., 1988; Kliks et al., 1989]. It was shown *in vitro* that in the presence of low titers of heterologous antibody, dengue virus replicates to higher titers in human monocytes [Kliks et al., 1989]. This hypothesis emphasizes the importance of heterologous antibody in enhancing the replication of the second virus in antigen-presenting cells. The enhancement results in profound activation of preexisting cross-reactive cytotoxic T cells and release of cytokines and cellular mediators, thus leading to subsequent immunopathological processes and dengue hemorrhagic fever/dengue shock syndrome [Innis, 1995; Gubler, 1998; Rothman and Ennis, 1999]. However, the link between T cell activation and pathology has never been established. The viral virulence hypothesis was based on the observation that some dengue viruses have greater epidemic potential than others [Rosen, 1977b; Gubler et al., 1978]. These 'virulent' virus strains have the advantage to replicate faster and to higher levels, and as a result cause higher levels of viremia. To study immunopathogenesis of dengue in immunocompetent mice, virus virulence and titer were taken into considerations. We established successfully this mouse model by intravenous injection of high titers of the hemorrhagic strain dengue virus serotype-2 16681 [Russell et al., 1967]. The infected mice became viremic and the virus disseminated. Moreover, the infection induced T cell activation.

Immunocompetent mice have not been used widely to study T cell responses to dengue virus. A/J mice inoculated intravenously with dengue virus developed transient thrombocytopenia and produced anti-platelet

Fig. 4. CD4⁺ as well as CD8⁺ T cells produce IFN- γ . Spleen cells were harvested from mice at day 5 of infection and cultured in wells containing plate-bound anti-CD28 antibody, rIL-2, and DV (3×10^7 PFU/100 μ l). Cells were stained with APC-conjugated anti-CD8 or anti-CD4, PE-conjugated anti-IFN- γ , and FITC-conjugated 1B1 or anti-CD44 antibodies. (A) CD8⁺ and CD4⁺ T cells produce IFN- γ . The numbers above the dot plot indicate percent CD8⁺ IFN- γ -positive

or CD4⁺ IFN- γ -positive cells in CD8⁺ or CD4⁺ T cell populations. (B) Phenotyping IFN- γ -positive cells. The percentages of IFN- γ producing CD8⁺ and CD4⁺ T cells expressing O-glycosylated CD43 or CD44^{hi} are indicated. Forty thousand cells were acquired and analyzed by CellQuest. Three mice were included in each experiment and the experiment was repeated three times. Data from one representative mouse are shown.

antibody after primary infection and upon challenge [Huang et al., 2000]. However, mouse T cell responses, cytokine production and immunopathology to dengue virus were not reported. So far as we know, we are the first to report that in dengue virus infected mice, T cells especially CD8⁺ T cells are activated and constitute the major cell population that infiltrate the liver. The T cell infiltration also seems to associate with liver injury.

The association of T cell activation, especially that of CD8⁺ T cells, with more severe clinical syndrome has been shown in several dengue clinical studies. As mentioned above, patients with dengue hemorrhagic fever had higher serum sCD8 compared to that with dengue fever [Kurane et al., 1991]. The percentages of CD8⁺ T cells and NK cells expressing CD69 are higher within 72 hr of fever onset in pediatric patients who later developed dengue hemorrhagic fever than in those developed dengue fever, including patients with primary and secondary infections with dengue virus serotype-1, 2, and 3 [Green et al., 1999]. Examining a dengue outbreak in Southern Taiwan, Lei et al. [2001] also reported that CD69 expression is high on T cells and the expression is higher on CD8⁺ T cells than on CD4⁺ T cells. These data indicate that early CD69 expression, especially on CD8⁺ T cells, correlates with severe outcome of dengue virus infection.

It has also been reported that the CD8⁺ T cells derived from dengue immune donors have functional activity. The CD8⁺ T cell clones derived from dengue immune donors recognizing epitopes on NS3 (non-structural protein 3) are cytotoxic [Livingston et al., 1995]. The Flavivirus cross-reactive T cells isolated from mice infected with Murray Valley encephalitis (MVE) virus not only have cytotoxic activity but also produce IFN- γ [Regner et al., 2001]. The present study in mice infected with high titers of dengue virus serotype-2 strain 16681 showed that the numbers of CD8⁺ as well as CD4⁺ T cells expressing CD69 are up early in the course of infection (Table I). The activated T cells are functionally active as demonstrated by their ability to produce IFN- γ (Fig. 4). Moreover, this study comparing the responses induced by UV-inactivated virus and that by infection with live virus showed that T cell responses are results of a true infection. These results demonstrated that CD8⁺ T cell responses in C57BL/6 mice injected with high titers of hemorrhagic strain of dengue virus serotype-2 model the CD8⁺ T cell responses in human disease. Thus, this animal model is useful for the study of the role of activated T cells in the pathogenesis of dengue.

