

Early activation of natural killer and B cells in response to primary dengue virus infection in A/J mice

Sujan Shresta,^a Jennifer L. Kyle,^{a,b} P. Robert Beatty,^c and Eva Harris^{a,*}

^aDivision of Infectious Diseases, School of Public Health, University of California at Berkeley, Berkeley, CA 94720-7360, USA

^bGraduate Group in Microbiology, University of California at Berkeley, Berkeley, CA 94720, USA

^cDepartment of Molecular and Cell Biology, University of California at Berkeley, Berkeley, CA 94720, USA

Received 18 April 2003; returned to author for revision 15 August 2003; accepted 30 September 2003

Abstract

Dengue virus (DEN) causes the most prevalent arthropod-borne viral illness in humans worldwide. Immune mechanisms that are involved in protection and pathogenesis of DEN infection have not been fully elucidated due largely to the lack of an adequate animal model. Therefore, as a first step, we characterized the primary immune response in immunocompetent inbred A/J mice that were infected intravenously with a non-mouse-adapted DEN type 2 (DEN2) strain. A subset (55%) of infected mice developed paralysis by 14 days post-infection (p.i.), harbored infectious DEN in the central nervous system (CNS), and had an elevated hematocrit and a decreased white blood cell (WBC) count. Immunologic studies detected (i) increased numbers of CD69⁺ splenic natural killer (NK) and B cells at day 3 p.i., (ii) DEN-specific IgM and IgG responses by days 3 and 7 p.i., respectively, and (iii) splenocyte production of IFN γ at day 14 p.i. We conclude that the early activities of NK cells, B cells and IgM, and later actions of IFN γ and IgG likely play a role in the defense against DEN infection.

© 2004 Elsevier Inc. All rights reserved.

Keywords: Dengue virus; Flavivirus; Immunity; Mouse model

Introduction

The four dengue virus (DEN) serotypes cause dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) in humans, with an estimated 100 million new cases of DF and over 250000 cases of DHF/DSS per year worldwide (Burke and Monath, 2001). DEN belongs to the *Flavivirus* genus of the *Flaviviridae* family of single-stranded, positive-polarity, enveloped RNA viruses, which also includes yellow fever (YF) and the Japanese, St. Louis, and West Nile encephalitis viruses (Burke and Monath, 2001; Chambers et al., 1990). Primary infection typically leads to DF, an acute febrile illness that is characterized by headache, retro-orbital pain, arthralgia, and myalgia, and some DF patients may experience neurologic complications, including vertigo, mental irritability, and depression (George and Lum, 1997). In comparison,

secondary infection by a different serotype, as well as some primary infections, may result in the severe, life-threatening DHF/DSS. Clinically, DHF/DSS manifests as DF with increased vascular permeability, thrombocytopenia, focal, or generalized hemorrhages, and in cases of DSS, shock (Halstead, 1988). In rare instances, DHF/DSS may involve severe central nervous system (CNS) symptoms, such as reduced consciousness, convulsions, and encephalitis (Cam et al., 2001; Ramos et al., 1998; Solomon et al., 2000). Uncontrolled urbanization and globalization have resulted in the geographic spread of the DEN-transmitting *Aedes aegypti* and *Aedes albopictus* mosquitoes, co-circulation of different DEN serotypes, and increased frequency of dengue epidemics, culminating in the emergence and spread of DHF/DSS (Gibbons and Vaughn, 2002; Rigau-Perez et al., 1998). Thus, dengue is now a major public health problem throughout the world, with no specific treatment or vaccine currently available.

The immune response to DEN infection is poorly defined, and the immune system may have both protective and pathogenic roles. In vitro studies suggest that the protective nature of the immune system involves interferons (IFNs)

* Corresponding author. Division of Infectious Diseases, School of Public Health, University of California at Berkeley, 140 Warren Hall, Berkeley, CA 94720-7360. Fax: +1-510-642-6350.

E-mail address: eharris@socrates.berkeley.edu (E. Harris).

(Diamond and Harris, 2001; Diamond et al., 2000b; Kurane et al., 1998), antibodies (Abs) (Falgout et al., 1990; Gentry et al., 1982; Kurane et al., 1986; Russell et al., 1967), and T cells (Kurane et al., 1989; Mathew et al., 1996). In addition, *in vitro* as well as clinical and epidemiological studies suggest a role for host cross-reactive Abs and DEN serotype-cross-reactive Abs and T cells in DEN pathogenesis (Halstead, 1988; Innis, 1997; Rothman and Ennis, 1999). However, without an animal model for DEN infection, the exact mechanisms by which the various immune components protect against DEN infection or contribute to DHF/DSS cannot be clarified. Therefore, to investigate the role of the immune system in DEN infection *in vivo*, an adequate animal model for primary DEN infection is an essential first step.

The role of the immune system *in vivo* has not been examined in the majority of published murine models for DEN infection because they are created using either outbred mouse strains whose immune system may vary from one mouse to another, or mouse brain-adapted DEN strains with uncertain relevance to human infection. Specifically, the Swiss albino mouse model (Chaturvedi et al., 1991) involves outbred mice with variable genetic backgrounds, although the IFN α , β , and γ receptor knock-out (Johnson and Roehrig, 1999) and BALB/c models (Atraseuskaya et al., 2003; Hotta et al., 1981) use mouse-adapted DEN. The chimeric severe combined immunodeficient (SCID) mice, transplanted with human peripheral blood mononuclear cells (PBMCs) (Wu et al., 1995), erythroleukemic K562 cells (Lin et al., 1998), or HepG2 hepatocarcinoma cells (An et al., 1999), are complicated by variable levels of human cell engraftment and the altered nature of the murine immune response to both human cells and DEN. To date, the most suitable murine model for investigating the role of the immune system in DEN infection has been the A/J inbred strain that is inoculated intravenously with a non-mouse-adapted DEN serotype 2 (DEN2) (Huang et al., 2000).

Since previously published models have not been extensively analyzed at the immunologic level, the current study was undertaken to characterize the primary immune response in A/J mice infected intravenously with a non-mouse-adapted DEN2 strain. This study describes the immunologic elements that are involved in the response of mice with primary DEN infection. Our results suggest that the early activities of natural killer (NK) cells, B lymphocytes, and IgM and later actions of IFN γ and IgG may be important for clearing primary DEN infection. In addition, this study extends published results by demonstrating that the symptomatic mice have (i) increased hematocrit levels, (ii) decreased white blood cell (WBC) counts, and (iii) infectious DEN in the spinal cord as well as the brain. These data provide valuable information for further optimization of a mouse model for DEN infection and disease. The immunologic data indicate that the mouse model for primary DEN infection has considerable potential for dissecting

the interactions between specific elements of the immune system and DEN.

Results

Virologic characteristics

We characterized the A/J mouse model at multiple levels to define the role of the immune system in protection against primary DEN infection. A/J mice were intravenously inoculated with 10^8 PFU of non-mouse-adapted DEN2 (Taiwanese strain PL046), and a subset (55%) of DEN-infected mice exhibited paralysis and death within 8–14 days post-infection (p.i.) (Fig. 1). These results are consistent with a previously published report using the same DEN strain and dose, mouse strain, and route of infection (Huang et al., 2000). The presence of infectious virus in both the brain and spinal cord from paralytic mice, but not from asymptomatic mice, was confirmed by indirect plaque assays (data not shown). The brain and spinal cord samples from all the paralytic mice harbored DEN2 RNA, as quantitated by real-time RT-PCR (Fig. 2). Serum, liver, spleen, lymph nodes, and lung from paralytic mice did not contain detectable levels of DEN by either plaque assays or real-time RT-PCR. These results indicate that a significant number of A/J mice infected with DEN2 develop paralysis and harbor infectious virus in the central nervous system (CNS).

Hematologic findings

Clinical parameters of DEN infection in humans include an elevated hematocrit and a decreased WBC count (Green et al., 1999a; Kalayanarooj et al., 1997; WHO, 1997);

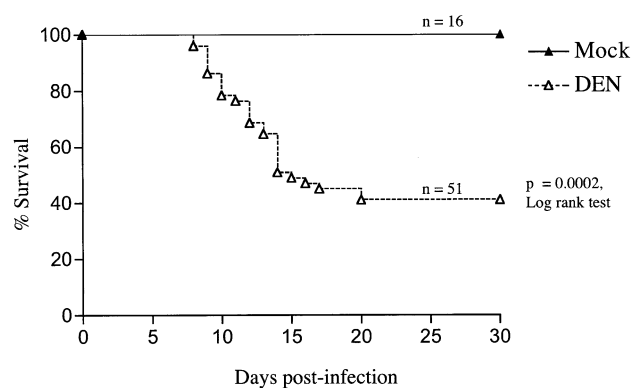


Fig. 1. Susceptibility of A/J mice to primary DEN infection. A/J mice were inoculated via tail-vein with 10^8 PFU of DEN2, PL046 strain, (DEN group; $n = 51$). The control mice (Mock) included mice injected with PBS ($n = 10$) or with heat-inactivated 10^8 PFU of DEN2 ($n = 6$). Mice were euthanized as soon as they developed paralysis, as per guidelines of the Office of Laboratory Animal Care at UC Berkeley. Kaplan–Meier analysis was performed, and the log rank test yielded $P = 0.0002$ for mock- versus DEN-infected mice. The survival curves shown represent combined data from three separate experiments.

