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Increased blood–brain barrier permeability is not a primary determinant for lethality of West Nile virus infection in rodents

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Blood–brain barrier (BBB) permeability was evaluated in mice and hamsters infected with West Nile virus (WNV, flavivirus) as compared to those infected with Semliki Forest (alphavirus) and Banzi (flavivirus) viruses. BBB permeability was determined by measurement of fluorescence in brain homogenates or cerebrospinal fluid (CSF) after intraperitoneal (i.p.) injection of sodium fluorescein, by macroscopic examination of brains after i.p. injection of Evans blue, or by measurement of total protein in CSF compared to serum. Lethal infection of BALB/c mice with Semliki Forest virus and Banzi virus caused the brain : serum fluorescence ratios to increase from a baseline of 2–4 % to as high as 11 and 15 %, respectively. Lethal infection of BALB/c mice with WNV did not increase BBB permeability. When C57BL/6 mice were used, BBB permeability was increased in some, but not all, of the WNV-infected animals. A procedure was developed to measure BBB permeability in live WNV-infected hamsters by comparing the fluorescence in the CSF, aspirated from the cisterna magnum, with the fluorescence in the serum. Despite a time-dependent tendency towards increased BBB permeability in some WNV-infected hamsters, the highest BBB permeability values did not correlate with mortality. These data indicated that a measurable increase in BBB permeability was not a primary determinant for lethality of WNV infection in rodents. The lack of a consistent increase in BBB permeability in WNV-infected rodents has implications for the understanding of viral entry, viral pathogenesis and accessibility of the CNS of rodents to drugs or effector molecules.

INTRODUCTION

Increased blood–brain barrier (BBB) permeability in West Nile virus (WNV)-infected rodents may be a primary determinant for mortality, or it may be incidental to viral infection (Bradbury, 2005; Diamond & Klein, 2004; Paterson, 2005; Wang *et al.*, 2004). The BBB separates the parenchyma of the central nervous system (CNS) from the general circulation. It is composed of specialized microvascular endothelial cells in association with astrocyte-foot processes on the abluminal side of the vessels (Beck *et al.*, 1984). While the barrier acts to exclude most molecules and cells from the brain, it also selectively transports molecules, e.g. ions, glucose and insulin, into the brain. For example, peripheral interleukin (IL)-1α can alter tight junctions, increase pinocytosis of cells, and perhaps allow entrance of tumour necrosis factor (TNF)-α, quinolinic acid, arachidonic acid metabolites or nitric oxide, thereby affecting pathogenic processes (Rosenberg & Fauci, 1990). Viruses can benefit from increased permeability by having greater access to the CNS. For example, mortality associated with infection by non-neuroinvasive strains of WNV or Sindbis virus can be enhanced by prior administration of lipopolysaccharide, which is known to increase BBB permeability (Lustig *et al.*, 1992) in addition to having strong inflammatory properties. Alternatively, viruses may infect the brain by alternative routes. Rabies virus, for example, infects peripheral tissues and neurons, and travels transneuronally to the CNS by retrograde axonal spread thereby bypassing entry through the BBB (Finke & Conzelmann, 2005). Protection from lethal WNV infection may depend on the accessibility of the brain to immune-reactive cells through the BBB or through alternative routes (Ransohoff *et al.*, 2003). The chemokines CXCL10 and CCL5 direct leukocyte recruitment into the brain and control of WNV disease (Klein *et al.*, 2005).

Cytokines such as TNF-α, IL-1β and IL-6 enhance the permeability of the BBB in cell culture models (de Vries *et al.*, 1996; Fiala *et al.*, 1997) and in rodents (Mayhan,

2002). More specifically, Toll-like receptor 3 (Tlr-3) regulates the entry of WNV into the brains of C57BL/6 mice challenged intraperitoneally (i.p.) with WNV, possibly by TNF-α production and TNF-α receptor 1 signalling (Wang *et al.*, 2004). Despite an increased WNV load outside the brain in Tlr-3 deficient mice (Tlr-$3^{-/-}$) compared with wild-type C57BL/6 mice, the WNV load within the brain is markedly reduced in Tlr-$3^{-/-}$ mice, suggesting that Tlr-3 facilitates entry of the virus into the brain. To test this directly, Evans blue administered i.p. was excluded from the brains of WNV-infected Tlr-$3^{-/-}$ knockout mice, but permeated into some brains of polyribocytidylic acid [poly(I:C)]-treated and WNV-infected C57BL/6 wild-type mice (Wang *et al.*, 2004).