The liver is not thought to be a major target organ of dengue infection, but the involvement of liver cells in pathogenesis of dengue virus infection has been indi-

Fig. 5. Dengue virus infection results in hepatic cellular infiltration. Liver tissues were removed from control mouse (A), mouse at 5 days after receiving one inoculation of virus (B) and mouse at 3 days after receiving a second inoculation of virus (C). Tissues were fixed in 10% buffered formaldehyde and stained with hematoxylin and eosin. The magnification was 200 $\times$.

cated by clinical signs of hepatomegaly, abnormal liver function, pathological findings, and detection of viral antigen [Bhamarapravati et al., 1967; Rosen et al., 1989; Hall et al., 1991; Lanciotti et al., 1992; Kangwanpong et al., 1995; Couvelard et al., 1999; Huerre et al., 2001]. In examining fatal dengue hemorrhagic fever cases, liver is the tissue most often the virus is isolated [Rosen et al., 1989; Innis, 1995; Bhamarapravati, 1997; Huerre et al., 2001]. Dengue patients were reported to have elevated AST levels and AST elevation is associated with hemorrhage [Kalayanarooj et al., 1997; Monath et al., 2000]. In dengue virus induced hepatitis, the level of AST is higher than ALT with a ratio of around 1–1.5 while other types of virally induced hepatitis have higher ALT levels than AST [Kuo et al., 1992; Nguyen et al., 1997; Lei et al., 2001]. Serum liver enzyme AST level is higher in dengue hemorrhagic fever patients than in dengue fever patients [Kalayanarooj et al., 1997; Monath et al., 2000; Wahid et al., 2000]. It has also been reported that dengue virus induces HepG2 apoptosis and production of RANTES [Lin et al., 2000a,b]. Together, these reports indicate that dengue virus infection can cause liver pathology. The mechanism of liver damage has never been examined.

Examining liver pathology of 100 fatal pediatric (age 5 months to 14 years) dengue hemorrhagic fever cases, Bhamarapravati et al. [1967] reported that cellular

infiltration was noted in 64 cases. Megakaryocytes, lymphocytoid cells, and rarely neutrophils were observed in sinusoids. Cellular infiltration in the portal areas was composed of young lymphocytes, plasmacytoid cells and some histiocytes [Bhamarapravati et al., 1967]. A recent liver histopathological report of five fatal pediatric (age 10–72 months) cases revealed that liver histopathology was detected in four out of five post-mortem examinations [Huerre et al., 2001], but little or no inflammatory response was found in four cases and moderate infiltration, mainly in the periportal area in only one case [Huerre et al., 2001]. Therefore, it appears that liver histopathology is common in patients who died of dengue hemorrhagic fever but it remains to be determined virus infecting liver cells, lymphocyte infiltration or both is the cause of liver pathology.

In this study, mice infected with dengue virus had significantly higher liver enzyme levels (days 3, 5, and 7, Table II) when the numbers of activated T cells were high (days 3, 5, and 7, Fig. 2). Analysis of infiltrating cells showed that they are activated lymphocytes and the majority are CD8⁺ T cells (Fig. 7B,D). In situ detection of DNA fragmentation by TUNEL assay showed that liver cells were apoptotic (Fig. 8), similar to what is reported in infected humans [Couvelard et al., 1999]. These data strongly indicate that liver injury in dengue virus infection is associated with activated

Fig. 6. The cellular infiltrates in the liver consist of $CD8^+$ T and $CD4^+$ T cells. Liver tissues were from control mouse (A, D), mouse at 5 days after receiving one inoculation of virus (B, E) and mouse at 3 days after receiving a second inoculation of virus (C, F). Cryosectioned tissues were stained with rat anti-mouse CD8 (A, B, C) or anti-CD4 antibody (D, E, F) followed by peroxidase-conjugated goat anti-rat IgG antibody. DAB was used as substrate for color development. Arrows point at positive cells. The magnification was $200\times$.

lymphocyte, especially $CD8^+$ T cell infiltration. However, these results do not rule out the contribution of direct viral damage to the liver.

Dengue virus infection induced specific antibody response. Specific IgM became significantly higher at day 3 and peaked at day 5 of infection while specific IgG was detectable at day 5–7 and peaked at day 14 (unpublished data). The infiltration in liver was more pronounced in mice receiving a second dose of dengue virus (Figs. 5C and 6C,F) at day 7 when T cells and specific IgM declined and before specific IgG elevated. The liver enzyme AST levels in these mice are much higher than in those receiving only one injection. The ratio of infiltrating $CD8^+$ to $CD4^+$ T cells was 3/2 in

mice receiving single inoculation and became 5.8/1 in mice receiving two viral inoculations (Fig. 7B,C), showing that $CD8^+$ T cells greatly expanded upon second inoculation. This correlation strengthened further the association between liver injury and $CD8^+$ T cell infiltration. It is worth noting that same observation was repeated in mice receiving lower dose of inoculum (1.5×10^7 PFU), indicating the phenomenon is not caused by high viral titer per se. Although it has not been documented that patients who later develop dengue hemorrhagic fever have been inoculated with dengue virus by multiple mosquito bites at different times within a short time frame, the chances of a person living in an endemic area being bitten by infected

