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Complement Activation Is Required for Induction of a Protective Antibody Response against West Nile Virus Infection

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Infection with West Nile virus (WNV) causes a severe infection of the central nervous system (CNS) with higher levels of morbidity and mortality in the elderly and the immunocompromised. Experiments with mice have begun to define how the innate and adaptive immune responses function to limit infection. Here, we demonstrate that the complement system, a major component of innate immunity, controls WNV infection in vitro primarily in an antibody-dependent manner by neutralizing virus particles in solution and lysing WNV-infected cells. More decisively, mice that genetically lack the third component of complement or complement receptor 1 (CR1) and CR2 developed increased CNS virus burdens and were vulnerable to lethal infection at a low dose of WNV. Both C3-deficient and CR1- and CR2-deficient mice also had significant deficits in their humoral responses after infection with markedly reduced levels of specific anti-WNV immunoglobulin M (IgM) and IgG. Overall, these results suggest that complement controls WNV infection, in part through its ability to induce a protective antibody response.

West Nile virus (WNV) is an enveloped *Flavivirus* with a single-stranded positive-sense RNA genome. WNV is endemic in parts of Africa, Europe, the Middle East, and Asia (34), and successive outbreaks in the United States have established its presence in the western hemisphere (51). The virus is maintained in a natural cycle between mosquitoes and birds, although humans, horses, and other vertebrate animals are infected as incidental hosts. Humans develop a febrile illness with a subset of cases progressing to a meningitis or encephalitis syndrome (26). Currently, no specific therapy or vaccine has been approved for human use.

Infection with WNV causes fatal encephalitis more frequently in the immunocompromised (2, 34, 53). Although a complete understanding of this increased risk remains unclear, experiments with mice have begun to define the sequence of events during WNV pathogenesis and the response by the innate and adaptive immune systems to control infection (17, 37, 65). After subcutaneous infection, replication occurs in the skin, possibly in Langerhans dendritic cells (33, 40, 41, 70). Subsequently, infectious virus is detected in draining lymph nodes and shortly afterwards enters the circulation via the efferent lymphatic system. Viremia ensues, and after spread to visceral organs (e.g., kidney and spleen), WNV disseminates to the brain and spinal cord within 3 to 5 days (16, 71, 72) of initial infection depending on the mouse age, strain, and amount of input virus. An intact immune system response is required for protection from lethal infection, as genetic or acquired deficiencies of macrophages or lymphocytes result in higher central nervous sytem (CNS) virus burdens and more severe encephalitis (6, 16, 27). Previously, our laboratory and others have demonstrated that humoral immunity, particularly immunoglobulin M (IgM) and IgG (13, 16, 18, 21) and T-cell-mediated immunity (58, 66, 68) have essential roles in limiting WNV infection in the CNS in mice.

The complement system is a family of ~30 serum and cell surface proteins that are involved in pathogen recognition and clearance (10). It is an integral part of the host innate immune response against pathogens; its role in mediating protection against bacterial infection has been described extensively (5, 10, 69) and is highlighted by an enhanced susceptibility to infection by encapsulated bacteria that is associated with complement deficiencies (43). Increasing evidence has suggested that the complement system may play a key role in protection against viruses (11, 29, 31, 36, 45, 46, 63, 64) by several possible mechanisms. (i) The C5-C9 membrane attack complex lyses enveloped viral particles and infected cells. (ii) Proinflammatory peptides (C3a and C5a) are generated by complement activation leading to recruitment and activation of monocytes and granulocytes. (iii) The proteolytic fragments of C3 (C3b, C3bi, C3d, and C3dg) bind to and opsonize viral particles, facilitating clearance by cells that express complement receptors. (iv) C3 facilitates antigen uptake and presentation and immune cell priming. In response to these possible antiviral mechanisms, several families of viruses have evolved specific strategies to sabotage complement activation and neutralization by producing or incorporating complement-modulating or complement-blocking molecules (24, 35, 52, 62).

Although complement activation inhibits infection of several enveloped DNA and RNA viruses, including herpesvirus (11), influenza virus (36), vesicular stomatitis virus (45), and Sindbis virus (30–32) to varying degrees, its role in flavivirus infection and pathogenesis remains somewhat controversial. Early clinical studies suggested that enhanced complement activation

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correlated with the development of dengue hemorrhagic fever and shock syndrome (7) and that the addition of complement and IgM enhanced flavivirus infection in macrophages (8, 9). In contrast, other experiments suggest that complement is protective as it augments the inhibitory capacity in vivo of anti-flavivirus antibodies. Passive administration of antibodies against nonstructural protein 1 (NS1) prevented lethal yellow fever or dengue virus infection in mice even though NS1 was absent from the virion (22, 25, 28, 54). Because $F(ab')_2$ fragments did not protect in vivo, complement-mediated cytolysis was proposed to explain the inhibitory effects of anti-NS1 antibodies (55).

In this study, we directly assess the function of complement activation in the control of WNV infection. We find that complement controls WNV infection through multiple mechanisms including its effector activity and ability to trigger WNV-specific antibody responses.

MATERIALS AND METHODS

Cells, viruses, and antibodies. BHK21-15, C6/36 *Aedes albopictus*, and MC57GL mouse fibrosarcoma cells were cultured as previously described (12, 14, 58). The WNV strain (3000.0259) was isolated in New York in 2000 (20). All cell culture and in vivo studies used a stock (2×10^8 PFU/ml) of this virus that was propagated (passage 1) once in C6/36 cells. Viruses were diluted in Hank's balanced salt solution (HBSS) and 1% heat-inactivated fetal bovine serum (FBS) for injection into mice. Hybridoma cells that produce monoclonal antibodies (MAbs) against WNV envelope protein (for E1, IgG2a; E8, IgG1; and E16, IgG2b) (47) or severe acute respiratory syndrome (SARS) coronavirus open reading frame 7a (ORF7a) protein (2E11, IgG2a) (44) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated FBS.

Complement neutralization and lysis of WNV-infected cells. Complement neutralization assays were performed as a modification of a plaque reduction assay that was described previously (14). Briefly, 10^2 PFU of WNV was mixed with increasing concentrations (0, 5, 10, and 25% final concentration [vol/vol] of baby rabbit complement (Cedarlane Labs, Ontario, Canada) or mouse serum in the absence or presence of MAbs (20 μg/ml final concentration) against WNV E (E1 and E8) or the human SARS coronavirus ORF7a (2E11) proteins, and incubated for 1 h at 37°C. These virus-complement-antibody mixtures (in a 170-μl volume) were added to BHK21-15 cell monolayers in six-well plates for an additional hour and then rinsed with warm phosphate-buffered saline (PBS) to remove unbound virus, antibody, and complement. Subsequently, a 1% low-melting-point agarose (SeaPlaque; Cambrex BioSciences, Rockland, ME) solution containing α-MEM and 4% FBS was added. After a 3-day incubation, cells were fixed with 2 ml of 10% formaldehyde, the agarose plugs were removed, a crystal violet (1% [wt/vol] in 20% ethanol) solution was added, and the plaques were scored visually with a light box.

The method for measuring complement-mediated lysis of infected cells was a modification of a protocol to quantitate complement-mediated lysis of Sindbis virus-infected cells (61). Briefly, MC57GL cells were infected at a multiplicity of infection (MOI) of 5 or mock infected. Thirty hours later, cells were detached with Hank's balanced salt solution (Sigma Chemical Co., St. Louis, MO) supplemented with 3 mM EDTA (HBSS-EDTA) and replated in 12-well plates. Six hours later, cells were incubated with medium alone or with MAbs (20 μg/ml final concentration) against WNV E (E1 and E16) or SARS ORF7a (2E11) proteins for 30 min at room temperature. MC57GL cells were then washed extensively with warm medium to remove unbound antibody and then incubated with increasing concentrations of baby rabbit complement (0, 5, 10, and 25% [vol/vol]) in DMEM supplemented with 10% heat-inactivated FBS for 3 h at 37°C. Subsequently, propidium iodide (Molecular Probes, Eugene, OR) at a final concentration of 2 μg/ml was added for 15 min at room temperature in the dark, and cells were removed with HBSS-EDTA and fixed lightly in a diluted paraformaldehyde (PFA) solution (1/40 dilution of 4% PFA in PBS stock solution). Cells were immediately processed by flow cytometry (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ), and cell death was assessed on the FL2 channel. Routinely, we observed a baseline of between 2 and 4% "background" cell death in untreated cells.

Mouse experiments. All mice were age matched between 8 and 12 weeks and inoculated subcutaneously with WNV by footpad injection after anesthetization with xylazine and ketamine. Genetically C3-deficient mice (on a mixed 129 Sv × C57BL/6J background) were obtained from H. Virgin (Washington University, St. Louis, MO) (35) and 129 Sv × C57BL/6J F1, 129 Sv, and C57BL/6J control mice were purchased from Jackson Laboratories (Bar Harbor, ME). Complement receptor 1 (CR1) and CR2 (CR1/2)-deficient mice (42) were backcrossed for eight generations onto a pure C57BL/6 background and obtained from H. Molina (Washington University, St. Louis, MO). Mouse experiments were approved and performed according to the guidelines of the Washington University School of Medicine Animal Safety Committee.

Passive antibody transfer experiments. Serum was isolated from immune (day 28 after infection) wild-type mice, heat inactivated for 30 min at 56°C, pooled, and stored aliquoted at −80°C. The E16-neutralizing MAb (IgG2b) against the WNV E protein (47) was purified from hybridoma culture supernatants by protein A affinity and size exclusion chromatography, buffer exchanged into PBS, and filtered with a 0.2-μm syringe filter. For passive transfer experiments, mice were administered a volume of 250 μl that contained immune serum (0.05 to 50 μl diluted into HBSS and 1% heat-inactivated FBS) or E16 antibody (500 μg) intraperitoneally immediately after footpad inoculation with 10^2 PFU of WNV.