BBB permeability can be assessed by measuring the leakage of systemic proteins, such as fibrinogen or albumin, into the cerebrospinal fluid (CSF) or CNS. This has been done with human immunodeficiency virus (Dallasta *et al.*, 1999), simian immunodeficiency virus encephalitis (Luabeya *et al.*, 2000) and a mouse model of experimental allergic encephalomyelitis induced by infection with an avirulent strain of Semliki Forest virus (SFV) (Eralinna *et al.*, 1996). BBB permeability can also be assessed by measuring the leakage of indicator dyes or fluorescent molecules into the brain or CSF (Olsen *et al.*, 2007). The question addressed in this study, using these methods, is whether increased BBB permeability in WNV-infected rodents is necessary for a lethal infection to occur.

METHODS

Animals and viruses. Adult female Syrian golden hamsters, BALB/c mice and C57BL/6 mice greater than 7 weeks of age were used (Charles River Laboratories). Animals were randomized to treatment groups. Animal use was in compliance with the Utah State University Institutional Animal Care and Use Committee in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility. Banzi virus (BANV) (H336 strain) and SFV (Original strain) were obtained from the American Type Culture Collection (Manassas, VA). The prototypic NY99 WNV was obtained from Robert Lanciotti (Centers for Disease Control). Viruses were diluted appropriately in minimal essential medium (Gibco/BRL). Animals were injected subcutaneously with WNV and i.p. with BANV and SFV.

Collection of CSF from hamsters. CSF was collected from the cisterna magna of live hamsters (Morrey *et al.*, 2006). Animals were anaesthetized with ketamine HCl, and placed in a stereotaxic device with the neck maximally flexed to fully expose the atlanto-occipital fossa. Anaesthesia was maintained throughout the remainder of the procedure using isoflurane inhalation anaesthesia (2 % isoflurane, 1 l O_2 min^{-1}). A 5–7 mm incision was made from the shoulders to the dorsal aspect of the skull. Collection of CSF was made using a 30 gauge needle attached to one end of a short length of Tygon (Saint-Gobain) microbore tubing [0.01 inch (0.254 mm) inner diameter], with a syringe attached to the other end. The needle in the arm of the stereotaxic device was inserted into the neck 4 mm ventral of the crest of the skull and on the midline. While maintaining a mild suction with the syringe, the needle was slowly advanced until fluid was observed entering the tube. A 30–70 μl volume of CSF was collected per animal. Red blood cells were counted to determine the extent of blood contamination and to eliminate contaminated CSF from the dataset.

BBB permeability assay using fluorescein. For evaluation of BBB permeability to small molecular mass compounds in mice, the animals were injected with 10 mg sodium fluorescein (Sigma Aldrich) in 0.1 ml sterile saline, administered i.p. (Olsen *et al.*, 2007). Animals were anaesthetized with ketamine HCl (100–200 mg kg^{-1}) i.p. 45 min after the sodium fluorescein injection in order to collect blood. Blood was collected into serum separator tubes (Sarstedt) by retro-orbital bleeding. The serum was stored at −70 °C until processing. Transcardial perfusion with PBS (250 ml) was performed to remove blood from the intravascular compartment. The brain was removed, weighed, homogenized in 1 ml sterile PBS and then stored at −70 °C until processing. Protein was precipitated from brain and serum samples with trichloroacetic acid (TCA) to remove potential background fluorescence. To prevent precipitation of sodium fluorescein in serum, the samples were diluted 1 : 10 in sterile PBS prior to an additional 1 : 10 dilution in 20 % TCA. Brain samples were first centrifuged at 1250 ***g*** for 5 min, after which the resulting supernatant was diluted 1 : 10 in 20 % TCA. All samples were incubated at 4 °C for 24 h. Samples were centrifuged at 10 000 ***g*** for 15 min to remove precipitated protein. The supernatant was removed and diluted with equal volumes of borate buffer (0.05 M, pH 10), resulting in a final concentration of 10 % TCA and 0.025 M borate buffer. Samples were analysed on a 96-well plate fluorometer (Molecular Devices) using an excitation wavelength of 480 nm, and fluorescence was measured at 538 nm. A standard curve for quantification of sodium fluorescein in the samples was generated by simultaneously analysing samples of known sodium fluorescein concentration in 10 % TCA and 0.025 M borate buffer. The degree of BBB permeability was measured as the percentage (w/v) of sodium fluorescein in a gram of brain tissue per the amount of sodium fluorescein in a millilitre of serum.