Fig. 7. Activated CD8⁺ T cells constitute the major T cell population infiltrating the liver in DV-infected mice. Liver tissues were removed from mouse at 16 hr after Con A injection (A), mouse at 5 days after receiving one inoculation of virus (B, D) and mouse at 3 days after receiving a second inoculation of virus (C, E). Single cell suspension was prepared and mononuclear cells were separated on Ficoll-Paque

gradient. The mononuclear cells were stained with PE-conjugated anti-CD8 and APC-conjugated anti-CD4 antibodies (A, B, C) or PE-conjugated anti-CD8 and FITC-conjugated anti-CD44 antibodies (D, E). The numbers above the dot plots indicate the percentage of CD4⁺ or CD8⁺ T cells in the total mononuclear cells isolated (A, B, C) or CD8⁺ T cells expressing CD44^{hi} phenotype (D, E).

mosquitoes more than once are high. This model predicts that upon receiving a second dose of virus, the rapid expansion of CD8⁺ T cells can very well result in infiltration to the liver. Experiments are underway to investigate the direct causal relationship between CD8⁺ T cell infiltration and liver injury.

Clinical studies have shown that serum IFN- γ levels are high in dengue hemorrhagic fever patients [Kurane et al., 1989, 1991]. This present study showed that IFN- γ levels are high at the peak of T cell activation (Fig. 3) and the extent of cellular infiltration correlates with the amounts of IFN- γ secreted (Table III). However, it

TABLE II. Liver Enzyme Levels Increase in Mouse Sera After Infection

Liver enzyme ^a (U/L)	Inoculum ^b	Control ^c	Days after 1st inoculation ^d			Days after 2nd inoculation
			3	5	7	3
ALT	Live DV	25.7 $\pm$ 10.4	38.3 $\pm$ 16.2*	36.7 $\pm$ 15.6**	30.5 $\pm$ 5.5	33.0 $\pm$ 4.9*
	UV-DV		37.2 $\pm$ 4.1*	36.0 $\pm$ 18.3	27.0 $\pm$ 7.1	
	Mock		30.4 $\pm$ 1.7	29.3 $\pm$ 9.9	32.0 $\pm$ 2.8	
AST	Live DV	43.3 $\pm$ 5.4	67.1 $\pm$ 28.0*	94.8 $\pm$ 32.3**	82.2 $\pm$ 17.1**	164.3 $\pm$ 75.2**
	UV-DV		64.8 $\pm$ 5.2**	58.0 $\pm$ 10.0	63.0 $\pm$ 7.1	
	Mock		48.4 $\pm$ 9.2	35.3 $\pm$ 19.7	42.0 $\pm$ 5.7	

^aALT and AST levels were determined by Abbott Alcyon 300i. The enzyme levels are presented as units per liter (U/L).

^bMice were inoculated with live dengue virus (Live DV), UV-inactivated virus (UV-DV), or mock supernatant (mock).

^cControls were normal mice without inoculation.

^dSera were collected from mice at days 3, 5, and 7 after one inoculation and day 3 after second inoculation. Five to six mice were included in each experiment.

* $P < 0.05$, comparing the level of ALT or AST of live dengue virus, UV-inactivated dengue virus, or mock culture supernatant inoculated mice with that of control uninfected mice.

** $P < 0.005$, comparing the level of ALT or AST of live dengue virus, UV-inactivated dengue virus, or mock culture supernatant inoculated mice with that of control uninfected mice.

Fig. 8. Liver cell undergo apoptosis after dengue virus infection. DNA fragmentation was detected by in situ TUNEL staining. DNase I treated tissue was used as positive control (A). Uninfected normal (B) and dengue virus-infected (C) mouse liver sections were treated with FITC-conjugated TdT-mediated dUTP nick end labeling mixture and peroxidase-conjugated anti-FITC antibody. DAB was used as substrate and methyl green as counterstain.

remains to be investigated whether high IFN- γ level is an indication of T cell activation or directly related to liver injury.

In summary, by injecting high titers of dengue virus serotype-2 hemorrhagic strain to immunocompetent C57BL/6 mice, a mouse model to study T cell activation is developed. As Rothman et al. pointed out that various immune mechanism(s) is important to different stages of dengue infection [Rothman and Ennis, 1999], the results of this study revealed the possibility that liver

TABLE III. Correlation Between IFN- γ Production and Cellular Infiltration in the Liver

Experiment	Infiltration scores ^a	IFN- γ produced by spleen cells (ng/ml) ^b
I	++	21.0
	+	12.4
	–	2.8 ^c
II	++	19.6
	+	7.2
	–	3.2 ^c

^aScoring of cellular infiltration. ++, 10–30 cells per focus; +, lower than 10 cells per focus; –, no significant infiltration.

^bSpleen cells were harvested from mice at 5 days after virus inoculation and stimulated with inactivated virus in vitro as described in materials and methods. Supernatants were collected at 24 hr of incubation and IFN- γ concentration was determined by ELISA.

^cSpleen cells were collected from mice inoculated with inactivated virus.

injury in dengue is related to CD8⁺ T cell activation and infiltration.

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