Quantitation of virus burden in mice. To analyze the kinetics of virus production in the tissues and serum of infected mice, groups of mice were infected with WNV and euthanized on day 2, 4, 6, 8, or 10 after infection. Before organs were harvested, blood was collected by phlebotomy of the axillary vein; serum was recovered after centrifugation, aliquoted, and stored at −80°C. After cardiac perfusion with PBS, organs were removed, weighed, and homogenized, and plaque assays were performed as previously described (16). Viral RNA was prepared from thawed aliquots of serum by using a Qia-Amp viral RNA recovery kit (QIAGEN, Palo Alto, CA) and quantitated by real-time fluorogenic reverse transcriptase PCR (RT-PCR) using an ABI 7000 sequence detection system (Applied Biosystems, Foster City, CA) as described previously (38).

Quantitation of antibodies. Antibody titer and isotype were determined using an enzyme-linked immunosorbent assay against purified WNV antigen as described previously (16). Briefly, soluble WNV E protein that was generated from baculovirus-infected Hi-5 insect cells (48) was adsorbed overnight at 4°C to Maxi-Sorp microtiter plates (Nalge Nunc International, Rochester, NY). Nonspecific binding was blocked after incubation with blocking buffer (PBS, 0.05% Tween 20, 3% bovine serum albumin, and 3% horse serum) for 1 h at 37°C. Plates were then incubated with serial dilutions of heat-inactivated serum from infected mice for 1 h at 4°C. After being extensively washed, plates were incubated with biotin-conjugated goat anti-mouse IgG (whole IgG, IgG1, IgG2a, IgG2b, IgG2c, and IgG3; Southern Biotech, Birmingham, AL) or IgM (Sigma Chemical) and horseradish peroxidase-conjugated streptavidin (Sigma Chemical) at 4°C and developed after addition of tetramethylbenzidine substrate (Sigma Chemical). Optical densities were determined spectrophotometrically at 450 nm (Spectrafluor Plus; Tecan, Inc.).

Flow cytometry. MC57GL cells were infected at an MOI of 5 as described above. Thirty hours later, cells were detached and incubated in DMEM with 5% FBS at 4°C with 25 μg/ml of E1 (anti-WNV E) or 2E11 (anti-SARS ORF7a) for 1 h. After being washed three times, cells were incubated with a 1/500 dilution of Alexa 647 goat anti-mouse IgG in DMEM with 5% FBS for 30 min at 4°C. After additional washing was carried out, cells were fixed with 1% PFA in PBS and analyzed with a FACSCAN flow cytometer with Cellquest software (Becton Dickinson).

Histopathology and immunohistochemistry. For pathological analyses, CNS tissues were harvested after perfusion with PBS and 4% paraformaldehyde, incubated in 4% paraformaldehyde for 24 h at 4°C, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined for pathological changes. Serial sections of these tissues were also stained for WNV antigens as described previously (16), with the exception that a pool of anti-WNV MAbs was used (E18, E22, and E31).

Statistical analyses. All data were analyzed with Prism software (GraphPad Software, San Diego, CA). For survival analysis, Kaplan-Meier survival curves were analyzed by the log rank and Mantel-Haenszel tests. For virus burden and antibody experiments, statistical significance was determined using the Mann-Whitney test.

RESULTS

Complement-mediated neutralization of WNV. To determine whether complement directly neutralizes WNV, we modified an existing plaque reduction assay. Rabbit instead of

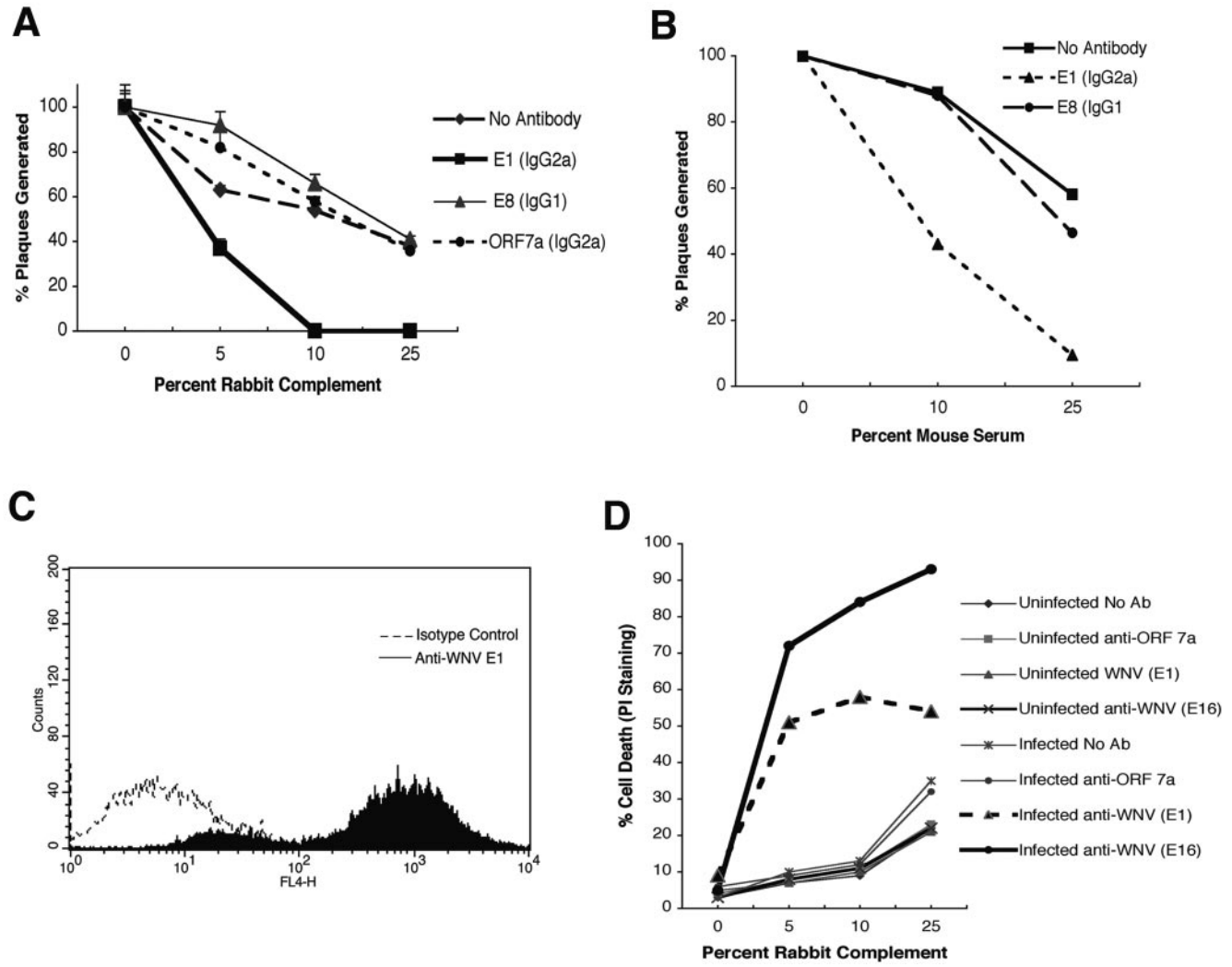

FIG. 1. Complement-mediated neutralization of WNV virions and lysis of WNV-infected cells. (A) Neutralization of WNV. Increasing concentrations of rabbit complement was preincubated with WNV virions (30 min at 37°C) in the presence or absence of MAbs against the WNV E (E1, IgG2a; E8, IgG1) or an unrelated viral protein (2E11, IgG2a, and anti-ORF7a of SARS coronavirus) prior to addition to a monolayer of hamster kidney (BHK21-15) epithelial cells. After addition of an agarose overlay and 72-h incubation, plaques were scored visually. The data shown are from one representative experiment of three and was performed in triplicate. In the absence of complement (0%), the number of plaques recorded (means ± standard deviation) was as follows: no antibody, 86 ± 10; anti-ORF7a, 83 ± 8, E1, 98 ± 15; and E8, 89 ± 3. (B) Increasing concentrations of freshly obtained mouse serum were preincubated with WNV in the presence or absence of MAbs against WNV. The experiment and data analysis were performed as described above. (C) Expression of WNV E protein on the surface of infected MC57GL cells. Cells were infected at an MOI of 5 with WNV and were processed 30 h later by flow cytometry as described in Materials and Methods with a MAb against WNV E protein (E1, IgG2a) or the SARS ORF7a protein (negative control; 2E11, IgG2a). The flow cytometric data are expressed as the log of the fluorescence intensity. One representative experiment of four is shown. (D) Lysis of WNV-infected cells. MC57GL mouse fibroblasts were mock infected or infected with WNV and incubated (60 min at 37°C) with increasing concentrations of rabbit complement in the presence or absence of complement-fixing MAbs against WNV (E1 or E16) or an unrelated viral protein (2E11). Subsequently, propidium iodide was added, and cell lysis was determined by flow cytometry. The data shown are from one representative experiment of three and was performed in duplicate.

mouse complement was used initially because of its enhanced lytic activity (3, 49). Rabbit complement was preincubated with WNV virions in the presence or absence of MAbs against the WNV structural protein E prior to addition to a monolayer of hamster kidney (BHK21-15) epithelial cells. In the absence of antibodies, a dose-dependent reduction in viral plaques was observed; approximately 60% of infectious virus was neutralized after incubation with a 25% (vol/vol) solution of complement (Fig. 1A). In the presence of a complement-fixing MAb (E1, IgG2a) against the WNV E protein, neutralization was markedly enhanced such that infectivity of the virus was abolished completely after exposure to a 10% (vol/vol) solution of complement. Control antibodies that lacked either binding to WNV virions (2E11, anti-ORF7a of SARS coronavirus, IgG2a) or the ability to efficiently fix complement (E8, IgG1) demonstrated no enhancement of complement-mediated neutralization. When wild-type mouse serum was used as the source of complement, a similar pattern of antibody-dependent neutralization of WNV infectivity was observed; as expected, because of the decreased lytic activity of mouse com-

plement (19), lower levels of inhibition were detected (Fig. 1B). Thus, complement directly neutralized WNV by antibody-dependent and antibody-independent mechanisms, although the degree of neutralization was greater in the presence of complement-fixing anti-WNV antibodies and highly lytic complement.