The sera and brain homogenates from mice were available in sufficient volumes for measurement of fluorescence using a fluorometer. However, CSF samples collected from live hamsters were in limiting volumes, so modifications were made to accommodate small volumes. This was accomplished by using a sensitive Typhoon Trio+ imager (GE Healthcare) in a 96-well format. Hamsters were injected i.p. with 10 mg sodium fluorescein in a volume of 0.1 ml. Forty-five minutes later, serum and CSF were obtained from each animal and assayed for fluorescence directly without protein precipitation. CSF from uninfected controls, CSF from WNV-infected hamsters and serum samples were diluted 1 : 10, 1 : 80 and 1 : 100, respectively, in 15 % ethanol in PBS to a volume of 40 μl per well of a 96-well plate. Similarly, standard curves were prepared from serial dilutions of either dextran-fluorescein or Na-fluorescein. A Typhoon Trio+ imager was used to quantify the fluorescence using a 526 nm SP fluorescein emission filter, 265V photomultipler tube (PMT) initial voltage setting, 488 nm blue laser, high sensitivity setting, 100 μm pixel size, and a +3 mm focal plane. The array analysis routine of ImageQuant TL software (GE Healthcare) was used to analyse the data. If a single well exceeded 3.8 million fluorescence units (FU), the plate was rescanned at a lower PMT voltage, e.g. 230 V. Alternatively, if the lower dilutions on the curve samples did not show numbers significantly above zero, or at least 10 FU the plate was rescanned at a higher PMT voltage, e.g. 300 V. After exporting the file to Microsoft Excel, the concentration of Na-fluorescein or dextran-fluorescein in samples was extrapolated from the standard curve and reported as μg ml^{-1} CSF or serum. The degree of BBB permeability was measured as the percentage of fluorescence in CSF as compared to serum for each animal.

Evans blue BBB permeability assay. Mice were injected i.p. with 800 μl or 1 % (w/v) Evans blue dye, and then perfused with PBS 1 h

later. The blue colouration of brains was a qualitative indicator of BBB permeability. The blue colourations of spleens, livers and kidneys were controls for effective tissue distribution of Evans blue.

Micro-protein assay. The protein concentrations of the CSF samples were determined by a modified bicinchoninic acid assay (Thermo Fisher Scientific). Modifications of the assay included scaling down the chemistry to use a 4 μl sample size and a 384 well micro-assay plate and reading on a Typhoon Trio+ imager.

RESULTS

A lethal infection of SFV, an alphavirus, in BALB/c mice increased the brain : serum ratio of fluorescence (Fig. 1a). Permeability began rising at 5 days post-injection (p.i.), when death among the mice had begun. By 7 days p.i., the brain : serum ratio of fluorescence of surviving mice was 7–10 % as compared with less than 4 % in uninfected mice. A lethal infection of BANV, a flavivirus like WNV, also increased the brain : serum ratio of fluorescence (Fig. 1b). Unlike SFV infection, the permeability began rising at 5 days p.i., which was 3 days (8 days p.i.) before animals started dying, which has been reported in a previous study (Olsen *et al.*, 2007). Unexpectedly, a lethal infection of WNV in BALB/c mice did not affect the BBB within the sensitivity of the fluorescence assay (Fig. 1c).