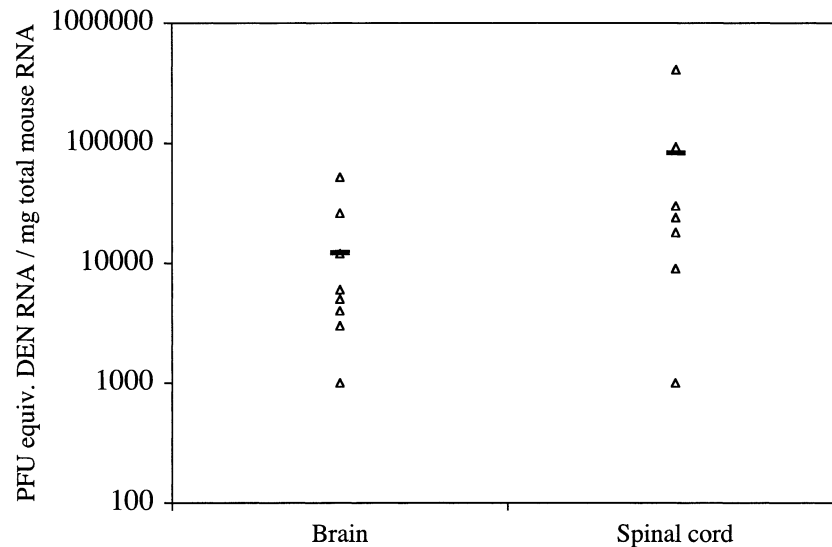


Fig. 2. Level of DEN RNA in the brain and spinal cord of DEN-infected A/J mice. DEN RNA was isolated from the brain and spinal cord from nine mice with paralysis and was quantified using real-time RT-PCR. Values are expressed as PFU equivalents of DEN RNA per milligram of total mouse RNA. Results from individual mice are shown as open triangles (Δ), while the mean is indicated by a dash (-). The paralytic mice from two independent experiments are represented, and the limit of detection of the assay was 1000 PFU equivalents of DEN RNA per milligram of total mouse RNA.

therefore, both the hematocrit and WBC count were monitored in the infected A/J mice. As compared to the mock- and DEN-infected mice with no signs of illness at day (d) 14 p.i., the DEN-infected mice with paralysis had a significantly increased hematocrit ($P < 0.001$) and a decreased WBC count ($P < 0.001$) (Fig. 3). In contrast, at both d3 and d7 p.i., all DEN-infected mice presented hematocrits and WBC counts that were similar to the mock-infected animals (data not shown). Thus, the observed hematologic abnormalities of symptomatic DEN-infected mice appear to be consistent with findings in DEN-infected humans.

Characterization of the immune response

To begin to characterize the primary immune response to DEN infection in A/J mice, the phenotype of immune cells in the spleen was assessed by flow cytometric analysis. As a control for specificity of the immune response, studies were conducted using mice that were infected with the 17D vaccine strain of yellow fever (YF), another flavivirus. Spleen cells were gated according to the forward and side scatter parameters, and the number of lymphocytes in the mock- and DEN-infected mice was found approximately equal at 3, 7, and 14 days p.i. (data not shown). Analyses of the gated cells for the expression of markers for dendritic cells (DCs), macrophages, and granulocytes revealed little to no difference in the number of CD11c⁺, F4/80⁺, and Gr1⁺ cells (data not shown). In contrast, 3 days after infection, the DEN-infected mice had two times more CD3⁻DX5⁺NK cells than the mock-infected mice ($P < 0.001$) (Fig. 4A), and more than 50% of these NK cells expressed the early activation marker CD69, although only 5–10% of NK cells

in the mock-infected animals expressed CD69 (Fig. 4B). Likewise, at d3 p.i., increased numbers of CD3⁻DX5⁺NK cells were present in the spleen of mice infected with live YF, as compared to that of mice injected with heat-inactivated YF (Fig. 4A). However, the numbers of CD69⁺-activated NK cells in the heat-inactivated YF- and live YF-infected mice were similar and were significantly lower than in DEN-infected mice (Fig. 4B). In addition to NK cells, significant numbers of B and T cells in the spleen of DEN-infected mice, but not in the mock-infected controls, expressed CD69 at d3 p.i. (Fig. 5). Ten times more CD19⁺CD69⁺B cells (Fig. 5A) and five times more CD3⁺CD69⁺T (both CD4⁺ and CD8⁺ subsets) cells were detected in DEN-infected mice than in mock-infected mice (Fig. 5B), revealing a greater increase in the number of activated B cells as compared to the number of activated T cells. The overall percentages of CD69⁺CD19⁺, CD69⁺CD4⁺, and CD69⁺CD8⁺ lymphocytes in the spleen of DEN-infected mice were 6.30%, 2.46%, and 1.58%, respectively (Fig. 5C). In comparison, the spleen from mice infected with live YF did not contain significantly higher numbers and percentages of CD69⁺B or T cell subsets than the spleen from mice that were injected with heat-inactivated YF (Figs. 5A, B). The percentages of CD69⁺CD19⁺, CD69⁺CD4⁺, and CD69⁺CD8⁺ splenocytes in YF-infected mice were 0.74%, 1.26%, and 0.71%, respectively. By d7 p.i., the numbers of NK cells, CD69⁺NK cells, CD69⁺B cells, and CD69⁺T cells in DEN-infected mice declined to the levels observed in mock-infected mice (Figs. 4 and 5). These data indicate that primary DEN infection in mice results in an early increase in (i) the number and activation of splenic NK cells and (ii) the number of activated B and T cells in the spleen. Moreover, this activation of NK, B, and T cells is observed only in response to DEN, but not to YF

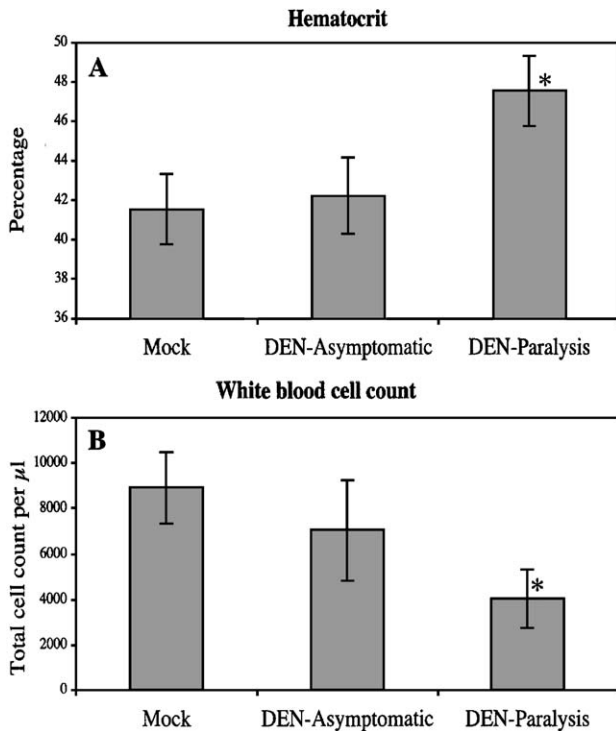


Fig. 3. Elevated hematocrit and reduced WBC count in paralytic A/J mice. Blood was collected from mice with paralysis between days 8–14 p.i., and the hematocrit (A) and WBC count (B) were determined manually. Values were compared to control samples obtained from mock-infected and asymptomatic DEN-infected mice at d14 p.i., because no difference was observed in results obtained from the control mice examined on days 8–14 p.i. Data are shown as the averages of results from two different experiments \pm SD, with $n = 12$, $n = 11$, and $n = 15$ for the mock, DEN-asymptomatic, and DEN-paralysis groups, respectively. The mock group of mice included those injected with PBS ($n = 10$) or with heat-inactivated DEN ($n = 5$). Two-tailed Student's *t* Tests were performed to obtain *P* values (*). Statistical values for the hematocrits (A): $P = 0.39$ (mock vs. DEN-asymptomatic); $P < 0.001$ (mock vs. DEN-paralysis), $P < 0.001$ (DEN-asymptomatic vs. DEN-paralysis). Statistical values for the WBC counts (B): $P < 0.05$ (mock vs. DEN-asymptomatic); $P < 0.001$ (mock vs. DEN-paralysis), $P < 0.001$ (DEN-asymptomatic vs. DEN-paralysis).