Complement-mediated lysis of WNV-infected cells. In addition to evaluating the degree by which complement directly neutralized WNV, we also assessed its capacity to trigger lysis of infected cells. MC57GL mouse fibroblasts cells that are infected with WNV express high levels of two viral glycoproteins, E and NS1, on their cell surfaces (Fig. 1C and data not shown) and thus could be targets for classical, lectin, or alternate pathway activation of complement. To define the efficiency of complement-mediated killing of WNV-infected cells, we used flow cytometry to measure the incorporation of propidium iodide, a DNA-binding agent that fluoresces and is normally excluded from live cells. MC57GL cells were mock infected or infected with WNV and incubated with complement in the presence or absence of complement-fixing MAbs against WNV. In the absence of antibodies, no specific cell lysis of WNV-infected cells was observed (Fig. 1D) even at high (25% [vol/vol] solution) concentrations of complement. In contrast, the addition of complement-fixing anti-WNV E MAbs (E1 and E16, IgG2a and IgG2b, respectively) resulted in the rapid killing of WNV-infected but not uninfected targets at low concentrations (5% solution) of complement. Importantly, addition of a complement-fixing antibody against an irrelevant viral antigen (2E11, anti-SARS ORF7a) did not significantly trigger lysis of infected cells. Collectively, these experiments suggest that WNV-infected cells are susceptible to lysis by complement, primarily in an antibody-dependent manner.

Decreased survival of C3-deficient mice after infection with WNV. The in vitro neutralization and killing studies demonstrated that complement fixation could directly limit WNV infection. However, the importance and mechanism of complement-dependent viral clearance are best judged with complement-deficient animals. To evaluate the function of complement against WNV in vivo, we compared morbidity and mortality after subcutaneous infection of wild-type and C3-deficient mice. Subcutaneous infection with 10^2 PFU of WNV resulted in 100% mortality of the C57BL/6 × 129 Sv C3-deficient mice (Fig. 2A) ($P < 0.001$). In contrast, only 30% of C57BL/6, 40% of 129 Sv, and 50% of C57BL/6 × 129 Sv F1 mice succumbed to infection at this dose. In each group, all mice showed clinical evidence of infection with fur ruffling, hunchbacked posture, and weight loss (data not shown). Significant mortality differences between C3-deficient and wild-type mice were also observed at other doses of WNV (data not shown).

Increased virus burden in C3-deficient mice. To elucidate the mechanism by which a deficiency in complement activation made mice vulnerable to lethal infection by WNV, wild-type and C3-deficient mice were infected with 10^2 PFU of WNV and virus burdens in the serum, spleen, spinal cord, and brain were measured at days 2, 4, 6, 8, and 10 days after infection (Fig. 2B to E).

(i) Serum. In both wild-type and C3-deficient mice, viremia was below the level of detection by direct plaque assay throughout the time course, with the exception of a single C3-deficient mouse (data not shown). However, when viral RNA in serum was measured by a more sensitive fluorogenic RT-PCR assay (16), additional information was obtained. The kinetics and magnitude of viremia were virtually identical between wild-type and C3-deficient mice; viral RNA was detected from day 2 to day 4 after infection but was cleared from circulation by day 6 (Fig. 2B).

(ii) Spleen. A different pattern was observed between wild-type and C3-deficient mice in the spleen. In wild-type mice, virus was detectable in spleen samples as early as day 2, persisted through day 8, and was ultimately cleared from all animals by 10 days after infection. In contrast, there was a delayed clearance phase in C3-deficient mice as levels of virus (10^3 PFU/g) persisted in the spleen throughout the course of the experiment (Fig. 2C). Thus, a lack of C3 resulted in a failure to rapidly clear virus infection from the spleen.

(iii) CNS. WNV was detected earlier and in greater levels in the spinal cord and brain of C3-deficient mice (Fig. 2D and E).

(a) Spinal cord. At day 4 after infection, 17% of C3-deficient mice had detectable levels of infectious virus in the lumbar-sacral spinal cord. By day 6, 67% of C3-deficient mice had significant levels (~10^5 PFU/g) in both the inferior and superior spinal cord. In contrast, infectious virus was not detected in wild-type mice until 8 days after infection. Even at the latter stages of the time course, the magnitude of viral infection in the spinal cord was increased: at day 10 after infection, C3-deficient mice had ~30-fold higher levels ($P = 0.03$) in the spinal cord than the wild-type counterparts.

(b) Brain. A similar pattern of infection was observed in the brain. At day 6 after infection, 50% of C3-deficient mice had infectious virus in the brain, whereas none was detected in wild-type mice until 8 days after infection. As the time course progressed, the gap in virus burden in the brain widened such that by day 10 after infection there were ~150-fold-higher levels ($P = 0.007$) of infectious virus in the C3-deficient mice. Overall, the virologic analysis demonstrates that C3 is essential for controlling the early spread of WNV into the CNS.

Histopathology after WNV infection in the brain. To understand the cellular basis for increased infection and mortality, we examined brain tissues for histopathological changes following infection and compared this to wild-type mice. Brains were harvested from equivalently moribund C3-deficient and wild-type mice on day 10 after infection. As observed previously, wild-type and C3-deficient mice had high levels of WNV antigen, primarily in cells that stained positive for neuronal antigens (59; data not shown). In wild-type mice, scattered neurons in the cortex, hippocampus, and the base of the brain stained positive for WNV antigen. In contrast, in C3-deficient mice, significantly enhanced staining was observed in neurons in all regions of the brain, with prominent staining in the cerebral cortex, hippocampus, and brain base (Fig. 3 and data not shown). The enhanced infection in the brain in C3-deficient mice was associated with increased numbers of dysmorphic and pyknotic neurons, indicative of the severity of injury to neurons.

Effect of C3 on the anti-WNV antibody response. We have previously shown that a depressed anti-WNV antibody response results in enhanced dissemination of WNV into the CNS (16, 18), To evaluate whether complement activation protected against WNV infection by virtue of its ability to facilitate virus-specific antibody production, we assessed the

