

Early activation of the host complement system is required to restrict central nervous system invasion and limit neuropathology during Venezuelan equine encephalitis virus infection

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Venezuelan equine encephalitis virus (VEEV) is a mosquito-borne RNA virus of the genus *Alphavirus*, family *Togaviridae*, that is responsible for sporadic outbreaks in human and equid populations in Central and South America. In order to ascertain the role that complement plays in resolving VEEV-induced disease, complement-deficient C3^{-/-} mice were infected with a VEEV mutant (V3533) that caused mild, transient disease in immunocompetent mice. In the absence of a functional complement system, peripheral inoculation with V3533 induced much more severe encephalitis. This enhanced pathology was associated with a delay in clearance of infectious virus from the serum and more rapid invasion of the central nervous system in C3^{-/-} mice. If V3533 was inoculated directly into the brain, however, disease outcome in C3^{-/-} and wild-type mice was identical. These findings indicate that complement-dependent enhancement of peripheral virus clearance is critical for protecting against the development of severe VEEV-induced encephalitis.

INTRODUCTION

Venezuelan equine encephalitis virus (VEEV) is a mosquito-borne alphavirus of the family *Togaviridae* that is endemic to Central and South America (Griffin, 2001). Although normally maintained in an enzootic transmission cycle between various rodent host species and the *Culex* mosquito vector, VEEV periodically emerges from its natural cycle to cause local epidemics in human and equid populations (Carrara *et al.*, 2005; Weaver & Barrett, 2004). The most recent major outbreak occurred in 1995 in Colombia and Venezuela, where 75 000–100 000 human cases were reported (Weaver *et al.*, 1996). VEEV infection in humans causes a spectrum of disease ranging from asymptomatic to flu-like illness to overt encephalomyelitis in approximately 4% of cases, with an overall case mortality rate of approximately 0.5–1% (Weaver *et al.*, 2004). In equid

populations, the development of overt encephalomyelitis is more common, and the overall mortality rate often exceeds 50% (Wang *et al.*, 2001). Although specific viral sequence determinants associated with epidemic emergence have been identified, outbreaks remain unpredictable (Anishchenko *et al.*, 2006). As a result, VEEV remains a significant public health threat in the region.

Much of our current understanding of VEEV pathogenesis and immunity comes from studies carried out in a well-characterized mouse model of infection. VEEV infection of the mouse closely replicates the lymphotropic aspects of infection in humans and the lymphotropic and encephalitic aspects of equid infection (Gleiser *et al.*, 1962). Subcutaneous (s.c.) injection of VEEV into the mouse footpad is followed by efficient replication in the skin-draining popliteal lymph node and rapid dissemination to the spleen and other secondary lymphoid organs (Aronson *et al.*, 2000; MacDonald & Johnston, 2000). Replication at these sites leads to the development of a serum viraemia within the first 12 h of infection. Virus invasion of the central nervous system (CNS) first occurs through the olfactory neuroepithelium, a mucosal surface that is densely innervated with olfactory sensory neurons (Charles *et al.*, 1995). It is thought that these cells are infected following diffusion of virus through the permeable

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fenestrations of adjacent capillaries. The virus then disseminates into the olfactory bulb of the brain by centripetal spread, usually within 36 h of infection (Charles *et al.*, 1995). Once the virus has crossed into the CNS, it replicates predominantly within neurons, triggering the death of the animal from a paralyzing encephalomyelitis within 6–8 days post-infection (p.i.) (Charles *et al.*, 2001; Grieder *et al.*, 1995). The mortality rate in both inbred and outbred strains of mice is 100%, and results from a combination of virus-mediated cytolysis of infected neurons and the detrimental effects of the host immune response within the CNS (Charles *et al.*, 2001; Wang *et al.*, 2001).

Engagement of both the innate and adaptive arms of the immune response is required for successful control of VEEV infection. The type I interferon system plays a critical role in limiting early virus replication and dissemination (White *et al.*, 2001). Antiviral antibodies can limit virus dissemination in the periphery, as well as aid in clearance of virus from infected neurons (Levine *et al.*, 1991; Mathews & Roehrig, 1982). The role of $\alpha\beta$ T cells during VEEV infection is unclear, as they can contribute to control of VEEV infection within the CNS, as well as to VEEV-induced immune pathology (Brooke *et al.*, 2010; Charles *et al.*, 2001; Paessler *et al.*, 2007). Further efforts to identify the components of a successful immune response to VEEV infection have been limited by the extreme lethality of the virus in mice.