(17D) infection, although both viruses induce an increase in NK cell number.

To investigate the biological significance of early NK cell activation in the spleen, the levels of IFN γ and TNF α were measured in supernatants from unstimulated 24-h splenocyte cultures by ELISA. A significant amount of IFN γ was present in the spleen culture supernatants from DEN-infected mice on d14 p.i., but none (limit of detection = 15 pg/ml) was detected from d3 and d7 p.i. splenocytes (Fig. 6A). IFN γ was absent in the culture supernatants of spleen cells harvested from the mock-infected mice at d3, d7, and d14 p.i. (Fig. 6A). To determine the cellular source of IFN γ production, CD4 $^{+}$ and CD8 $^{+}$ T cells were lysed via treatment of splenocytes with Ab and complement. Fig. 6B shows decreased levels of IFN γ in splenocytes that were treated either with anti-CD4 and complement, or with both anti-CD4 and anti-CD8 and complement, indicating that CD4 $^{+}$ T cells are the major

producers of IFN γ . In contrast, splenocytes treated with anti-CD8 and complement did not demonstrate a decrease in IFN γ production. In comparison, TNF α was undetectable in spleen cell cultures from either mock- or DEN-infected mice at all three times post-infection (data not shown; limit of detection = 100 pg/ml). These results indicate that splenocytes produce IFN γ , but not TNF α , in response to DEN infection later in the course of infection, and that CD4 $^{+}$ T cells, but not CD8 $^{+}$ T cells, are the major source of this IFN γ secretion. Since the kinetics of IFN γ production by spleen cells does not appear to correlate with the early activation of NK cells in the spleen, IFN γ production might not be a major effector function of NK cells during early DEN infection.

To explore the significance of the early activation of B cells in the spleen of DEN-infected mice, the Ab response

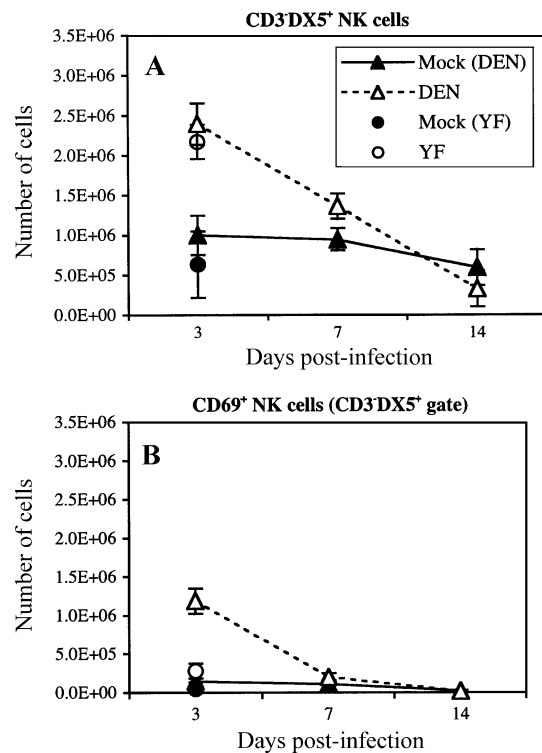


Fig. 4. Early increase in the numbers of CD3 $^{-}$ DX5 $^{+}$ NK cells and CD3 $^{-}$ DX5 $^{+}$ CD69 $^{+}$ -activated NK cells in the spleen of mice with primary DEN infection. Cells were isolated from mock (▲)- and DEN (△)-infected mouse spleens at d3, d7, and d14 p.i., counted by hemocytometer, and triply stained with fluorescent Abs against cell surface markers CD3, DX5, and CD69 for flow cytometric analysis. The percentage of fluorescence-positive cells out of 50 000 gated events was multiplied by the total spleen cell number, and results were graphed as the average number of lymphocytes ($n = 4-6$ mice) \pm SD. The mock-infected group included mice injected with PBS alone ($n = 2-3$ mice) or heat-inactivated DEN ($n = 2-3$ mice). Similar results were obtained in two additional experiments. As a control for specificity, splenocytes were isolated from A/J mice that were intravenously inoculated with 10^8 PFU of the YF vaccine strain 17D (○) (YF group; $n = 3$ mice) or 10^8 PFU of heat-inactivated YF (●) (mock group; $n = 3$) at d3 p.i. Cells were processed for flow cytometric analysis exactly as performed for cells from DEN-infected mice. This experiment was performed twice with similar results.

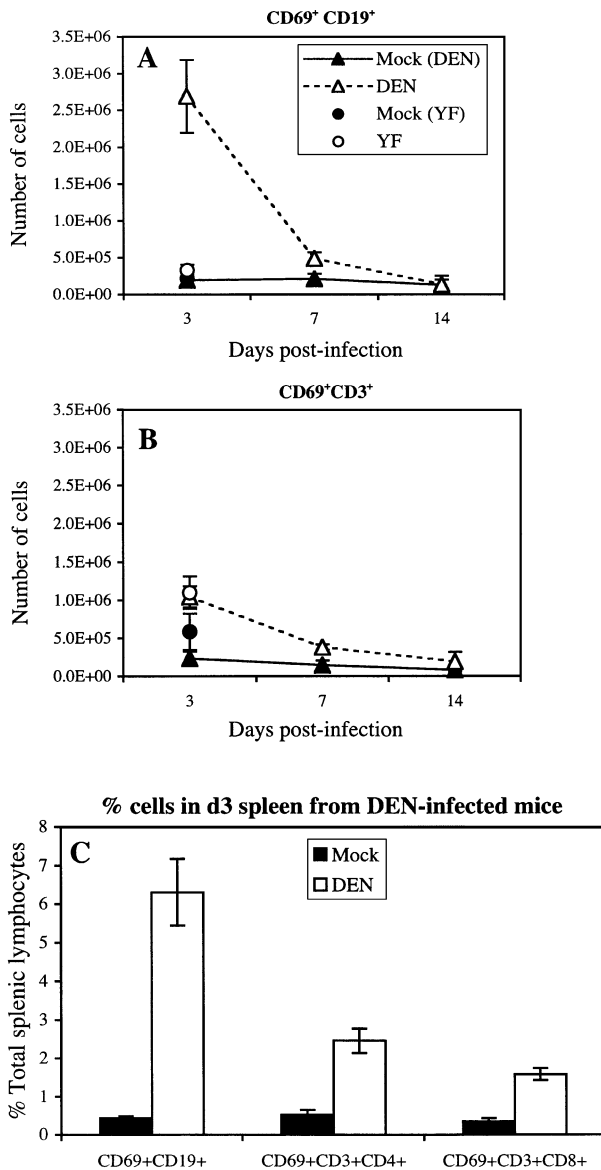


Fig. 5. A larger increase in the number and percentage of CD69⁺CD19⁺ B cells than of CD69⁺CD3⁺ T cells in the spleen of DEN-infected mice at d3 p.i. Cells from mock (▲)- and DEN (Δ)-infected mouse spleens at d3, d7, and d14 p.i. were doubly or triply stained with fluorescent Abs against cell surface markers CD69, CD19, CD3, CD4, and/or CD8, and analyzed by flow cytometry. Mock-infected mice were injected with either PBS ($n = 2-3$ mice) or heat-inactivated DEN ($n = 2-3$ mice). A minimum of 50,000 events was gated, and data were plotted as the mean number (panels A and B) or percentage (panel C) of positive events \pm SD ($n = 4-6$ mice). Similar data were reproduced in three independent experiments. In a control experiment, spleen cells were isolated from A/J mice that were injected with 10^8 PFU of the YF vaccine strain 17D (○) (YF group; $n = 3$ mice) or 10^8 PFU of heat-inactivated YF (●) (mock group; $n = 3$) at d3 p.i. Cells were prepared for flow cytometry exactly as described for cells from DEN-infected mice. This experiment was conducted twice with similar results.