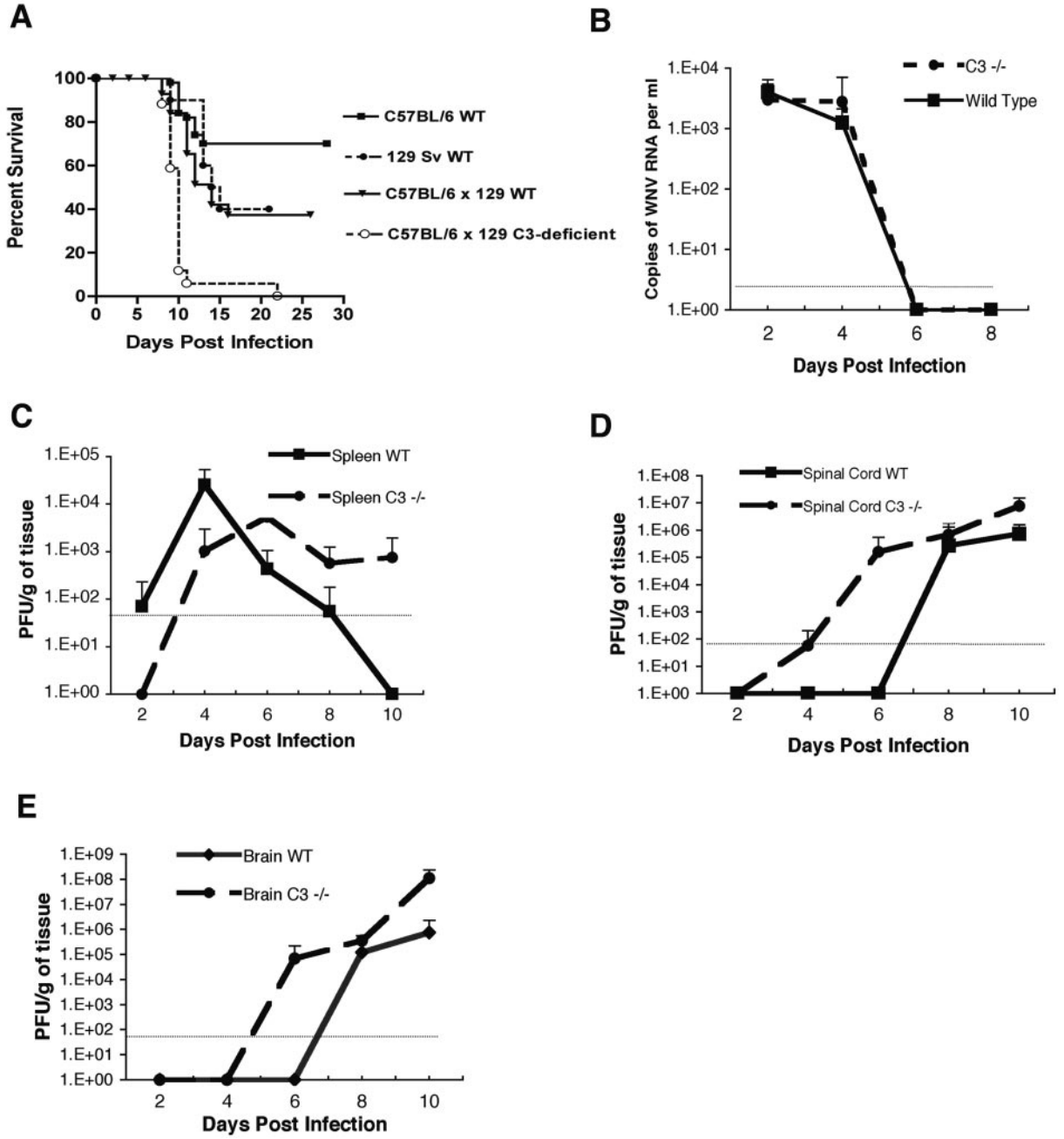

FIG. 2. WNV infection in C3-deficient mice. (A) Survival data of wild-type and C3-deficient mice after inoculation with WNV. 129 Sv, C57BL/6, 129 Sv × C57BL/6 F1, and C3-deficient (129 Sv × C57BL/6) mice were inoculated via footpad with 10^2 PFU of WNV and followed for 28 days. The survival curves were constructed using data from between three and five independent experiments. The number of animals was 10 for 129 Sv, 49 for C57BL/6, 41 for 129 Sv × C57BL/6, and 26 for C3-deficient mice. Survival differences between wild-type and C3-deficient mice were statistically significant ($P < 0.0001$). (B) Levels of viral RNA in serum. Viral RNA levels were determined from serum of wild-type 129 Sv × C57BL/6 or C3-deficient mice after WNV infection at the indicated days by a real-time fluorogenic RT-PCR assay. Data are expressed as genomic equivalents of WNV RNA per milliliter of serum and reflect the average of at least five independent mice per time point. The dotted line represents the limit of sensitivity of the assay. (C to E) Infectious virus levels in tissues. Virus levels were measured from the spleen (C), spinal cord (D), and brain (E) of wild-type and C3-deficient mice by a viral plaque assay in BHK21 cells after tissues were harvested at the indicated days after inoculation. Data are shown as the average PFU per gram of tissue and reflect 5 to 10 mice per time point for either wild-type or C3-deficient mice. The dotted line represents the limit of sensitivity of the assay.

kinetics of the specific IgM and IgG response in wild-type and C3-deficient mice.

(i) IgM. Equivalent levels of anti-WNV IgM levels were observed in wild-type and C3-deficient mice through day 6 after infection. Subsequently, in wild-type C57BL/6 × 129 mice, anti-WNV IgM titers rose dramatically, peaking (1:3,200) at day 8 after infection (Fig. 4A) and declining thereafter (data not shown). In contrast, C3-deficient C57BL/6 ×

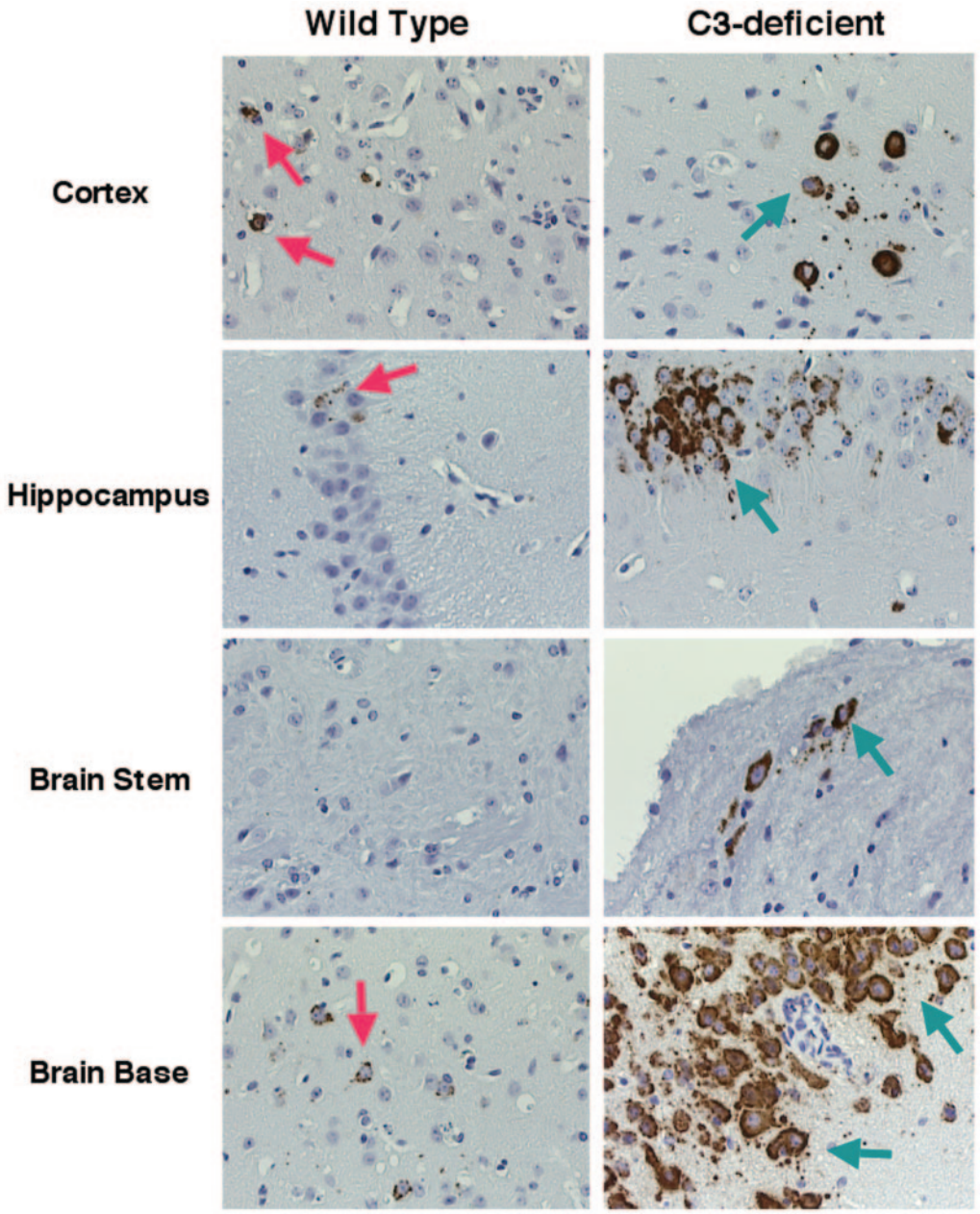

FIG. 3. Immunohistochemistry of the brain after WNV infection in wild-type and C3-deficient mice. The brains of infected wild-type (left) and C3-deficient (right) mice were harvested 10 days after infection with WNV, sectioned, and stained with mouse anti-WNV MAbs. Typical sections from the cortex, hippocampus, brain stem, and the amygdala in the base of the brain are shown after several independent brains from either wild-type or C3-deficient mice were reviewed. Red arrows denote infected neurons in wild-type mice, and blue arrows show infected neurons in C3-deficient mice.

129 mice produced significantly less (1:450) WNV-specific IgM at day 8 after infection ($P = 0.02$).

(ii) IgG. WNV specific IgG was first detected at 6 days after infection in wild-type mice, rising to a titer of 1:2,100 by day 10. C3-deficient mice demonstrated a delayed and blunted anti-WNV IgG response (Fig. 4B). Virus-specific IgG was not detected in C3-deficient mice until day 8 after infection, and the levels were significantly lower (1:300 at day 10) than those produced in wild-type mice (day 8, $P = 0.04$; day 10, $P = 0.01$). Collectively, these experiments demonstrate that a deficiency in C3 results in a defect in the production WNV-specific IgM and IgG. Because of the importance of the IgG isotype in complement-mediated effector function, isotype analysis of WNV-specific IgG was also assessed in wild-type and C3-deficient mice (Fig. 4C). At day 10 after infection, wild-type C57BL/6 × 129 mice demonstrated all circulating IgG isotypes against WNV; however, the response was dominated by the complement-fixing IgG2 subtypes, as significantly higher levels of IgG2a, IgG2b, and IgG2c than IgG1 and IgG3 were observed. Although overall titers were markedly lower in