We evaluated BBB permeability in C57BL/6 mice because a previous report demonstrated that WNV infection elevated BBB permeability in this strain (Wang *et al.*, 2004). Before evaluating BBB permeability in C57BL/6 mice, however, we compared the lethality of WNV using the same viral challenge in both 6–8-week-old BALB/c and C57BL/6 female mice (Fig. 2). There was essentially no difference in the survival curves between these two mouse strains. Therefore, the differences in BBB permeability between the two mouse strains would not account for a difference in strain mortality. When evaluated in C57BL/6 mice, the lethal WNV infection appeared to increase BBB permeability, beginning at about the time that animals started to die (6 days p.i.) and probably after the virus had infected the brain (Morrey *et al.*, 2006; Oliphant *et al.*, 2005). Some

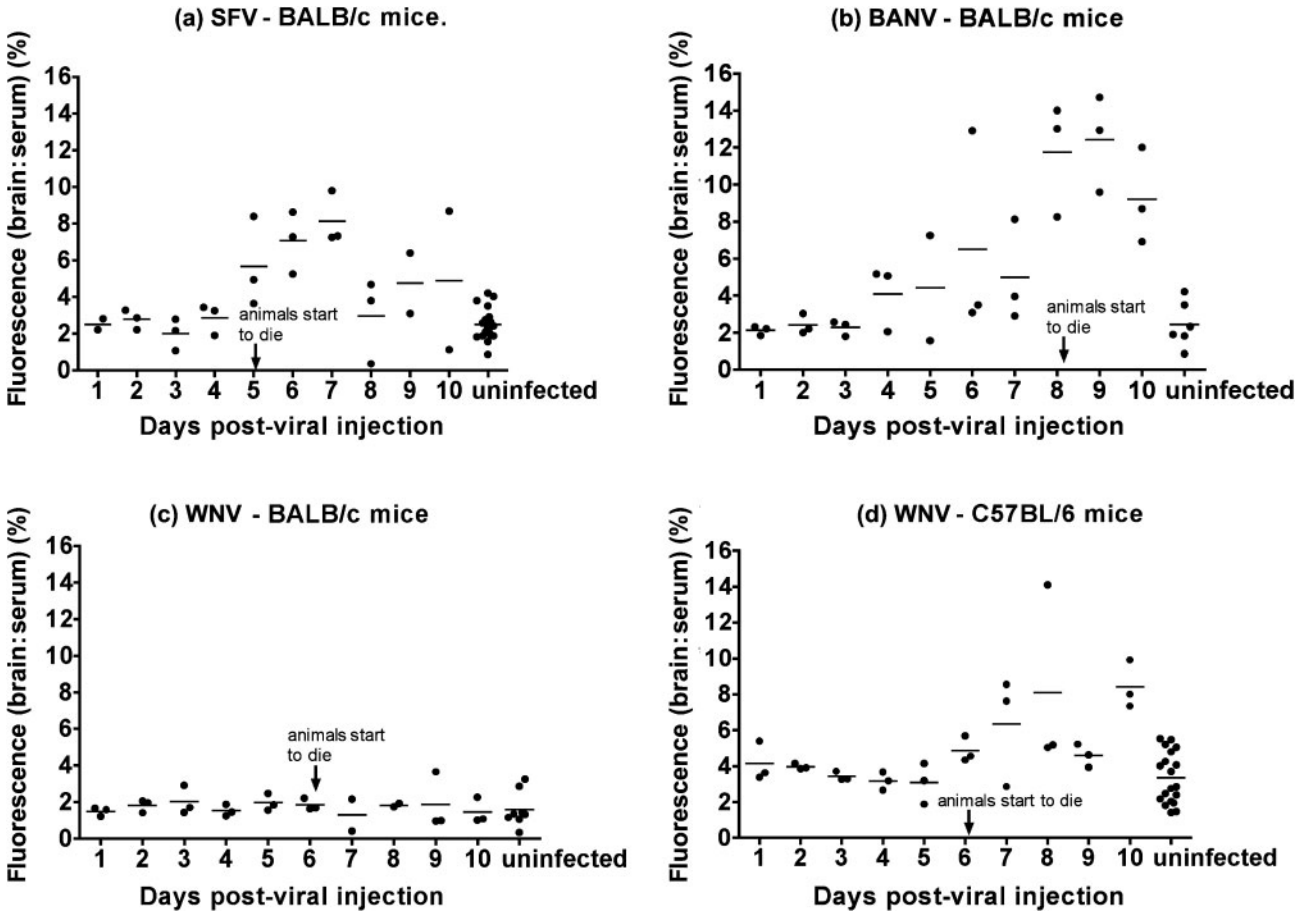

Fig. 1. Measurement of BBB permeability using fluorescein in BALB/c or C57BL/6 mice inoculated subcutaneously (s.c.) with BANV (flavivirus), SFV (alphavirus) and WNV (flavivirus). Mice were challenged i.p. with 10^4 50 % cell culture infectious doses ($CCID_{50}$) SFV or 10 $CCID_{50}$ BANV or s.c. with 10^5 $CCID_{50}$ WNV. Forty-five minutes after i.p. injection with sodium fluorescein, the sera and brains were processed to measure fluorescence, and ratios of brain : serum fluorescence were calculated.

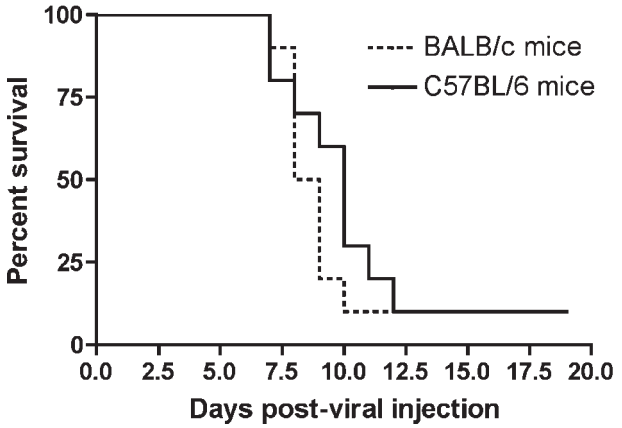

Fig. 2. Comparative survival of female BALB/c and female C57BL/6 mice (6–7-weeks-old) inoculated s.c. with 10^4 $CCID_{50}$ WNV.

of the mice had slightly elevated brain : serum ratios of fluorescence during this period of infection, but many animals had values within the normal range (Fig. 1d).