was examined. Isotype-specific ELISAs using DEN-infected and uninfected C6/36 cellular antigen were performed to measure the levels of DEN antigen-specific IgM and IgG. Similar results were obtained using an ELISA against purified envelope (E) protein (data not shown). DEN anti-

gen-specific IgM was detected in d3, d7, and d14 sera from both the mock- and DEN-infected mice, after subtracting binding to uninfected C6/36 cellular antigen (Fig. 7A). IgM binding to DEN antigen in sera from mock-infected mice at d3, d7, and d14 p.i. was low (mean titer \pm SD = $1/150 \pm 1/100$), but higher than negative controls consisting of no serum or serum from B cell-deficient mice (data not shown). As compared with the mock-infected mice, the IgM titers in sera from DEN-infected mice were significantly higher at all three time-points, and the titers of IgM Abs were greater in d7 (mean titer \pm SD = $1/1150 \pm 1/499$) and d14 sera (mean titer \pm SD = $1/2250 \pm 1/1140$) than in d3 sera (mean titer \pm SD = $1/900 \pm 1/623$), with the highest mean titers in d14 sera. In contrast, DEN-specific IgG Abs were undetectable in the mock-infected sera and d3 sera from DEN-infected mice and were present only in d7 and d14 sera from DEN-infected mice, with the highest titers in d14 sera (Fig. 7B). Together, these results demonstrate that (i) the mock-infected A/J mice have natural IgM Abs that react with DEN antigen, (ii) IgM

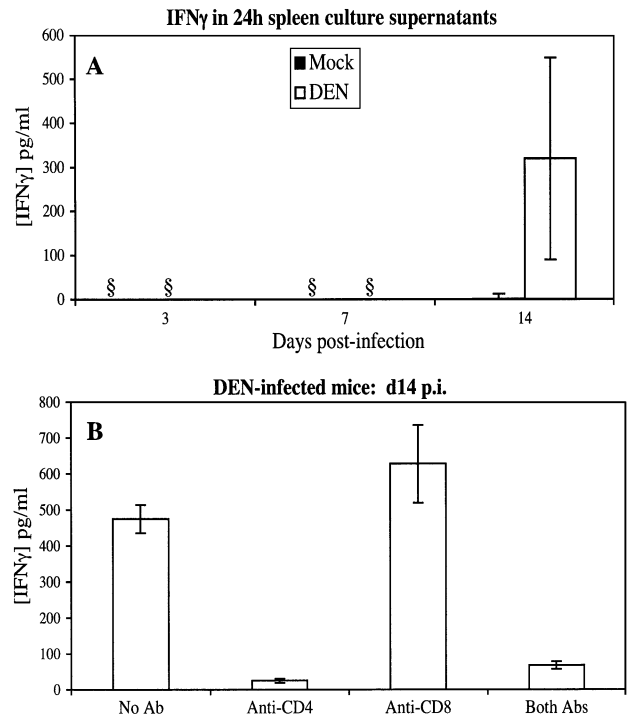


Fig. 6. IFN γ production by splenocytes from DEN-infected mice. Panel A: Ex vivo splenic leukocytes from mock- or DEN-infected A/J mice at d3, d7, and d14 p.i. were cultured for 24 h in the absence of stimuli, and IFN γ production in the culture supernatant was quantitated by ELISA. Results from two experiments were combined and graphed as the average \pm SD ($n = 4-8$ mice). The mock group included mice that received PBS ($n = 2$ mice) or heat-inactivated DEN ($n = 2$ mice). The symbol § indicates values that are below the limit of detection (15 pg/ml). Panel B: CD4⁺ and CD8⁺ T cells were lysed in splenocytes isolated from DEN-infected A/J mice at d14 p.i., as described in Methods, and were then cultured for 24 h in the absence of stimuli. The level of IFN γ in the culture supernatant was measured via ELISA. This experiment was performed twice ($n = 3$ mice). Anti-CD4 = splenocytes treated with anti-CD4 + complement; anti-CD8 = splenocytes treated with anti-CD8 + complement; both Abs = splenocytes treated with anti-CD4 + anti-CD8 + complement.

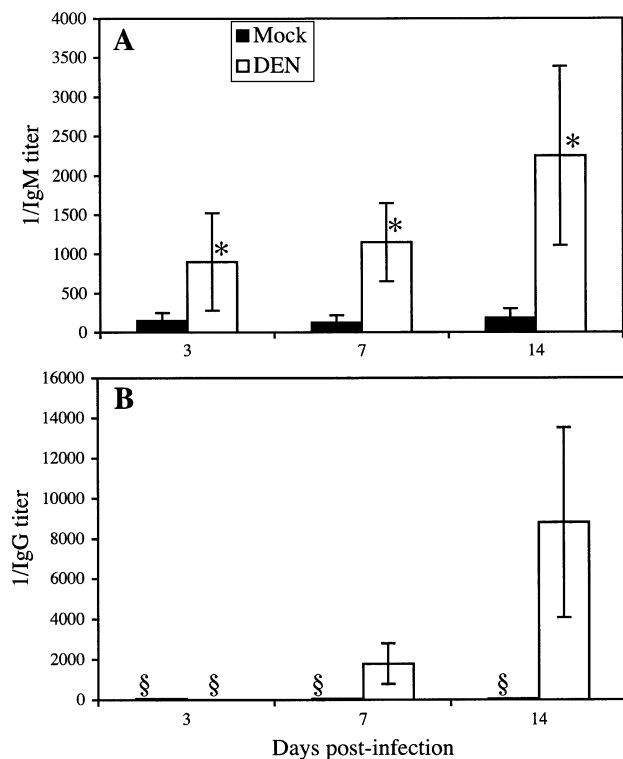


Fig. 7. Production of DEN antigen-specific Ab titers. Sera from PBS-injected (Mock) or DEN-infected (DEN) A/J mice were used to measure DEN antigen-specific Ab titers by ELISA, as described in Methods. Results from two experiments were combined and plotted as the mean \pm SD ($n = 4$ –6 mice in the mock group and $n = 6$ –10 mice in the DEN group). Asterisks (*) in panel A indicate statistically significant differences in the means between the mock- and DEN-infected mice, as determined by two-tailed Student's t tests: $P < 0.05$, d3 sera; $P < 0.001$, d7 sera; $P < 0.001$, d14 sera. In panel B, § symbols represent 1:100 serum dilutions that are below twice the background level.

Abs against DEN are produced by d3 p.i. and anti-DEN IgG Abs are produced later, between d3 and d7 p.i., and (iii) levels of both anti-DEN IgM and anti-DEN IgG Abs remain high at d14 p.i.

Discussion

Although various murine models for DEN infection have been reported, the A/J model is the only immunocompetent strain with several desirable features for the analysis of the immune response to DEN infection in vivo. Unlike the models that have been created using outbred mice, mouse brain-adapted DEN, or an intracranial route of infection, the A/J model involves an inbred mouse strain with a uniform genetic background, a non-mouse brain-adapted DEN strain that has not acquired mutations during mouse brain passage, and an intravenous infection route that may be more physiologically relevant (Huang et al., 2000). In this study, we used the A/J model to characterize for the first time the primary immune response to DEN infection in vivo, and found that DEN infection induced an early increase in the

number and activation of NK cells, early B and T cell activation, DEN-specific IgM and IgG production, and late IFN γ production by CD4⁺T cells.

Virologic characteristics and hematologic findings

A subset of A/J mice in this study developed paralysis between d8 and d14 p.i., and the brain as well as the spinal cord contained infectious virus. Similar to published mouse models for DEN infection, our infected mice exhibited paralysis within 2 weeks of infection, and tissue surveys for DEN infection showed that the brain increasingly accumulated a high viral load as the infection proceeded. Compared with other models, such as the intraperitoneal infection of IFN receptor-deficient 129/Sv/Ev mice with a mouse-adapted DEN (Johnson and Roehrig, 1999), the level of DEN in the infected A/J brain was lower at d14 p.i. and was undetectable in the spleen of infected mice at an early time after infection (data not shown), suggesting that differences in the viral and mouse strains and route of infection may control the level of viral replication and site of DEN tropism. In addition, viral dose was an important parameter of infection in our model, because paralysis was observed in mice challenged with 10^8 PFU, but not with 10^7 PFU (data not shown). However, A/J mice inoculated with 10^8 PFU of 16681, another non-mouse-adapted DEN2 strain, failed to show clinical symptoms (data not shown), implying a role for viral determinants in the outcome of infection. Comparison of PL046 with other known DEN2 sequences, together with mutational analysis, should help identify the virulence determinants in PL046.

DEN-infected A/J mice with paralysis had an elevated hematocrit and decreased WBC count, as compared to mock-infected and asymptomatic DEN-infected mice. Dehydration may have contributed to increased hematocrit, as DEN-infected paralytic mice have a compromised ability to move; however, an increased hematocrit was also observed in the HepG2-SCID mouse chimera model for DEN infection (An et al., 1999). This study using A/J mice is the first mouse model to show decreased WBC counts in response to disease onset. Further studies, including differential counts, flow cytometry, platelet counts, and serum albumin level, are required to understand the significance of this observation and to extend the relevance of these findings to human disease. Still, the A/J model may exhibit some key features of human DEN infection, and it may be used to elucidate the mechanisms that are responsible for causing hematologic dysfunctions, including decreased WBC count and elevated hematocrit.