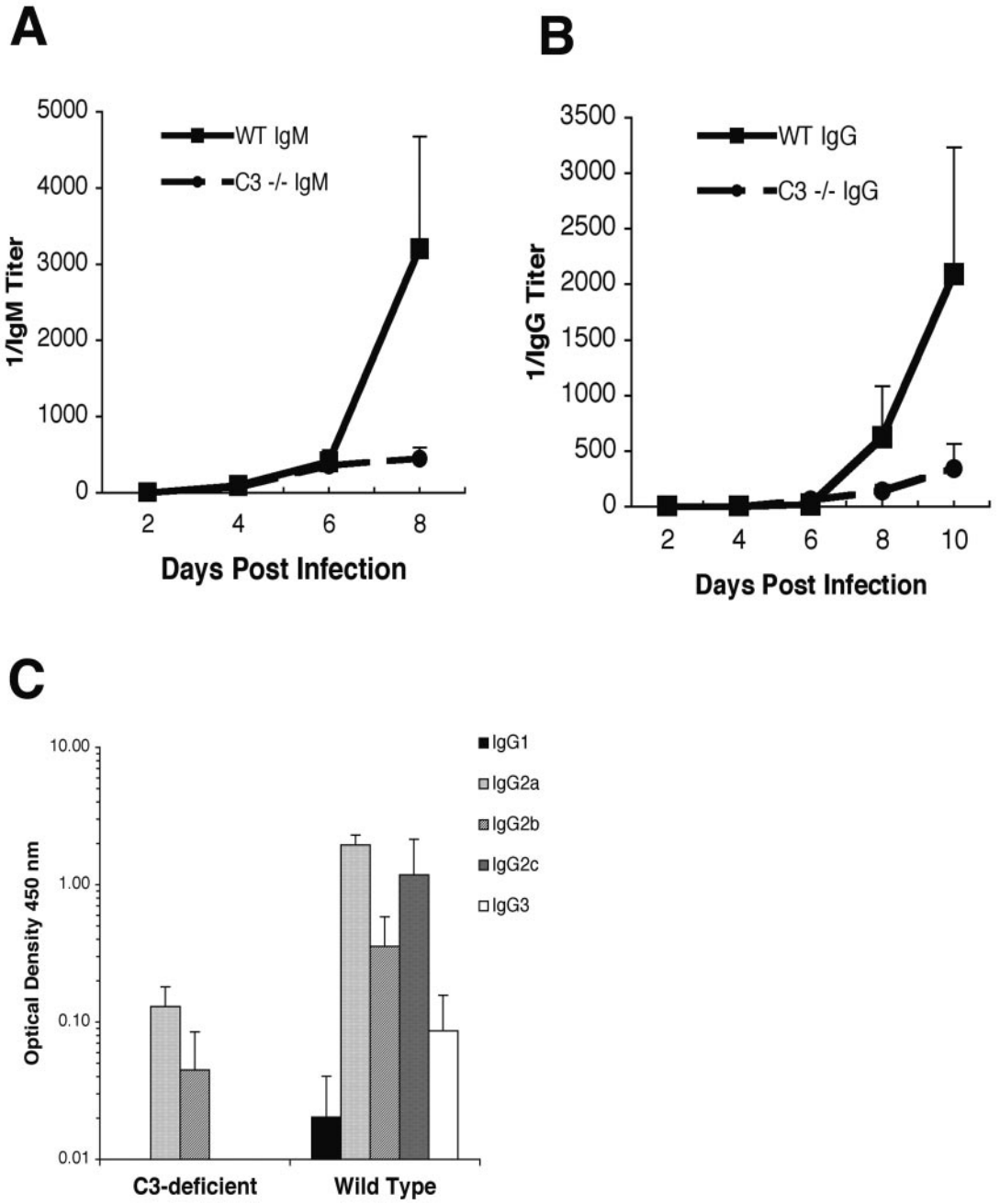

FIG. 4. Development of specific antibodies against WNV in wild-type and C3-deficient mice. Serum was collected from wild-type or C3-deficient mice at the indicated days after infection with 10^2 PFU of WNV. The development of specific IgM (A) or IgG (B) antibodies against WNV was determined after incubating serum with adsorbed control or purified WNV E protein. Serum was collected from between 5 and 10 wild-type or C3-deficient mice per time point, and individual experiments were performed in duplicate. (C) WNV-specific IgG isotype analysis of wild-type and C3-deficient mice. Serum samples were obtained from five wild-type and four C3-deficient mice at day 10 after WNV infection, diluted 1/50, and analyzed for IgG isotype by enzyme-linked immunosorbent assay. The data are expressed in a logarithmic scale as units of optical density after subtraction of the background (0.05), and the error bars represent standard deviations. For wild-type mice, the levels of IgG2a, IgG2b, and IgG2c were statistically different from levels of IgG1 or IgG3 ($P < 0.03$). For C3-deficient mice, the levels of IgG2a were statistically different from levels of IgG1 or IgG3 ($P = 0.01$).

C57BL/6 × 129 C3-deficient mice, the response at day 10 was composed almost exclusively of IgG2 antibodies against WNV.

Complement-dependent humoral response: experiments in CR1/2-deficient mice. The data from the previous sections suggest that a lack of complement activation led to a blunted WNV-specific antibody response after day 6, uncontrolled CNS infection, and increased mortality. However, complement activation could inhibit WNV infection through additional effector mechanisms, aside from the depressed antibody production. To test which effect was dominant, C57BL/6 mice that

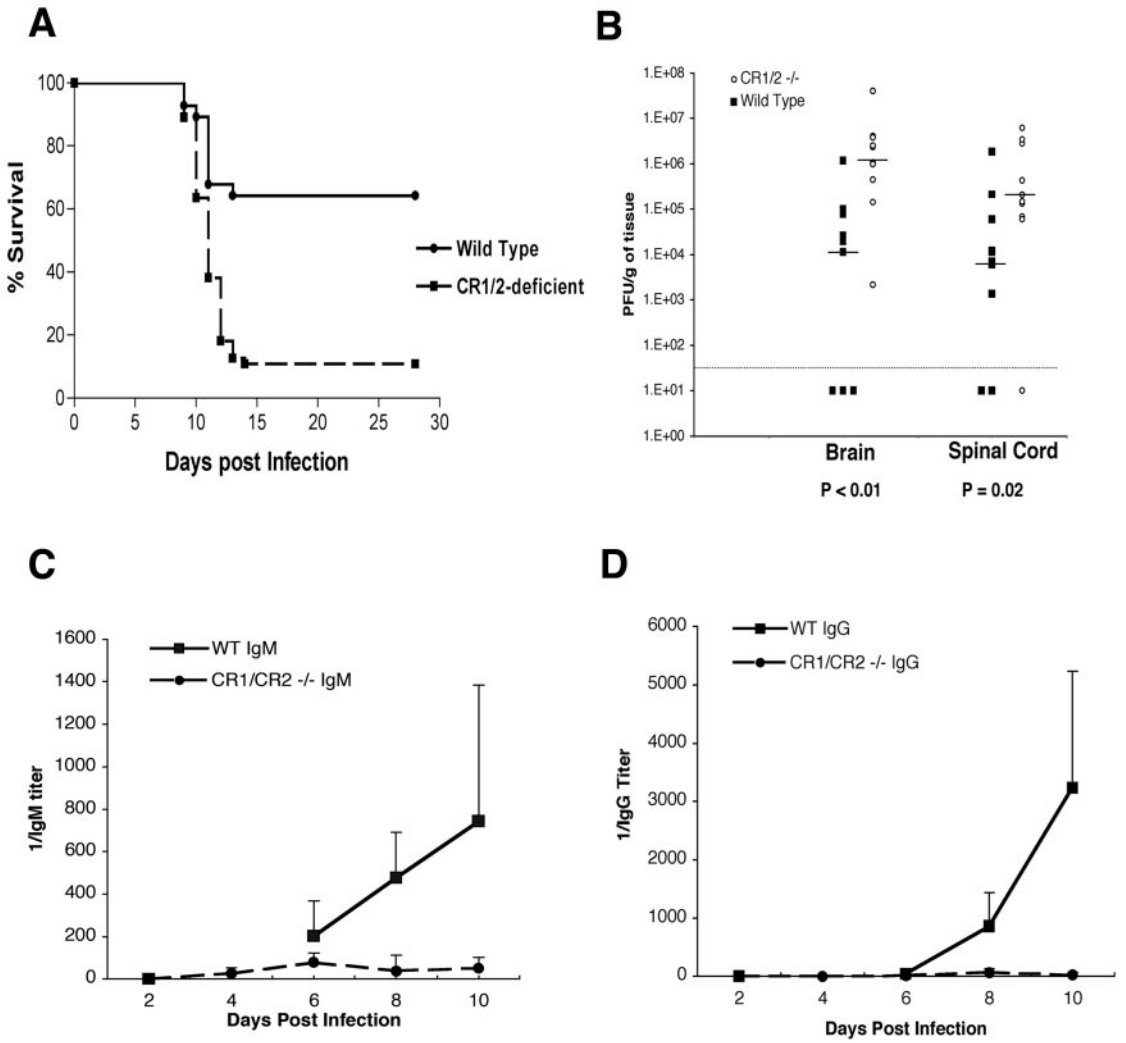

FIG. 5. WNV infection and humoral response in wild-type and CR1/2-deficient C57BL/6 mice. (A) Survival data of wild-type and CR1/2-deficient mice after infection with WNV. Wild-type and CR1/2-deficient C57BL/6 mice were inoculated via footpad with WNV and followed for 28 days. The survival curves were constructed using data from between three and five independent experiments. Survival differences between wild-type and CR1/2-deficient mice were statistically significant ($P < 0.0001$). (B) Virus levels were measured by plaque assay from brain and spinal cord tissues of wild-type and CR1/2-deficient mice at day 10 after infection with WNV. The data are shown as a scatter plot with each shaded square (wild-type) or open circle (CR1/2-deficient) corresponding to an individual data point. The horizontal bars reflect the average of the log viral titer, and the *P* values for statistical significance are shown. The dotted line represents the limit of sensitivity of the assay. (C and D) Development of specific IgM and IgG against WNV in wild-type and CR1/2-deficient mice. Serum samples were collected from wild-type or CR1/2-deficient mice at the indicated days after infection with WNV. The development of specific IgM or IgG antibodies against WNV was determined after incubating serum with adsorbed control or purified WNV E protein. Serum samples from between 5 and 10 wild-type or CR1/2-deficient mice per time point were used, and individual experiments were performed in duplicate. The differences between wild-type and CR1/2-deficient were statistically significant at day 8 (IgM, $P < 0.001$; IgG, $P = 0.01$) and day 10 (IgM, $P = 0.01$; IgG, $P = 0.008$).

were deficient for the complement receptors CR1 (CD21) and CR2 (CD35) were infected with WNV and evaluated for lethality, virus burden in the CNS, and WNV-specific antibody responses. Because C3d binding to CR2 augments antibody production by lowering the threshold for B-cell activation (23), CR1/2-deficient mice have defects in IgM and IgG titers after immunization with a soluble protein antigen but do not have deficiencies in complement-mediated effector (opsonization or lysis) function (1, 42).