To provide further confirmation that BBB permeability is not altered in BALB/c mice by WNV infection, a different dye, Evans blue, was used. At 1, 3, 5, 7 and 9 days after viral challenge, BALB/c mice were injected i.p. with 800 µl or 1 % (w/v) Evans blue dye, and then cardiac-perfused with PBS 1 h later. None of the brains from WNV-infected mice were a blue colour at any time during the course of infection, and all appeared the same as those of normal mice not receiving Evans blue (Fig. 3). Tissues from a WNV-infected mouse injected i.p. with Evans blue at 9 days after viral challenge are shown in Fig. 3 along with those of a sham-infected mouse with no Evans blue injected. The lack of blue colour in the brains of WNV-infected BALB/c mice suggested no gross change in BBB permeability. However, the blue colour of spleens, livers and kidneys indicated that the Evans blue dye was well distributed throughout the peripheral tissues

The hamster was chosen as a second laboratory species for evaluation because the hamster presents various neurological disease signs similar to other species (Morrey *et al.*, 2004; Xiao *et al.*, 2001), and because surgical manipulation is more feasible as compared to the mouse (Morrey *et al.*, 2006). To evaluate BBB permeability in WNV-infected hamsters, we used a previously described procedure (Frankmann, 1986) in which CSF was collected from WNV- or sham-infected hamsters after i.p. injection of sodium fluorescein at 10 days p.i. The percentage fluorescence of CSF : serum ratios correlated with the percentage fluorescence of brain : serum ratios of the same hamsters (R^2=0.79) (Fig. 4), which helped to validate the use of CSF for evaluating BBB permeability.