Characterization of the immune response

Increased number and activation of NK cells

Phenotypic analysis of spleen cells identified the immune cell types that expressed CD69, a transient cell activation marker, early in response to DEN infection. Spleen cells

from DEN-infected mice at d3 p.i. had higher numbers of NK cells and activated NK cells, in the absence of an increase in the numbers of total splenocytes. These results support a published observation that peripheral blood mononuclear cell (PBMC) samples obtained from dengue patients early in the course of illness had significantly more CD69⁺NK cells than those from patients with other febrile illnesses (Green et al., 1999a). Thus, both the mouse and human findings imply a potential role for activated NK cells early in DEN infection. In fact, a role for NK cells during initial DEN infection has previously been implied by studies demonstrating that human blood NK cells are cytotoxic against DEN-infected targets (Kurane et al., 1984), and that infection of human cells with DEN leads to an upregulation of MHC class I cell surface expression, perhaps as a means to evade the early NK cell recognition of non-MHC I-expressing cells (Lobigs et al., 1996). Interestingly, A/J mice possess lower numbers of NK cells in the blood and spleen than C57BL/6 mice (Whyte and Miller, 1998), which are more resistant to infection with both DEN and MCMV (Brown et al., 2001; Lee et al., 2001). Thus, NK cells may be important in controlling DEN infection in vivo, as has been demonstrated for MCMV.

Early B cell activation and IgM production followed by later IgG production

Three days after infection, DEN-infected mice had a greater number of activated B cells in the spleen and a higher level of DEN antigen-specific IgM in sera, as compared to mock-infected animals. Early activation of B cells and IgM production have been documented in mice to be critical for clearing certain viruses, such as rotavirus (Blutt et al., 2002). Recently, the initial IgM-dependent early B cell response has been shown to play a key role in limiting West Nile virus (WNV) infection in mice as well (Diamond et al., 2003). Further studies are now required to determine the specificity and neutralizing ability of these anti-DEN IgM Abs, because humans with primary DEN infection generate an early DEN-specific IgM response with both a neutralizing and a cross-reactive nature (Innis, 1997). In particular, high levels of anti-DEN-NS1 IgM Abs have been found in patients with both primary and secondary DEN infections (Huang et al., 1999), and these Abs may cross-react with endothelial cellular antigens and damage the endothelial cells (Lin et al., 2003).

Notably, in our study, sera from mock-infected mice contained a low level of natural, pre-immune IgM Abs that bind to DEN. Similarly, natural IgMs that recognize certain influenza virus hemagglutinins have been demonstrated in a murine model of influenza virus infection (Baumgarth et al., 1999, 2000). These cross-reactive natural IgM Abs may provide a first line of defense by accelerating the development of the primary immune response, as demonstrated in experiments using natural serum IgM-deficient mice, whose major phenotype is a delayed IgG response (Ehrenstein et al., 1998). However, natural IgM Abs may also have a

pathogenic role. Specifically, lymphocytic choriomeningitis virus (LCMV)-induced hypergammaglobulinemia may be due to the isotype switching from natural IgM Abs (Hunziker et al., 2003). Thus, additional studies are needed to determine whether the observed natural IgM Abs that bind to DEN recognize other flaviviruses, as well as the specificity and neutralizing activity of these Abs.

Characterization of the IgG response in the A/J model revealed that no DEN-specific IgG Abs were found in sera from mock-infected mice, whereas anti-DEN IgG Abs were detected in d7 and d14 sera from DEN-infected mice, with a higher titer in d14 sera. These data agree with clinical findings, in which people develop a DEN-specific IgG response later in the course of a primary infection (Innis, 1997). Studies have shown that sera from infected individuals or anti-DEN monoclonal IgG Abs neutralize epitopes required for viral entry (Gentry et al., 1982; Russell and Nisalak, 1967) and opsonize DEN for complement-mediated lysis (Falgout et al., 1990) and Ab-dependent, cell-mediated cytotoxicity (ADCC) (Kurane et al., 1986). Several experiments have also supported a role for serotype-cross-reactive IgG Abs in the pathogenesis of DHF/DSS via the “Ab-dependent enhancement (ADE)” effect (Burke et al., 1988; Daughaday et al., 1981; Halstead and O’Rourke, 1977; Halstead et al., 1980). As a follow-up to these human studies, the A/J model could be used to examine the protective versus the pathogenic nature of both the IgM and IgG response to DEN infection. Initially, adoptive transfer experiments using naive versus DEN-immunized sera should determine whether pretreatment of mice with DEN-specific IgM or IgG Abs protects mice from DEN-induced disease.

IFN γ production

A significant level of IFN γ was detected in unstimulated spleen cell cultures from DEN-infected A/J mice on d14 p.i., but not from mock-infected mice, suggesting a role for IFN γ in primary DEN infection. IFN γ may also play both a protective and pathogenic role in DEN infection. The protective nature of IFN γ has been demonstrated by several studies: (i) IFN γ inhibits DEN infection in both transformed and non-transformed human cells (Diamond and Harris, 2001; Diamond et al., 2000b), and (ii) mice lacking receptors for both IFN α/β and IFN γ succumb to DEN infection, whereas wild-type counterparts survive (Johnson and Roehrig, 1999). The pathogenic nature of IFN γ has been implied by the findings that (i) some CD4⁺ cytotoxic T cell (CTL) clones with serotype-cross-reactivity produce significant levels of IFN γ (Gagnon et al., 1999), (ii) patients with DHF/DSS have higher levels of IFN γ , as compared to individuals with DF (Green et al., 1999b; Kurane et al., 1991b), and (iii) IFN γ enhances Fc γ receptor-mediated DEN infection of U937 cells, a human monocytic cell line (Kontny et al., 1988). Further studies using mice lacking IFN γ should help determine the precise role of this cytokine in DEN infection in vivo and are currently underway.

The lack of detectable IFN γ on d3, when the activation of splenic NK cells was observed, suggests that IFN γ production may not be a major effector mechanism of these activated NK cells and that direct antiviral activities of IFN γ at early times post-infection may be less important. Based on other viral infection models, IFN γ may be playing an immunomodulatory role, and T cells are the likely candidates to produce IFN γ at d14 p.i. (Hooper et al., 2002). Indeed, CD4⁺T cells, but not CD8⁺T cells, produced IFN γ at d14 p.i., and this result supports the observation that a higher frequency of CD44^{hi}CD4⁺T cells, which represent activated or memory CD4⁺T cells, was found in the d14 spleens of DEN-infected mice than in the spleens of mock-infected mice (data not shown). Importantly, several human studies have suggested a role for CD4⁺T cells in DEN infection. For example, PBMCs isolated from infected patients contain DEN-specific CD4⁺T cells that mediate cytotoxicity *in vitro* and secrete IFN γ after stimulation with DEN antigen (Bukowski et al., 1989; Kurane et al., 1991a). Again, further studies using wild-type and IFN γ -deficient mice should help determine the mechanisms by which CD4⁺T cells regulate the immune response to DEN infection *in vivo* through IFN γ production.

In summary, this study has extended the characterization of a more suitable mouse model for DEN infection. The new virologic, hematologic, and immunologic data, in combination, now set the stage for investigating both the host- and virus-specific mechanisms that control primary and sequential DEN infections. Understanding the cellular and molecular basis of DEN infection and disease should contribute to the development of effective therapeutics and vaccines as well as a better small animal model for vaccine testing.

Methods

Mice

Sex-matched, 4- to 5-week-old A/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and were housed under specific pathogen-free conditions in the biosafety level 2 (BSL2) animal facility at the University of California (UC), Berkeley. After 1 week of rest in the facility, 5- to 6-week-old mice were used for experiments. All experiments were approved and conducted as per the guidelines of the Office of Laboratory Animal Care at UC Berkeley.

DEN2 infection of mice

DEN2 strain PL046, a Taiwanese isolate, was originally obtained from Dr. Huan-Yao Lei (National Cheng Kung University, Taiwan). Viral stocks were grown in the *A. albopictus* C6/36 cell line using “complete L15,” which contained endotoxin-free L15 medium supplemented with 5% heat-inactivated fetal calf serum (FCS; Omega Scientific,

Tarzana, CA) for 5–7 days at 28 °C. Culture supernatants containing virus were harvested, frozen, and stored at –80 °C, as previously described (Diamond et al., 2000a, 2000b). Viral titer was determined by a standard plaque assay using BHK-21 cells (Diamond et al., 2000a, 2000b). Virus stocks from the same passage ($P = 2$) were used to infect all animals in this study. For injection into mice, frozen stocks containing 5×10^6 to 5×10^7 plaque forming units (PFU)/ml were thawed, concentrated by ultracentrifugation ($43\,000 \times g$ for 2.5 h), and resuspended in endotoxin-free, ice-cold phosphate-buffered saline (PBS). An inoculum containing 10^8 PFU in 100–200 μ l was injected into the tail vein of each experimental mouse, and the same volume of PBS or 10^8 PFU of heat-inactivated DEN (incubated at 100 °C for 5 min) was injected intravenously into each mock control mouse. In all experiments, no difference was observed between PBS- and heat-inactivated DEN-injected mice; thus, results from these two groups were pooled and designated as the mock values. The exact numbers of mice per group are indicated in the figure legends. Since 100% of mice inoculated with 10^7 PFU of DEN survived, and no significant difference was found in mice infected with 10^8 PFU versus 2×10^8 PFU, results from experiments using only 10^8 PFU are shown in all figures.