Subcutaneous infection with 10^2 PFU of WNV resulted in ~90% mortality of the CR1/2-deficient C57BL/6 mice (Fig. 5A; $P < 0.0001$), whereas only ~35% of congenic wild-type succumbed to infection. A similar difference in survival was observed when 10^4 PFU of WNV was inoculated into wild-type and CR1/2-deficient mice (data not shown). To assess the mechanism for increased mortality, virus burden was analyzed in the spleen, brain, and spinal cord of C57BL/6 wild-type and CR1/2-deficient mice. Unlike that observed with the C3-deficient mice, infectious virus was rapidly cleared from the spleen of CR1/2-deficient mice with the same kinetics as wild-type mice (data not shown). In contrast, and similar to what was seen in C3-deficient mice, higher levels of virus were observed in the brain (~200 fold; $P < 0.01$) and spinal cord (~50 fold; $P = 0.02$) of CR1/2-deficient mice at day 10 after infection

TABLE 1. Passive antibody protection in 8-week-old wild-type and C3-deficient mice

Mouse group and dose[a]	% Survival at 28 days (n)[b]	P value[c]
C3 deficient	0 (17)	
E16 MAb, 500 μg	100 (4)	0.005*
Immune serum, 0.5 μl	13 (8)	0.006*
Immune serum, 5 μl	44 (9)	<0.0001*
immune serum, 50 μl	100 (8)	<0.0001*
Wild type	50 (13)	
E16 MAb, 500 μg	100 (10)	<0.0001*
Immune serum, 0.05 μl	80 (10)	>0.3
Immune serum, 0.5 μl	100 (10)	0.03*
Immune serum, 5 μl	100 (10)	0.03*

[a] E16 is an anti-WNV E protein MAb that binds to domain III and strongly neutralizes WNV infection. Heat-inactivated immune serum was generated from surviving wild-type mice at day 28 after infection. Immune serum was diluted in HBSS with 1% heat-inactivated serum to obtain the indicated dose. A single dose of E16 or immune serum was passively transferred by the intraperitoneal route to C57BL/6 × 129 Sv wild-type or C3-deficient mice on the same day that 10^2 PFU of WNV was inoculated via footpad inoculation.

[b] The percentage of mice surviving infection at the termination of the experiment (28 days) is indicated.

[c] The P value is shown after performance of log rank analysis of the Kaplan-Meier survival curve. An asterisk indicates statistical significance.

(Fig. 5B). To establish whether the virologic and survival phenotype in CR1/2-deficient mice was associated with an attenuated humoral response, we evaluated the kinetics of the anti-WNV IgM and IgG response in wild-type and CR1/2-deficient C57BL/6 mice. In this genetic background, equivalent low levels of anti-WNV IgM levels were observed in wild-type and CR1/2-deficient mice through day 4 after infection. Subsequently, in wild-type mice, anti-WNV IgM titers rose significantly, whereas only a slight increase was observed in CR1/2-deficient mice (Fig. 5C). WNV-specific IgG was first detected at 8 days after infection in wild-type mice and increased significantly by day 10. In contrast, the absence of CR1/2 resulted in an almost completely blunted WNV IgG response (Fig. 5D), even at day 10 after infection.

Significance of the depressed WNV-specific antibody in C3-deficient mice. The increased susceptibility and blunted WNV-specific antibody responses in C3- and CR1/2-deficient mice suggest that the complement activation is necessary for the development of a protective humoral response during primary infection and that the attenuated antibody response directly results in increased viral infection and mortality. To address whether WNV-specific antibody by itself could protect C3-deficient mice from lethal infection, neutralizing MAbs or polyclonal antibodies were passively transferred to 8-week-old wild-type or C3-deficient mice (Table 1). For both wild-type and C3-deficient mice, a single high dose of the neutralizing MAb E16 (IgG2b; 500 μg) completely prevented WNV-induced mortality. In contrast, when limiting amounts of heat-inactivated immune serum were administered, greater protection was observed in wild-type mice: ~100-fold-higher doses were required to completely block lethality in C3-deficient mice. Naïve serum (50 μl or greater), however, provided no protection against mortality (reference 18 and data not shown). Thus, the severe phenotype in the C3-deficient mice could be overcome by the addition of sufficient quantities of exogenous immune antibody.

DISCUSSION

Because complement plays a variable role in the control the infection of other RNA and DNA viruses, we sought to evaluate its role in WNV infection. The net function of complement in flavivirus infection has remained controversial, as some studies suggest a protective effect, whereas others indicate that complement activation contributes to pathogenesis. Animals that were genetically deficient in C3 and thus unable to activate the lytic, opsonic, and priming functions of complement, were completely vulnerable to infection with low doses of WNV. In addition, mice that were deficient in CR1 and CR2 and thus impaired in their ability to generate antigen-specific IgM and IgG responses (1, 42) also demonstrated increased susceptibility to lethal WNV infection. Taken together, these data establish that in mice, complement activation is necessary for the efficient induction of a protective antibody response against WNV infection.

Infection in C3- and CR1/2-deficient mice. To our knowledge, this is the first report to show a definitive protective role for complement against infection by a flavivirus in vivo. Previous in vivo studies that addressed the role of complement in flavivirus infection were conflicting. In a retrospective clinical study, excessive consumption of complement proteins correlated with the most severe forms of dengue virus infection (7). Because cell culture studies indicated that complement could enhance infection in myeloid cells by facilitating entry through CR3 (CD11b/CD18), complement activation was suggested to be pathogenic (8, 9). However, lysis of flavivirus-infected cells by complement is thought to explain why passive transfer of antibodies against NS1, a cell- but not virion-associated protein, protects mice against lethal yellow fever virus or dengue virus infection (22, 25, 28, 54). Our mortality, virologic, and immunologic data clearly demonstrate that, at least for WNV, complement activation has a profound effect on coordinating a protective humoral response. Although C3 activation limits the infection of other enveloped viruses, the effect appears more dramatic with WNV. An absence of C3 in mice caused higher and prolonged virus burdens after influenza infection but did not increase mortality, and a deficiency of CR1/2 had little significant effect on influenza virus clearance (36). A 100% lethality after infection with vesicular stomatitis virus was observed in C3-deficient mice but only when high doses (10^7 PFU) were administered intravenously (45). Although C3-deficient mice had depressed specific antibody responses against herpes simplex virus, there was no increase in viral load or mortality compared to wild-type controls (11). A similar lack of effect on mortality was observed in C3-deficient mice with murine gammaherpesvirus 68 (35). In comparison, we show that the absence of C3 or CR1/2 resulted in 100 and 90% lethality, respectively, in response to a low dose (10^2 PFU) of WNV infection.

Although our in vitro experiments suggest that complement is capable of inactivating or lysing WNV particles, its apparent lack of effect on WNV levels in serum in vivo was unexpected. We suspect that this may be because mouse complement C4 lacks or has low classical pathway C5 convertase subunit activity in vitro and in vivo (3, 19, 60). Indeed, when mouse serum was used as the source of complement, less antibody-dependent neutralization of WNV infectivity in vitro was observed.

Alternatively, WNV RNA is not a truly accurate measure of the number of infectious particles in serum. Because infectious virus burden in serum was below the level of detection by the direct plaque assay, we cannot be certain that complement did not diminish the infectivity of the virus in serum.

Compared to wild-type mice, no significant increase in WNV infection was detected in C3-deficient mice during the first 4 days after infection. However, by 6 days after infection, significantly higher levels of infectious virus and viral RNA were detected in the brain and spinal cord of C3-deficient mice. At present, the precise mechanism for this early entry into the CNS in complement-deficient mice remains unclear. It does not appear to be due to increased hematogenous spread, as the kinetics and magnitude of viremia were similar in wild-type and C3-deficient mice. One possible explanation is that complement activation affects WNV CNS entry by lysing infected peripheral neurons. Complement is synthesized by several different types of neurons (4), and its activation has been recently shown to cause neuronal cell death through the formation of the membrane attack complex (56, 57). Alternatively, complement activation could trigger a distinct cytokine expression pattern during the initial phases of infection. A recent study suggested that the generation of tumor necrosis factor alpha in peripheral lymphoid tissues alters blood-brain barrier permeability and facilitates WNV entry into the CNS (15, 67). Future experiments that examine the kinetics of cytokine production in complement-deficient mice should test this hypothesis directly.

Antibody response in C3- and CR1/2-deficient mice. The initial antibody response against WNV, the development of specific IgM by day 4 and 6 after infection (18), was similar in wild-type and C3-deficient mice. In contrast to the early phase of anti-WNV IgM production, a deficiency in C3 blunted the amplification phase of the anti-WNV IgM and IgG responses. Our data are consistent with the hypothesis that complement activation plays an essential role in priming the humoral response, thus linking innate and adaptive immunity (5, 10, 46, 64). The enhancing effect of complement on the humoral response is believed to be T-cell independent (11, 45) and to occur through complement receptors CD21/CD35. C3d binding to CD21 may directly enhance antibody production by lowering the threshold for B-cell activation (23). Consistent with this, mice that were deficient in CD21/CD35 also exhibited markedly depressed and delayed IgM and IgG responses against WNV and increased mortality. Notably, in wild-type mice, the WNV-specific IgG response was dominated by IgG2 antibodies, which efficiently fix complement. Thus, one reason why C3- and CR1/2-deficient mice are sensitive to lethal WNV infection is because they generate low levels of specific and complement-fixing antibodies; in contrast to other viruses (11, 35), a strong antibody response is critical to the control of peripheral and CNS WNV infection in mice (16, 17) and likely in humans (39). As further evidence for this, passive transfer of either neutralizing MAbs or immune serum completely protected C3-deficient mice against lethal WNV infection. With mice, we suspect that the complement-dependent effector functions of antibody (e.g., opsonic and/or lytic potential) also participate in the control of WNV infection, albeit to a lesser degree, as ~100-fold-higher levels of immune serum were required to provide complete protection.