The host complement system is a complex network of over 30 soluble and cell-associated factors that contribute to both innate and adaptive control of microbial infection (Carroll, 2004). Activated complement components act to limit infection through a number of mechanisms including opsonization, direct killing of pathogens, and recruitment and regulation of inflammatory cells (Carroll, 2004; Kemper & Atkinson, 2007; Roozendaal & Carroll, 2006). Complement plays a critical protective role during infection with a number of viruses, including influenza A virus, West Nile virus, ectromelia virus and herpes simplex virus (Kopf *et al.*, 2002; Mehlhop & Diamond, 2006; Moulton *et al.*, 2008; Verschoor *et al.*, 2003). In contrast, complement activation enhances virus-induced pathology following infection with the alphaviruses Ross River virus and Sindbis virus (Hirsch *et al.*, 1978; Morrison *et al.*, 2007).

In this study, we used an established model of acute, non-lethal VEEV infection to ascertain the role of the complement system in VEEV pathogenesis and immunity. S.c. infection of wild-type C57BL/6J mice with the V3533 strain of VEEV results in the development of a mild, self-limiting illness with minimal signs of CNS complications (Brooke *et al.*, 2010). In contrast, infection of complement-deficient $C3^{-/-}$ mice with V3533 resulted in the development of severe encephalitis, suggesting that the complement system plays an important role in limiting VEEV-induced pathology. No differences in disease outcome were observed following intracranial infection, however,

indicating that complement was acting at a step prior to neuroinvasion. Further studies revealed delayed serum clearance and earlier neuroinvasion in $C3^{-/-}$ mice that could not be explained by defects in anti-VEEV antibody induction or the loss of C5-dependent complement function. Together, these findings demonstrate that complement-dependent enhancement of virus clearance from the periphery plays a critical role in limiting the severity of VEEV-induced encephalitis by restricting virus dissemination to the CNS.

RESULTS

$C3^{-/-}$ mice develop more severe encephalomyelitis following V3533 infection

Previous work has demonstrated that s.c. infection of C57BL/6J mice with V3533 results in the development of mild disease associated with virus neuroinvasion and replication within the CNS, followed by clearance and recovery (Brooke *et al.*, 2010). Using V3533 infection of C57BL/6J mice as a model of successful control of VEEV infection, we used congenic complement-deficient $C3^{-/-}$ mice to determine what role, if any, the host complement system plays during VEEV infection. $C3^{-/-}$ and C57BL/6J mice were infected s.c. with 10^6 p.f.u. V3533 in the footpad (Fig. 1). Weight loss in C57BL/6J mice was minimal, and clinical signs of disease were mild, consisting of hunched posture, ruffled fur and mild motor dysfunction in a subset of infected animals. In contrast, $C3^{-/-}$ mice uniformly lost significantly more weight and developed more severe signs of encephalitis, with all infected animals developing pronounced ataxia and in some cases hind limb paresis or paralysis. In both C57BL/6J and $C3^{-/-}$ mice, these signs of disease were transient, and all infected mice went on to recover. The significantly enhanced severity of encephalitic disease observed in $C3^{-/-}$ mice compared with that in with C57BL/6J mice clearly demonstrated that the host complement system was playing a protective role during V3533 infection.

Enhanced disease severity in $C3^{-/-}$ mice is associated with more extensive inflammation and pathology within the brain

In several different models of alphavirus infection of the CNS, disease is associated with extensive inflammatory-cell infiltration within the brain parenchyma (Charles *et al.*, 2001; Fazakerley & Buchmeier, 1993; Grieder *et al.*, 1995; Irani & Griffin, 1996). To determine whether the differences in weight loss and clinical course of wild-type and $C3^{-/-}$ mice following V3533 infection correlated with differences in inflammation and neuropathology within the brain, haematoxylin and eosin (H&E)-stained brain sections from V3533-infected mice were blinded and scored by an outside investigator (Fig. 2). In both wild-type and $C3^{-/-}$ mice, signs of inflammation first became apparent between 4 and 8