Quantitation of virus in infected mice

To assess viral burden in tissues of infected mice, organs were harvested, weighed, and homogenized using zirconia/silica beads (1.0 mm diameter) with a Mini-Beadbeater-8 apparatus (BioSpec Products, Bartlesville, OK), and microcentrifuged (10 min, 4 °C, 14000 rpm) to pellet debris. Serum was isolated from whole blood that was obtained via a cardiac puncture of mice immediately after euthanasia by isofluorane inhalation. The following three different assays were performed to measure the amount of infectious virus or viral RNA in supernatants of tissue homogenate and serum samples:

- (i) Direct plaque assay: Infectious virus in 1:10 serial dilutions of organ homogenates was titered using a standard plaque assay on BHK-21 cells, as above.
- (ii) Indirect plaque assay: Supernatants of tissue homogenates (75 or 7.5 μ l/well for liver) and sera (7.5 μ l/well) were incubated with C6/36 cells in a 24-well plate (3×10^5 cells/0.25 ml complete L15 medium/well) at 28 °C. Following an overnight incubation, an additional 0.5 ml/well of complete L15 medium was added. After 6–7 days, culture supernatant was harvested and titered in plaque assays using BHK-21 as described above.
- (iii) Real-time RT-PCR: For analysis of total viral RNA in tissues, samples were submerged into RNAlater (Qiagen, Valencia, CA) immediately after dissection and kept at 4 °C overnight, followed by long-term storage at –80 °C. Frozen samples were thawed and homogenized using the Mini-Beadbeater-8 just before the isolation of

total RNA, which was performed using the RNEasy minikit (Qiagen). Viral RNA from thawed aliquots of serum (10–15 µl) was isolated using a Qia-Amp viral RNA recovery kit (Qiagen). RNA samples, eluted in RNase-free water, were snap-frozen in liquid nitrogen for storage. Quantitative measurements of total viral RNA were obtained using Taqman reagents (One Step RT-PCR Kit; Applied Biosystems, Foster City, CA) and an ABI PRISM 7700 sequence detection system (Applied Biosystems) according to a published protocol (Houng et al., 2000). To control for RNA content and quality, the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in each sample was measured using a Taqman rodent GAPDH control kit (Applied Biosystems) in parallel real-time RT-PCR reactions. A four-point standard curve was run on each plate for determining the DEN and GAPDH RNA levels. To generate the DEN standard curve, viral RNA was isolated from a sample containing a known number of PFU, and real-time RT-PCR reactions were performed with serial dilutions of this RNA. The GAPDH standard curve was based on known amounts of total mouse RNA that was provided by the manufacturer (Applied Biosystems). Samples were run in duplicate, and viral RNA was quantitated as the PFU of DEN RNA per milligram of total mouse RNA. Each PFU of DEN contains approximately 1×10^3 to 5×10^3 copies of viral RNA, as measured by real-time PCR in comparison with known numbers of viral RNA molecules (Edgil et al., in press).

Preparation of spleen cell suspension

Spleens were harvested and placed in complete RPMI medium consisting of RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FCS (Omega Scientific), 10 mM HEPES, 200 mM L-glutamine, 10000 U/ml penicillin and streptomycin, 50 mM 2-mercaptoethanol, and 1% nonessential amino acids. Single-cell suspensions were made by mechanically disrupting the tissue between the frosted ends of two glass slides. Red blood cells were lysed using a buffered ammonium chloride (ACK) solution, and cell suspensions were washed and resuspended in complete RPMI 1640. Aliquots of cell suspensions were stained with trypan blue, and viable cells were counted using a hemocytometer. To test for the spontaneous secretion of cytokines, splenocytes were incubated in 96-well round-bottom plates (10^6 cells/well, in triplicate) for 24 h in complete RPMI 1640 (200 µl/well), followed by centrifugation ($450 \times g$ for 5 min) to pellet cells; then the supernatant was harvested and stored at -20°C . To identify the cell types that produced IFN γ , CD4 $^+$, and CD8 $^+$ T cell subsets in the spleen cell suspension were depleted via a standard complement-mediated lysis protocol. Briefly, splenocytes were incubated with anti-mouse CD4 (clone RL172) and anti-mouse CD8 (clone 3.155)

hybridoma supernatants (10^7 cells/ml, 1 h, 4°C), washed, and resuspended in complete RPMI 1640 (10^7 cells/ml). Cells were incubated with rabbit and guinea pig complement (1/16 of total cell volume, 1 h, 37°C), washed, and allowed to settle. Cell debris was removed, viable cells were counted via trypan blue exclusion, and cells were then plated in 96-well round-bottom plates, as described above.

Cytokine ELISAs

To determine IFN γ and TNF α levels in spleen culture supernatants and sera, standard sandwich ELISAs were performed. To detect mouse IFN γ , anti-mouse IFN γ clone R4-6A2 (Caltag Laboratories, Burlingame, CA) and biotinylated anti-mouse IFN γ clone XMG1.2 (Caltag Laboratories) were used as capture and detection Abs, respectively. To assay for mouse TNF α , anti-mouse TNF α clone 1F3F3D4 (eBioscience, San Diego, CA) was used as the capture Ab, in combination with biotinylated anti-mouse TNF α cocktail (clones MP6-XT3 and MP6-XT22; eBioscience) as the detection Abs. All procedures were performed according to manufacturers' protocols, and the limits of detection were 15 and 100 pg/ml for IFN γ and TNF α , respectively. Colorimetric changes of tetramethyl benzidine (TMB) enzyme substrate (Sigma Co., St. Louis, MO) were detected using a Bio-Tek ELx808 microplate reader (Bio-Tek Instruments, Winooski, VT) and KCjunior software (Bio-Tek Instruments).

Flow cytometry

Spleen cell suspensions were aliquoted ($1-2 \times 10^6$ cells/sample) and centrifuged at $220 \times g$, and the cell pellets were resuspended in 100 µl of supernatant from anti-mouse CD16 hybridoma (clone 24G2) to block Fc receptors (30 min, 4°C). Cells were then washed once in FACS buffer (PBS/2.5% FCS/0.05% NaN $_3$), followed by resuspension in FACS buffer containing fluorescein-labeled anti-mouse CD69 or CD11c ($0.1 \mu\text{g}/10^6$ cells; Pharmingen, San Diego, CA), Phycoerythrin (PE)-labeled anti-NK1.1 ($0.1 \mu\text{g}/10^6$ cells; Pharmingen), PE-Cy5-labeled anti-mouse CD3 ($0.1 \mu\text{g}/10^6$ cells; eBioscience), and TRI-COLOR-labeled anti-mouse CD4, CD8, CD19, F4/80, or Gr1 ($0.05 \mu\text{g}/10^6$ cells; Caltag Laboratories). Samples were incubated on ice for 30 min in the dark, washed twice with 200 µl of FACS buffer, resuspended in 200 µl of 1% paraformaldehyde, and stored at 4°C until they were analyzed using a Coulter EPICS XL-MCL (Beckman Coulter, Miami, FL) flow cytometer. Data were acquired and saved in the list mode and then reanalyzed using FlowJo software (Tree Star, San Carlos, CA).

ELISA for virus-specific antibody

C6/36 cells infected with DEN2 (PL046 strain) for 5 days and uninfected C6/36 cells were washed in PBS three times, freeze-thawed five times using dry ice/ethanol and

cold water baths, and coated overnight at 4 °C onto Nunc Maxisorp immunoplates (Nalge Nunc International, Roskilde, Denmark). Each 96-well plate was coated with both DEN-infected C6/36 (48 wells) and uninfected C6/36 (48 wells) lysates. The plates were washed twice with PBS/0.05% Tween-20, blocked with PBS/10% FCS/0.05% NaN₃ (2 h; RT), and washed three times in PBS/0.05% Tween-20. Twofold serum dilutions starting at 1:100 were added to the plates and incubated (3 h; RT), followed by five washes with PBS/0.05% Tween-20. Bound Ab was detected with IgM- or IgG-specific horseradish peroxidase-labeled goat anti-mouse antibodies (0.5 µg/ml; Southern Biotechnology Associates, Birmingham, AL), using TMB as the substrate (Sigma Co.). After stopping the reactions with 2 M H₂SO₄, the OD was measured at 450 nm in a Bio-Tek EL₈808 microplate reader (Biotek Instruments). The OD value for uninfected C6/36 cellular antigen was first subtracted from that of the corresponding wells coated with DEN2-infected C6/36 lysates to obtain the adjusted OD value, and then Ab titers were determined as the greatest serum dilution with an absorption of twice the background levels.