The CSF : serum ratio of fluorescence was determined over the course of WNV-infection in hamsters (Fig. 5a). Starting

Fig. 3. Measurement of BBB permeability using Evans blue in BALB/c mice inoculated s.c. with WNV ($10^{5.1}$ $CCID_{50}$). At 1, 3, 5, 7 and 9 days after viral challenge, mice were injected i.p. with 800 µl or 1 % (w/v) Evans blue dye, and then cardiac perfused with PBS 1 h later. Tissues from a WNV-infected mouse and a sham-infected mouse injected i.p. with Evans blue at 9 days after challenge are shown. The lack of blue colour in brains indicated no detectable BBB permeability at any time during the course of WNV infection. The blue colours of spleens, livers and kidneys were a control for tissue distribution of Evans blue.

at 6 days p.i., there was a trend towards increased permeability, at about the same time animals started dying, but the differences compared to the uninfected controls were not statistically significant. Additionally, the values of many WNV-infected animals were within the range of normal values. To further corroborate the fluorescence results, serum protein leakage was examined in the CSF. Leakage of peripheral proteins, such as fibrinogen or albumin, into the CSF or CNS has been used previously to assess BBB permeability (Dallasta *et al.*, 1999). Since albumin constitutes 55 % of all serum protein, the total protein concentration was determined as an indicator of permeability. A micro-method was developed to measure total protein in the CSF of infected hamsters during the course of infection (Fig. 5b). Only one animal at 4 days p.i. and one animal at 13 days p.i. appeared to have slightly increased CSF : serum protein ratios compared to the

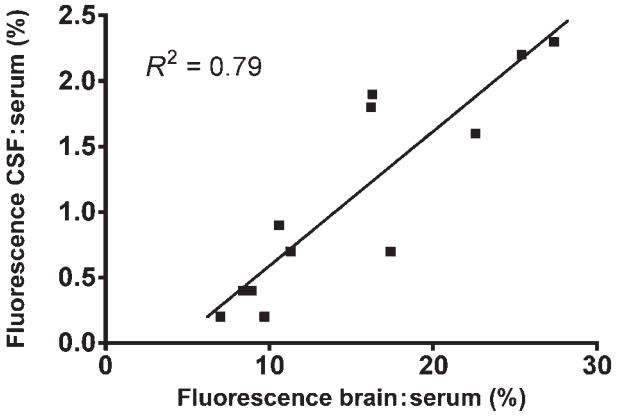

Fig. 4. Correlation of brain : serum fluorescence compared to CSF : serum fluorescence for measurement of BBB permeability in hamsters. Hamsters were challenged s.c. with $10^{5.3}$ $CCID_{50}$ of WNV or sham. At 10 days p.i. 45 min after fluorescein injection, the brain homogenate, CSF and serum were collected and measured for fluorescence using the Typhoon Trio+ imager.

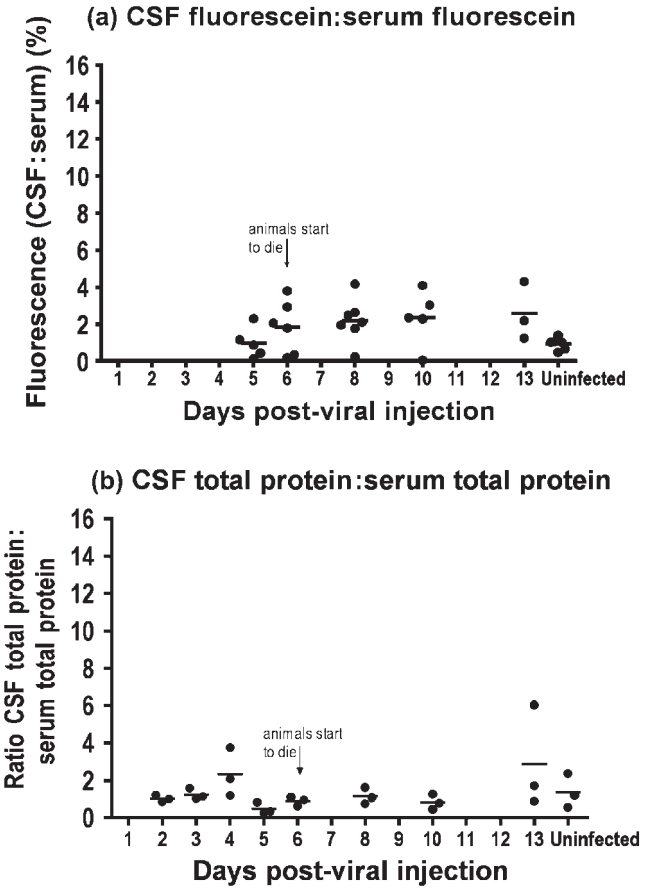

Fig. 5. Temporal BBB permeability in hamsters infected with WNV as determined by fluorescein and total protein in CSF compared to sera. Hamsters were injected s.c. with $10^{5.3}$ $CCID_{50}$ (Morrey *et al.*, 2002). At various days p.i., sodium fluorescein was injected, and sera and CSF were collected during recovery surgery. (a) Ratio of CSF : serum fluorescence. (b) Total protein in CSF : serum.