Studies with immunodeficient mice have provided insight into the mechanism of pathogenesis and protection against WNV infection and may have implications for human disease. In the mouse model, complement, anti-WNV IgM and IgG, and $CD8^+$ T cells have important roles in preventing the dissemination of virus into the CNS. Our studies here document that complement is absolutely required to generate a rapid and effective antibody response against WNV; a deficiency leads to enhanced CNS viral loads and increased mortality. It is intriguing to consider that severe human WNV infection, which occurs infrequently (~1/50 cases) even in the elderly and immunocompromised population, could be more common in patients with dysfunctional complement responses against WNV early during infection. Although complete deficiencies of complement proteins are rare, a heterozygous deficiency of C4 has a frequency of approximately 20% in the Caucasian population (73), and has been associated with an increased risk for hepatitis C virus-induced cirrhosis (50). As therapies against WNV become available, it will be important to target high-risk populations. Natural history studies with humans are currently under way to identify both clinical and laboratory risk factors for severe WNV disease. Based on the studies presented here, a depressed level of complement proteins or activity may be an independent risk factor for morbidity and mortality.

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REFERENCES

1. **Ahearn, J. M., M. B. Fischer, D. Croix, S. Goerg, M. Ma, J. Xia, X. Zhou, R. G. Howard, T. L. Rothstein, and M. C. Carroll.** 1996. Disruption of the Cr2 locus results in a reduction in B-1a cells and in an impaired B cell response to T-dependent antigen. Immunity **4:**251–262.
2. **Asnis, D. S., R. Conetta, A. A. Teixeira, G. Waldman, and B. A. Sampson.** 2000. The West Nile virus outbreak of 1999 in New York: the Flushing Hospital experience. Clin. Infect. Dis. **30:**413–418.
3. **Atkinson, J. P., K. McGinnis, and D. Shreffler.** 1980. Development and characterization of a hemolytic assay for mouse C4. J. Immunol. Methods **33:**351–368.
4. **Barnum, S. R.** 2002. Complement in central nervous system inflammation. Immunol. Res. **26:**7–13.
5. **Barrington, R., M. Zhang, M. Fischer, and M. C. Carroll.** 2001. The role of complement in inflammation and adaptive immunity. Immunol. Rev. **180:**5–15.
6. **Ben-Nathan, D., I. Huitinga, S. Lustig, N. van Rooijen, and D. Kobiler.** 1996. West Nile virus neuroinvasion and encephalitis induced by macrophage depletion in mice. Arch. Virol. **141:**459–469.
7. **Bokisch, V. A., F. H. Top, Jr., P. K. Russell, F. J. Dixon, and H. J. Muller-Eberhard.** 1973. The potential pathogenic role of complement in dengue hemorrhagic shock syndrome. N. Engl. J. Med. **289:**996–1000.
8. **Cardosa, M. J., S. Gordon, S. Hirsch, T. A. Springer, and J. S. Porterfield.** 1986. Interaction of West Nile virus with primary murine macrophages: role of cell activation and receptors for antibody and complement. J. Virol. **57:**952–959.
9. **Cardosa, M. J., J. S. Porterfield, and S. Gordon.** 1983. Complement receptor mediates enhanced flavivirus replication in macrophages. J. Exp. Med. **158:**258–263.