Hematocrit and WBC count

Immediately after a cardiac puncture, blood was collected using a 1-ml plastic transfer pipette and placed into an EDTA-coated Microtainer tube (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ). The anticoagulated blood sample was thoroughly mixed and processed using the Unopette microcollection system (Becton Dickinson Vacutainer Systems) for a manual WBC count on a hemocytometer. Thin blood smears were checked to verify the absence of clots, and clotted samples were discarded. The remaining blood sample was drawn into a micro-hematocrit capillary tube (Fisher Scientific, Pittsburgh, PA), which was then plugged at one end with Cha-seal (Fisher Scientific) and spun in a micro-hematocrit centrifuge for 5 min. After spinning, the percentage of total volume occupied by the packed red blood cells was read on a micro-hematocrit capillary tube reader (Sherwood Medical, St. Louis, MO).

Statistical analyses

Statistical analyses were performed in Microsoft Excel X (Microsoft Corporation, Seattle, WA), and values are shown as mean ± standard deviation (SD). To plot the outcome of infected mice, Kaplan–Meier analysis was performed using GraphPad Prism software, version 3.0cx (GraphPad Software, Inc., San Diego, CA).

Acknowledgments

We thank Dr. Huan-Yao Lei for providing DEN2 (PL046 strain) and Drs. Michael Diamond, Joel Ernst, and David

Raulet for advice and helpful discussions. We thank Drs. Michael Diamond and Marija Helt for critical reading of the manuscript as well.

This work was supported by NIH (NRSA 1F32 AI51070-01 to SS), the Ellison Medical Foundation (EMF 1D-1A-0031 to EH), and the UC Berkeley Committee on Research (to EH).

References

- An, J., Kimura-Kuroda, J., Hirabayashi, Y., Yasui, K., 1999. Development of a novel mouse model for dengue virus infection. *Virology* 263, 70–77.
- Atrasheuskaya, A., Petzelbauer, P., Fredeking, T.M., Ignatyev, G., 2003. Anti-TNF antibody treatment reduces mortality in experimental dengue virus infection. *FEMS Immunol. Med. Microbiol.* 35, 33–42.
- Baumgarth, N., Herman, O.C., Jager, G.C., Brown, L., Herzenberg, L.A., 1999. Innate and acquired humoral immunities to influenza virus are mediated by distinct arms of the immune system. *Proc. Natl. Acad. Sci. U.S.A.* 96, 2250–2255.
- Baumgarth, N., Chen, J., Herman, O.C., Jager, G.C., Herzenberg, L.A., 2000. The role of B-1 and B-2 cells in immune protection from influenza virus infection. *Curr. Top. Microbiol. Immunol.* 252, 163–169.
- Blutt, S.E., Warfield, K.L., Lewis, D.E., Conner, M.E., 2002. Early response to rotavirus infection involves massive B cell activation. *J. Immunol.* 168, 5716–5721.
- Brown, M.G., Dokun, A.O., Heusel, J.W., Smith, H.R., Beckman, D.L., Blattenberger, E.A., Dubbelde, C.E., Stone, L.R., Scalzo, A.A., Yokoyama, W.M., 2001. Vital involvement of a natural killer cell activation receptor in resistance to viral infection. *Science* 292, 934–937.
- Bukowski, J.F., Kurane, I., Lai, C.J., Bray, M., Falgout, B., Ennis, F.A., 1989. Dengue virus-specific cross-reactive CD8⁺ human cytotoxic T lymphocytes. *J. Virol.* 63, 5086–5091.
- Burke, D.S., Monath, T.P., 2001. Flaviviruses. In: Knipe, D.M., Howley, P.M. (Eds.), 4th ed. *Fields Virology*, vol. 1. Lippincott Williams and Wilkins, Philadelphia, pp. 1043–1126.
- Burke, D.S., Nisalak, A., Johnson, D.E., Scott, R.M., 1988. A prospective study of dengue infections in Bangkok. *Am. J. Trop. Med. Hyg.* 38, 172–180.
- Cam, B.V., Fonsmark, L., Hue, N.B., Phuong, N.T., Poulsen, A., Heegaard, E.D., 2001. Prospective case-control study of encephalopathy in children with dengue hemorrhagic fever. *Am. J. Trop. Med. Hyg.* 65, 848–851.
- Chambers, T.J., Hahn, C.S., Galler, R., Rice, C.M., 1990. Flavivirus genome organization, expression, and replication. *Annu. Rev. Microbiol.* 44, 649–688.
- Chaturvedi, U.C., Dhawan, R., Khanna, M., Mathur, A., 1991. Breakdown of the blood–brain barrier during dengue virus infection of mice. *J. Gen. Virol.* 72 (Pt. 4), 859–866.
- Daughaday, C.C., Brandt, W.E., McCown, J.M., Russell, P.K., 1981. Evidence for two mechanisms of dengue virus infection of adherent human monocytes: trypsin-sensitive virus receptors and trypsin-resistant immune complex receptors. *Infect. Immun.* 32, 469–473.
- Diamond, M.S., Harris, E., 2001. Interferon inhibits dengue virus infection by preventing translation of viral RNA through a PKR-independent mechanism. *Virology* 289, 297–311.
- Diamond, M.S., Edgil, D., Roberts, T.G., Lu, B., Harris, E., 2000a. Infection of human cells by dengue virus is modulated by different cell types and viral strains. *J. Virol.* 74, 7814–7823.
- Diamond, M.S., Roberts, T.G., Edgil, D., Lu, B., Ernst, J., Harris, E., 2000b. Modulation of dengue virus infection in human cells by alpha, beta, and gamma interferons. *J. Virol.* 74, 4957–4966.