control values, but all of the other WNV-infected animals had ratios at normal levels.

To determine if the lethality of WNV infection was correlated with levels of BBB permeability, the fluorescence of i.p. injected fluorescein (Fig. 6a) and concentration of total protein (Fig. 6b) in the CSF of hamsters were quantified. The hamsters were then monitored for survival. BBB permeability values did not correlate with mortality of these hamsters (Fig. 6), suggesting that increased BBB permeability was not an important determinant for mortality in hamsters.

DISCUSSION

WNV infection has been reported to increase BBB permeability, at least in some individual animals (Lustig *et al.*, 1992; Wang *et al.*, 2004). Access of immune reactive cells into the CNS of infected animals mediates recovery from an otherwise lethal infection (Sitati & Diamond, 2006). It was not known, however, if increased BBB permeability was necessary to achieve a lethal infection. Our studies suggest that a change in BBB permeability is not a requisite for the development of a lethal WNV infection. The survival curves were essentially the same between BALB/c and C57BL/6 mice, yet there was no detectable increase in BBB permeability examined using two common methods, i.e. increased brain fluorescence from i.p. administration of fluorescein and macroscopic blue colouration of the brain from i.p. administration of Evans blue. As demonstrated in a previous study (Wang *et al.*, 2004), WNV can increase the BBB permeability of WNV-infected C57BL/6 mice. However, we observed that only a subset of mice of this strain had increased permeability. Moreover, the levels and frequency of brain fluorescence of WNV-infected mice were lower than those for the positive control mice using another flavivirus (BANV) or an alphavirus (SFV) (Olsen *et al.*, 2007). If BBB permeability were required for a lethal infection, then the onset of increased permeability would occur before the onset of death, which it did not. Taken together, our results suggest that increased BBB permeability in WNV-infected mice is not essential for a lethal infection.

Investigation with the WNV-hamster model (Morrey *et al.*, 2004) also substantiated this hypothesis. The ability to sample the CSF from hamsters during recovery surgery allowed us to correlate BBB permeability with survival or death of infected hamsters. Infected animals were injected with fluorescein and the fluorescence in the CSF was determined, where increased fluorescence would indicate increased permeability. This method was validated by