10. **Carroll, M. C.** 2004. The complement system in regulation of adaptive immunity. Nat. Immunol. **5:**981–986.
11. **Da Costa, X. J., M. A. Brockman, E. Alicot, M. Ma, M. B. Fischer, X. Zhou, D. M. Knipe, and M. C. Carroll.** 1999. Humoral response to herpes simplex virus is complement-dependent. Proc. Natl. Acad. Sci. USA **96:**12708–12712.
12. **Diamond, M., T. Roberts, D. Edgil, B. Lu, J. Ernst, and E. Harris.** 2000. Modulation of dengue virus infection in human cells by alpha, beta, and gamma interferons. J. Virol. **74:**4957–4966.
13. **Diamond, M. S.** 2003. Evasion of innate and adaptive immunity by flaviviruses. Immunol. Cell Biol. **81:**196–206.
14. **Diamond, M. S., D. Edgil, T. G. Roberts, B. Lu, and E. Harris.** 2000. Infection of human cells by dengue virus is modulated by different cell types and viral strains. J. Virol. **74:**7814–7823.
15. **Diamond, M. S., and R. S. Klein.** 2004. West Nile virus: crossing the blood-brain barrier. Nat. Med. **10:**1294–1295.
16. **Diamond, M. S., B. Shrestha, A. Marri, D. Mahan, and M. Engle.** 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.
17. **Diamond, M. S., B. Shrestha, E. Mehlhop, E. Sitati, and M. Engle.** 2003. Innate and adaptive immune responses determine protection against disseminated infection by West Nile Encephalitis virus. Viral Immunol. **16:**259–278.
18. **Diamond, M. S., E. Sitati, L. Friend, B. Shrestha, S. Higgs, and M. Engle.** 2003. Induced IgM protects against lethal West Nile virus infection. J. Exp. Med. **198:**1–11.
19. **Ebanks, R. O., and D. E. Isenman.** 1996. Mouse complement component C4 is devoid of classical pathway C5 convertase subunit activity. Mol. Immunol. **33:**297–309.
20. **Ebel, G. D., A. P. Dupuis III, K. Ngo, D. Nicholas, E. Kauffman, S. A. Jones, D. Young, J. Maffei, P. Y. Shi, K. Bernard, and L. Kramer.** 2001. Partial genetic characterization of West Nile virus strains, New York State, 2000. Emerg. Infect. Dis. **7:**650–653.
21. **Engle, M., and M. S. Diamond.** 2003. Antibody prophylaxis and therapy against West Nile Virus infection in wild type and immunodeficient mice. J. Virol. **77:**12941–12949.
22. **Falgout, B., M. Bray, J. J. Schlesinger, and C. J. Lai.** 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.
23. **Fearon, D. T., and R. H. Carter.** 1995. The CD19/CR2/TAPA-1 complex of B lymphocytes: linking natural to acquired immunity. Annu. Rev. Immunol. **13:**127–149.
24. **Frade, R., M. Barel, B. Ehlin-Henriksson, and G. Klein.** 1985. gp140, the C3d receptor of human B lymphocytes, is also the Epstein-Barr virus receptor. Proc. Natl. Acad. Sci. USA **82:**1490–1493.
25. **Gould, E. A., A. Buckley, A. D. Barrett, and N. Cammack.** 1986. Neutralizing (54K) and non-neutralizing (54K and 48K) monoclonal antibodies against structural and non-structural yellow fever virus proteins confer immunity in mice. J. Gen. Virol. **67:**591–595.
26. **Granwehr, B. P., K. M. Lillibridge, S. Higgs, P. W. Mason, J. F. Aronson, G. A. Campbell, and A. D. Barrett.** 2004. West Nile virus: where are we now? Lancet Infect. Dis. **4:**547–556.
27. **Halevy, M., Y. Akov, D. Ben-Nathan, D. Kobiler, B. Lachmi, and S. Lustig.** 1994. Loss of active neuroinvasiveness in attenuated strains of West Nile virus: pathogenicity in immunocompetent and SCID mice. Arch. Virol. **137:**355–370.
28. **Henchal, E. A., L. S. Henchal, and J. J. Schlesinger.** 1988. Synergistic interactions of anti-NS1 monoclonal antibodies protect passively immunized mice from lethal challenge with dengue 2 virus. J. Gen. Virol. **69:**2101–2107.
29. **Hicks, J. T., F. A. Ennis, E. Kim, and M. Verbonitz.** 1978. The importance of an intact complement pathway in recovery from a primary viral infection: influenza in decomplemented and in C5-deficient mice. J. Immunol. **121:**1437–1445.
30. **Hirsch, R. L., D. E. Griffin, and J. A. Winkelstein.** 1980. The role of complement in viral infections. II. The clearance of Sindbis virus from the bloodstream and central nervous system of mice depleted of complement. J. Infect. Dis. **141:**212–217.
31. **Hirsch, R. L., D. E. Griffin, and J. A. Winkelstein.** 1980. Role of complement in viral infections: participation of terminal complement components (C5 to C9) in recovery of mice from Sindbis virus infection. Infect. Immun. **30:**899–901.
32. **Hirsch, R. L., J. A. Winkelstein, and D. E. Griffin.** 1980. The role of complement in viral infections. III. Activation of the classical and alternative complement pathways by Sindbis virus. J. Immunol. **124:**2507–2510.
33. **Ho, L. J., J. J. Wang, M. F. Shaio, C. L. Kao, D. M. Chang, S. W. Han, and J. H. Lai.** 2001. Infection of human dendritic cells by dengue virus causes cell maturation and cytokine production. J. Immunol. **166:**1499–1506.
34. **Hubalek, Z., and J. Halouzka.** 1999. West Nile fever—–a reemerging mosquito-borne viral disease in Europe. Emerg. Infect. Dis. **5:**643–650.
35. **Kapadia, S. B., B. Levine, S. H. Speck, and H. W. Virgin.** 2002. Critical role of complement and viral evasion of complement in acute, persistent, and latent gamma-herpesvirus infection. Immunity **17:**1–20.
36. **Kopf, M., B. Abel, A. Gallimore, M. Carroll, and M. F. Bachmann.** 2002. Complement component C3 promotes T-cell priming and lung migration to control acute influenza virus infection. Nat. Med. **8:**373–378.
37. **Kramer, L. D., and K. A. Bernard.** 2001. West Nile virus infection in birds and mammals. Ann. N. Y. Acad. Sci. **951:**84–93.
38. **Lanciotti, R. S., A. J. Kerst, R. S. Nasci, M. S. Godsey, C. J. Mitchell, H. M. Savage, N. Komar, N. A. Panella, B. C. Allen, K. E. Volpe, B. S. Davis, and J. T. Roehrig.** 2000. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J. Clin. Microbiol. **38:**4066–4071.
39. **Libraty, D. H., A. Nisalak, T. P. Endy, S. Suntayakorn, D. W. Vaughn, and B. L. Innis.** 2002. Clinical and immunological risk factors for severe disease in Japanese encephalitis. Trans. R. Soc. Trop. Med. Hyg. **96:**173–178.
40. **Libraty, D. H., S. Pichyangkul, C. Ajariyakhajorn, T. P. Endy, and F. A. Ennis.** 2001. Human dendritic cells are activated by dengue virus infection: enhancement by gamma interferon and implications for disease pathogenesis. J. Virol. **75:**3501–3508.
41. **Marovich, M., G. Grouard-Vogel, M. Louder, M. Eller, W. Sun, S. J. Wu, R. Putvatana, G. Murphy, B. Tassaneetrithep, T. Burgess, D. Birx, C. Hayes, S. Schlesinger-Frankel, and J. Mascola.** 2001. Human dendritic cells as targets of dengue virus infection. J. Investig. Dermatol. Symp. Proc. **6:**219–224.
42. **Molina, H., V. M. Holers, B. Li, Y. Fung, S. Mariathasan, J. Goellner, J. Strauss-Schoenberger, R. W. Karr, and D. D. Chaplin.** 1996. Markedly impaired humoral immune response in mice deficient in complement receptors 1 and 2. Proc. Natl. Acad. Sci. USA **93:**3357–3361.
43. **Morgan, B. P., and M. J. Walport.** 1991. Complement deficiency and disease. Immunol. Today **12:**301–306.
44. **Nelson, C. A., A. Pekosz, C. A. Lee, M. S. Diamond, and D. H. Fremont.** 2005. Structure and intracellular targeting of the ORF7a protein of SARS-associated coronavirus. Structure (Cambridge) **13:**75–85.
45. **Ochsenbein, A. F., D. D. Pinschewer, B. Odermatt, M. C. Carroll, H. Hengartner, and R. M. Zinkernagel.** 1999. Protective T cell-independent antiviral antibody responses are dependent on complement. J. Exp. Med. **190:**1165–1174.
46. **Ochsenbein, A. F., and R. M. Zinkernagel.** 2000. Natural antibodies and complement link innate and acquired immunity. Immunol. Today **21:**624–630.
47. **Oliphant, T., M. Engle, G. Nybakken, C. Doane, S. Johnson, L. Huang, S. Gorlatov, G. D. Ebel, L. D. Kramer, D. H. Fremont, and M. S. Diamond.** Unpublished data.
48. **Oliphant, T., M. Engle, G. Nybakken, C. Doane, S. Johnson, L. Huang, S. Gorlatov, E. Mehlhop, A. Marri, K. M. Chung, G. D. Ebel, L. D. Kramer, D. H. Fremont, and M. S. Diamond.** Development of a humanized monoclonal antibody with therapeutic potential against West Nile virus. Nat. Med., in press.
49. **Ong, G. L., and M. J. Mattes.** 1989. Mouse strains with typical mammalian levels of complement activity. J. Immunol. Methods **125:**147–158.
50. **Pasta, L., G. Pietrosi, C. Marrone, G. D'Amico, M. D'Amico, A. Licata, G. Misiano, S. Madonia, F. Mercadante, and L. Pagliaro.** 2004. C4BQ0: a genetic marker of familial HCV-realted liver cirrhosis. Dig. Liver Dis. **36:**471–477.
51. **Petersen, L. R., A. A. Marfin, and D. J. Gubler.** 2003. West Nile virus. JAMA **290:**524–528.
52. **Saifuddin, M., C. J. Parker, M. E. Peeples, M. K. Gorny, S. Zolla-Pazner, M. Ghassemi, I. A. Rooney, J. P. Atkinson, and G. T. Spear.** 1995. Role of virion-associated glycosylphosphatidylinositol-linked proteins CD55 and CD59 in complement resistance of cell line-derived and primary isolates of HIV-1. J. Exp. Med. **182:**501–509.
53. **Sampson, B. A., C. Ambrosi, A. Charlot, K. Reiber, J. F. Veress, and V. Armbrustmacher.** 2000. The pathology of human West Nile virus infection. Hum. Pathol. **31:**527–531.
54. **Schlesinger, J. J., M. W. Brandriss, and E. E. Walsh.** 1985. Protection against 17D yellow fever encephalitis in mice by passive transfer of monoclonal antibodies to the nonstructural glycoprotein gp48 and by active immunization with gp48. J. Immunol. **135:**2805–2809.
55. **Schlesinger, J. J., M. Foltzer, and S. Chapman.** 1993. The Fc portion of antibody to yellow fever virus NS1 is a determinant of protection against YF encephalitis in mice. Virology **192:**132–141.
56. **Shen, Y., J. A. Halperin, L. Benzaquen, and C. M. Lee.** 1997. Characterization of neuronal cell death induced by complement activation. Brain Res. Brain Res. Protoc. **1:**186–194.
57. **Shen, Y., J. A. Halperin, and C. M. Lee.** 1995. Complement-mediated neurotoxicity is regulated by homologous restriction. Brain Res. **671:**282–292.
58. **Shrestha, B., and M. S. Diamond.** 2004. The role of $CD8^+$ T cells in the control of West Nile virus infection. J. Virol. **78:**8312–8321.
59. **Shrestha, B., D. I. Gottlieb, and M. S. Diamond.** 2003. Infection and injury of neurons by West Nile encephalitis virus. J. Virol. **77:**13203–13213.
60. **Spath, G. F., L. A. Garraway, S. J. Turco, and S. M. Beverley.** 2003. The role(s) of lipophosphoglycan (LPG) in the establishment of Leishmania major infections in mammalian hosts. Proc. Natl. Acad. Sci. USA **100:**9536–9541.
61. **Stanley, J., S. J. Cooper, and D. E. Griffin.** 1986. Monoclonal antibody cure

and prophylaxis of lethal Sindbis virus encephalitis in mice. J. Virol. **58:**107–115.
62. **Takefman, D. M., B. L. Sullivan, B. E. Sha, and G. T. Spear.** 1998. Mechanisms of resistance of HIV-1 primary isolates to complement-mediated lysis. Virology **246:**370–378.
63. **Verschoor, A., M. A. Brockman, D. M. Knipe, and M. C. Carroll.** 2001. Cutting edge: myeloid complement C3 enhances the humoral response to peripheral viral infection. J. Immunol. **167:**2446–2451.
64. **Volanakis, J. E.** 2002. The role of complement in innate and adaptive immunity. Curr. Top. Microbiol. Immunol. **266:**41–56.
65. **Wang, T., and E. Fikrig.** 2004. Immunity to West Nile virus. Curr. Opin. Immunol. **16:**519–523.
66. **Wang, T., E. Scully, Z. Yin, J. H. Kim, S. Wang, J. Yan, M. Mamula, J. F. Anderson, J. Craft, and E. Fikrig.** 2003. IFN-γ-producing γδ T cells help control murine West Nile virus infection. J. Immunol. **171:**2524–2531.
67. **Wang, T., T. Town, L. Alexopoulou, J. F. Anderson, E. Fikrig, and R. A. Flavell.** 2004. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. Nat. Med. **10:**1366–1373.
68. **Wang, Y., M. Lobigs, E. Lee, and A. Mullbacher.** 2003. $CD8^+$ T cells mediate recovery and immunopathology in West Nile virus encephalitis. J. Virol. **77:**13323–13334.
69. **Wessels, M. R., P. Butko, M. Ma, H. B. Warren, A. L. Lage, and M. C. Carroll.** 1995. Studies of group B streptococcal infection in mice deficient in complement component C3 or C4 demonstrate an essential role for complement in both innate and acquired immunity. Proc. Natl. Acad. Sci. USA **92:**11490–11494.
70. **Wu, S. J., G. Grouard-Vogel, W. Sun, J. R. Mascola, E. Brachtel, R. Putvatana, M. K. Louder, L. Filgueira, M. A. Marovich, H. K. Wong, A. Blauvelt, G. S. Murphy, M. L. Robb, B. L. Innes, D. L. Birx, C. G. Hayes, and S. S. Frankel.** 2000. Human skin Langerhans cells are targets of dengue virus infection. Nat. Med. **6:**816–820.
71. **Xiao, S. Y., H. Guzman, H. Zhang, A. P. Travassos da Rosa, and R. B. Tesh.** 2001. West Nile virus infection in the golden hamster (Mesocricetus auratus): a model for West Nile encephalitis. Emerg. Infect. Dis. **7:**714–721.
72. **Xiao, S. Y., H. Zhang, H. Guzman, and R. B. Tesh.** 2001. Experimental yellow fever virus infection in the golden hamster (Mesocricetus auratus). II. Pathology. J. Infect. Dis. **183:**1437–1444.
73. **Yang, Y., K. Lhotta, E. K. Chung, P. Eder, F. Neumair, and C. Y. Yu.** 2004. Complete complement components C4A and C4B deficiencies in human kidney diseases and systemic lupus erythematosus. J. Immunol. **173:**2803–2814.