- Diamond, M.S., Shrestha, B., Marri, A., Mahan, D., Engle, M., 2003. B cells and antibody play critical roles in the immediate defense of disseminated infection by West Nile encephalitis virus. *J. Virol.* 77, 2578–2586.
- Edgil, D., Diamond, M.S., Holden, K.L., Paranjape, S.M., Harris, E., in press. Translation efficiency determines differences in cellular infection among dengue virus type 2 strains. *Virology*.
- Ehrenstein, M.R., O'Keefe, T.L., Davies, S.L., Neuberger, M.S., 1998. Targeted gene disruption reveals a role for natural secretory IgM in the maturation of the primary immune response. *Proc. Natl. Acad. Sci. U.S.A.* 95, 10089–10093.
- Falgout, B., Bray, M., Schlesinger, J.J., Lai, C.J., 1990. Immunization of mice with recombinant vaccinia virus expressing authentic dengue virus nonstructural protein NS1 protects against lethal dengue virus encephalitis. *J. Virol.* 64, 4356–4363.
- Gagnon, S.J., Ennis, F.A., Rothman, A.L., 1999. Bystander target cell lysis and cytokine production by dengue virus-specific human CD4(+) cytotoxic T-lymphocyte clones. *J. Virol.* 73, 3623–3629.
- Gentry, M.K., Henchal, E.A., McCown, J.M., Brandt, W.E., Dalrymple, J.M., 1982. Identification of distinct antigenic determinants on dengue-2 virus using monoclonal antibodies. *Am. J. Trop. Med. Hyg.* 31, 548–555.
- George, R., Lum, L.C.S., 1997. Clinical spectrum of dengue infection. In: Gubler, D.J., Kuno, G. (Eds.), *Dengue and Dengue Hemorrhagic Fever*. CAB International, New York.
- Gibbons, R.V., Vaughn, D.W., 2002. Dengue: an escalating problem. *BMJ* 324, 1563–1566.
- Green, S., Pichyangkul, S., Vaughn, D.W., Kalayanaraj, S., Nimmannitya, S., Nisalak, A., Kurane, I., Rothman, A.L., Ennis, F.A., 1999a. Early CD69 expression on peripheral blood lymphocytes from children with dengue hemorrhagic fever. *J. Infect. Dis.* 180, 1429–1435.
- Green, S., Vaughn, D.W., Kalayanaraj, S., Nimmannitya, S., Suntayakorn, S., Nisalak, A., Lew, R., Innis, B.L., Kurane, I., Rothman, A.L., Ennis, F.A., 1999b. Early immune activation in acute dengue illness is related to development of plasma leakage and disease severity. *J. Infect. Dis.* 179, 755–762.
- Halstead, S.B., 1988. Pathogenesis of dengue: challenges to molecular biology. *Science* 239, 476–481.
- Halstead, S.B., O'Rourke, E.J., 1977. Antibody-enhanced dengue virus infection in primate leukocytes. *Nature* 265, 739–741.
- Halstead, S.B., Porterfield, J.S., O'Rourke, E.J., 1980. Enhancement of dengue virus infection in monocytes by flavivirus antisera. *Am. J. Trop. Med. Hyg.* 29, 638–642.
- Hooper, D.C., Sauder, C., Scott, G.S., Dietzschold, B., Richt, J.A., 2002. Immunopathology and immunoprotection in CNS virus infections: mechanisms of virus clearance from the CNS. *Curr. Top. Microbiol. Immunol.* 265, 163–182.
- Hotta, H., Murakami, I., Miyasaki, K., Takeda, Y., Shirane, H., Hotta, S., 1981. Inoculation of dengue virus into nude mice. *J. Gen. Virol.* 52, 71–76.
- Houng, H.H., Hritz, D., Kanesa-athan, N., 2000. Quantitative detection of dengue 2 virus using fluorogenic RT-PCR based on 3'-noncoding sequence. *J. Virol. Methods* 86, 1–11.
- Huang, J.H., Wey, J.J., Sun, Y.C., Chin, C., Chien, L.J., Wu, Y.C., 1999. Antibody responses to an immunodominant nonstructural 1 synthetic peptide in patients with dengue fever and dengue hemorrhagic fever. *J. Med. Virol.* 57, 1–8.
- Huang, K.-J., Li, S.-Y.J., Chen, S.-C., Liu, H.-S., Lin, Y.-S., Yeh, T.-M., Liu, C.-C., Lei, H.-Y., 2000. Manifestation of thrombocytopenia in dengue-2-virus-infected mice. *J. Gen. Virol.* 81, 2177–2182.
- Hunziker, L., Recher, M., Macpherson, A.J., Ciurea, A., Freigang, S., Hengartner, H., Zinkernagel, R.M., 2003. Hypergammaglobulinemia and autoantibody induction mechanisms in viral infections. *Nat. Immunol.* 4, 343–349.
- Innis, B.L., 1997. Dengue and dengue hemorrhagic fever. In: Gubler, D.J., Kuno, G. (Eds.), *Antibody Response to Dengue Virus Infection*. CAB International, Wallingford, Oxon, UK., pp. 221–243.
- Johnson, A.J., Roehrig, J.T., 1999. New mouse model for dengue virus vaccine testing. *J. Virol.* 73, 783–786.
- Kalayanaraj, S., Vaughn, D.W., Nimmannitya, S., Green, S., Suntayakorn, S., Kunentrasai, N., Viramitrachai, W., Ratanachu-ekke, S., Kiatpolpoj, S., Innis, B.L., Rothman, A.L., Nisalak, A., Ennis, F.A., 1997. Early clinical and laboratory indicators of acute dengue illness. *J. Infect. Dis.* 176, 313–321.
- Kontny, U., Kurane, I., Ennis, F.A., 1988. Gamma interferon augments Fc gamma receptor-mediated dengue virus infection of human monocytic cells. *J. Virol.* 62, 3928–3933.
- Kurane, I., Hebblewaite, D., Brandt, W., Ennis, F.A., 1984. Lysis of dengue-infected cells by natural cell-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity. *J. Virol.* 52, 223–230.
- Kurane, I., Hebblewaite, D., Ennis, F.A., 1986. Characterization with monoclonal antibodies of human lymphocytes active in natural killing and antibody-dependent cell-mediated cytotoxicity of dengue virus-infected cells. *Immunology* 58, 429–436.
- Kurane, I., Innis, B.L., Nisalak, A., Hoke, C., Nimmannitya, S., Meager, A., Ennis, F.A., 1989. Human T cell responses to dengue virus antigens. Proliferative responses and interferon gamma production. *J. Clin. Invest.* 83, 506–513.
- Kurane, I., Brinton, M.A., Samson, A.L., Ennis, F.A., 1991a. Dengue virus-specific, human CD4+CD8-cytotoxic T-cell clones: multiple patterns of virus cross-reactivity recognized by NS3-specific T-cell clones. *J. Virol.* 65, 1823–1828.
- Kurane, I., Innis, B.L., Nimmannitya, S., Nisalak, A., Meager, A., Janus, J., Ennis, F.A., 1991b. Activation of T lymphocytes in dengue virus infections. High levels of soluble interleukin 2 receptor, soluble CD4, soluble CD8, interleukin 2, and interferon-gamma in sera of children with dengue. *J. Clin. Invest.* 88, 1473–1480.
- Kurane, I., Zeng, L., Brinton, M.A., Ennis, F.A., 1998. Definition of an epitope on NS3 recognized by human CD4+ cytotoxic T lymphocyte clones cross-reactive for dengue virus types 2, 3, and 4. *Virology* 240, 169–174.
- Lee, S.H., Girard, S., Macina, D., Busa, M., Zafer, A., Belouchi, A., Gros, P., Vidal, S.M., 2001. Susceptibility to mouse cytomegalovirus is associated with deletion of an activating natural killer cell receptor of the C-type lectin superfamily. *Nat. Genet.* 28, 42–45.
- Lin, Y.L., Liao, C.L., Chen, L.K., Yeh, C.T., Liu, C.I., Ma, S.H., Huang, Y.Y., Huang, Y.L., Kao, C.L., King, C.C., 1998. Study of dengue virus infection in SCID mice engrafted with human K562 cells. *J. Virol.* 72, 9729–9737.
- Lin, C.F., Lei, H.Y., Shiau, A.L., Liu, C.C., Liu, H.S., Yeh, T.M., Chen, S.H., Lin, Y.S., 2003. Antibodies from dengue patient sera cross-react with endothelial cells and induce damage. *J. Med. Virol.* 69, 82–90.
- Lobigs, M., Blanden, R.V., Mullbacher, A., 1996. Flavivirus-induced up-regulation of MHC class I antigens: implications for the induction of CD8+ T-cell-mediated autoimmunity. *Immunol. Rev.* 152, 5–19.
- Mathew, A., Kurane, I., Rothman, A.L., Zeng, L.L., Brinton, M.A., Ennis, F.A., 1996. Dominant recognition by human CD8+ cytotoxic T lymphocytes of dengue virus nonstructural proteins NS3 and NS1.2a. *J. Clin. Invest.* 98, 1684–1691.
- Ramos, C., Sanchez, G., Pando, R.H., Baquera, J., Hernandez, D., Mota, J., Ramos, J., Flores, A., Llausas, E., 1998. Dengue virus in the brain of a fatal case of hemorrhagic dengue fever. *J. Neurovirol.* 4, 465–468.
- Rigau-Perez, J.G., Clark, G.G., Gubler, D.J., Reiter, P., Sanders, E.J., Vornadam, A.V., 1998. Dengue and dengue haemorrhagic fever. *Lancet* 352, 971–977.
- Rothman, A.L., Ennis, F.A., 1999. Immunopathogenesis of dengue hemorrhagic fever. *Virology* 257, 1–6.
- Russell, P.K., Nisalak, A., 1967. Dengue virus neutralization by the plaque reduction neutralization test. *J. Immunol.* 99, 291–294.
- Russell, P.K., Udomsakdi, S., Halstead, S.B., 1967. Antibody response in dengue and dengue hemorrhagic fever. *Jpn. J. Med. Sci. Biol.* 20, 103–108.
- Solomon, T., Dung, N.M., Vaughn, D.W., Kneen, R., Thao, L.T., Raengsakulrach, B., Loan, H.T., Day, N.P., Farrar, J., Myint, K.S., Warrell,

- M.J., James, W.S., Nisalak, A., White, N.J., 2000. Neurological manifestations of dengue infection. *Lancet* 355, 1053–1059.
- WHO, 1997. Dengue hemorrhagic fever. Diagnosis, Treatment, Prevention and Control. World Health Organization, Geneva, 2nd ed.
- Whyte, A.L., Miller, S.C., 1998. Strain differences in natural killer cell-mediated immunity among mice: a possible mechanism for the low natural killer cell activity of A/J mice. *Immunobiology* 199, 23–38.
- Wu, S.J., Hayes, C.G., Dubois, D.R., Windheuser, M.G., Kang, Y.H., Watts, D.M., Sieckmann, D.G., 1995. Evaluation of the severe combined immunodeficient (SCID) mouse as an animal model for dengue viral infection. *Am. J. Trop. Med. Hyg.* 52, 468–476.