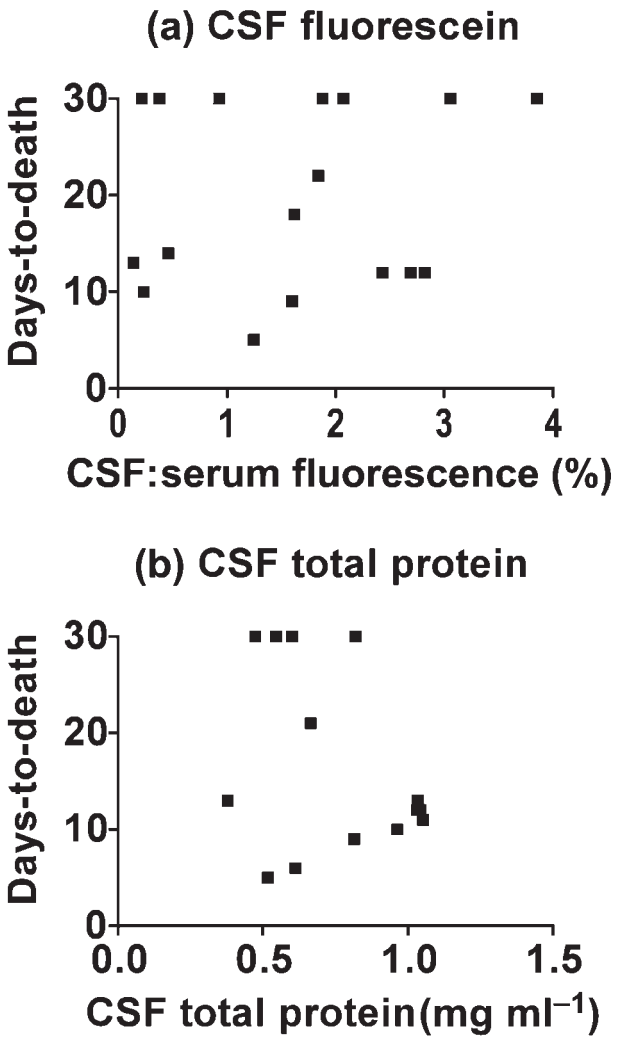

Fig. 6. Lack of correlation of mortality with BBB permeability as measured by (a) detection of i.p. injected fluorescein in the CSF, and (b) by total protein concentration in the CSF. Hamsters were injected s.c. with $10^{5.3}$ $CCID_{50}$ WNV (Morrey *et al.*, 2002). Serum and CSF were collected at 5, 6 and 8 days after viral challenge. The animals were then observed for mortality until 30 days after challenge. The data from samples collected 5, 6, and 8 days after challenge were combined since there was no correlation with mortality for any of the days.

correlating it with the widely used method of measuring fluorescence in the brains of necropsied animals ($R^2=0.79$). As observed in the mouse model, some, but not all WNV-infected hamsters had increased BBB permeability as measured with CSF. More importantly, the BBB permeability, as measured in the CSF with total protein and fluorescence of i.p. injected fluorescein, did not correlate with mortality. Thus, although some mice and hamsters have a slightly increased BBB permeability after WNV infection, increased BBB permeability is not necessary for a lethal outcome.

The reason for this diminished BBB permeability with WNV as compared with other encephalitides might be explained by the differences in disease pathology. We have observed that Western equine encephalitis virus (WEEV), an alphavirus, caused considerable neuropathology (ventral horn vacuolation, myelin sheath swelling, axonal swelling, neuronal degeneration and localized gliosis) in the spinal cord at the onset of paralysis in hamsters injected in the spinal cord with WEEV (unpublished data). In contrast, WNV caused extensive terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-apoptosis staining with only minimal lymphocyte infiltration, gliosis, or mild neuron necrosis in the spinal cord at the onset of paralysis in hamsters injected in the spinal cord with WNV (unpublished data). The reduced ability of WNV to increase BBB permeability in all animals may be because WNV kills neurons directly by apoptosis (Samuel *et al.*, 2006; Shrestha *et al.*, 2003; Yang *et al.*, 2002), but not primarily by inflammatory responses that increase the BBB permeability (Mayhan, 2002).

$CD4^+$ and $CD8^+$ T cells are required for clearance of WNV from the CNS (Sitati & Diamond, 2006), but increased BBB permeability may not be required for T cells to access the CNS (Kleine & Benes, 2006; Ransohoff *et al.*, 2003). Since BBB permeability is not measurably increased in many rodents infected with WNV, particularly in BALB/c mice, how might T cells traffic into the CNS to clear WNV from the CNS of surviving animals? Different model systems have shown that activated lymphocytes can enter the CNS of normal individuals (Kleine & Benes, 2006). More specifically, a first wave of low-efficiency migration may cross from blood to CSF through the choroid plexus where the BBB is not completely intact, from blood to the subarachnoid space through meningeal vessels, or from blood to parenchymal perivascular spaces (Ransohoff *et al.*, 2003). It is possible, therefore, that lymphocytic cells may have access to the CNS of WNV-infected mice without depending on increased BBB permeability.

In conclusion, even though some individual WNV-infected rodents may have increased BBB permeability (Diamond & Klein, 2004; Lustig *et al.*, 1992; Paterson, 2005; Wang *et al.*, 2004), the enhanced permeability is not a primary determinant for lethality of WNV in rodents. The lack of WNV-induced BBB permeability has implications for understanding viral entry into the CNS, viral pathogenesis and accessibility of drugs or effector molecules into the CNS of WNV-infected rodents.

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