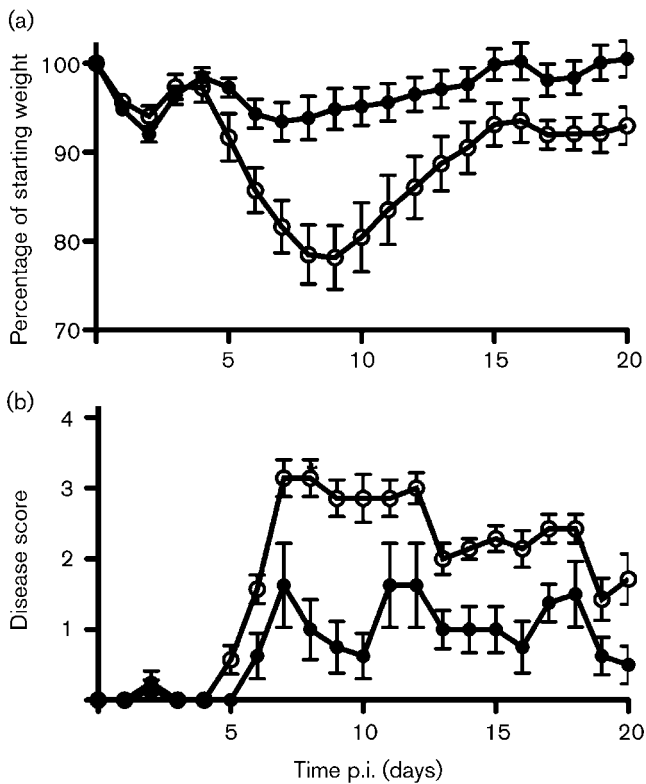


Fig. 1. Complement activation limits disease severity following V3533 infection. C57BL/6J (●) or C3^{-/-} (○) mice (seven or eight mice per group) were infected with 10⁶ p.f.u. V3533 in the left rear footpad. Weight loss (a) and disease score (b) were assessed following infection. Mice were scored according to the scale described in Methods. Each data point represents the arithmetic mean \pm SEM. Differences between groups were significant for both weight loss ($P=0.016$) and disease score ($P=0.0014$) by repeated-measures analysis of variance (ANOVA) testing. The results are representative of three independent experiments.

days p.i. By day 8, focal and diffuse inflammatory-cell infiltration was observable in the olfactory bulb, cerebellum, brainstem and occasionally the cortex of both wild-type and C3^{-/-} mice (Fig. 2c, d). At this time point, the cellular density of inflammatory cells within the glomerular layer of the olfactory bulb was significantly higher in C3^{-/-} mice compared with that in the wild-type mice ($P=0.01$, two-tailed t -test) (Fig. 2a). The glomerular layer is the site of synapse between sensory neurons of the olfactory neuro-epithelium and olfactory tract neurons within the CNS, and is thought to be the site at which VEEV first enters the CNS during infection. Throughout the rest of the brain, the mean number of inflammatory foci was higher in C3^{-/-} mice relative to wild-type mice, but this difference did not achieve significance (Fig. 2b). By day 12 p.i., when V3533-infected mice had begun to recover, inflammatory infiltration was still observed throughout the brains of both mouse strains, although the overall cellularity and numbers of foci tended to be higher in the C3^{-/-} mice. Thus, the differences in

outward disease signs between wild-type and C3^{-/-} mice following V3533 infection correlated with the severity of inflammation within the brain, primarily the olfactory bulb.

Viral burdens within the CNS of C3^{-/-} mice are higher and less variable than those of wild-type mice

V3533 infection of C57BL/6J mice results in virus replication within the brain and spinal cord, followed by clearance of infectious virus by 8 days p.i. (Brooke *et al.*, 2010). Given the differences in disease outcome between C57BL/6J and C3^{-/-} mice following V3533 infection, we next asked whether the more severe disease in C3^{-/-} mice was associated with a larger viral burden within the CNS or with a defect in virus clearance. To answer this question, C57BL/6J and C3^{-/-} mice were infected s.c. in the footpad with 10⁶ p.f.u. V3533. The animals were sacrificed at 0.5, 1, 2, 4, 6 and 8 days p.i. and viral burdens in serum, spleen, draining popliteal lymph node, brain and spinal cord were assessed by plaque assay (Fig. 3).

In C57BL/6J mice, virus was first detected in the brain on day 2, with peak titres being reached between days 4 and 6. On day 4, titres were highly variable, ranging from below the limit of detection up to 10⁶ p.f.u. g⁻¹, but by day 6, the titres were much more consistent, with a geometric mean of 8.65×10^3 p.f.u. g⁻¹. In the spinal cord, virus was detectable only on days 4 and 6, with no virus being detectable in a subset of mice at each time point (two of nine mice at day 4, three of seven mice at day 6). In both brain and spinal cord, infectious virus was undetectable by day 8. In contrast, C3^{-/-} mice had detectable virus in both the brain and spinal cord within 24 h of infection. Virus titres in both tissues peaked at day 4, with mean titres in both brain and spinal cord higher than those observed in C57BL/6J mice (brain: $P=0.0022$, spinal cord: $P=0.0552$; two-tailed t -test). Virus titres in the brains of C3^{-/-} mice at day 4 were also much less variable than those observed in C57BL/6J mice, as all mice tested had titres of at least 10⁵ p.f.u. g⁻¹. Clearance kinetics were similar between C57BL/6J and C3^{-/-} mice, although on day 8 some C3^{-/-} animals still had detectable virus titres in the brain (four of seven mice) and spinal cord (one of seven mice). Thus, the more severe disease observed in C3^{-/-} mice was associated with more rapid neuroinvasion and with higher and more consistent viral burdens within the CNS.

C3^{-/-} mice exhibit a delay in virus clearance from the serum relative to wild-type mice

To assess the effect of host complement on early replication of V3533 in the periphery, we compared viral burdens within the serum of C57BL/6J and C3^{-/-} mice, as well as the draining popliteal lymph node and spleen, two anatomical sites of replication thought to contribute to serum viraemia (Fig. 3). In the serum, peak titres in both C57BL/6J and C3^{-/-} mice occurred at 12 h p.i. and were similar, but the

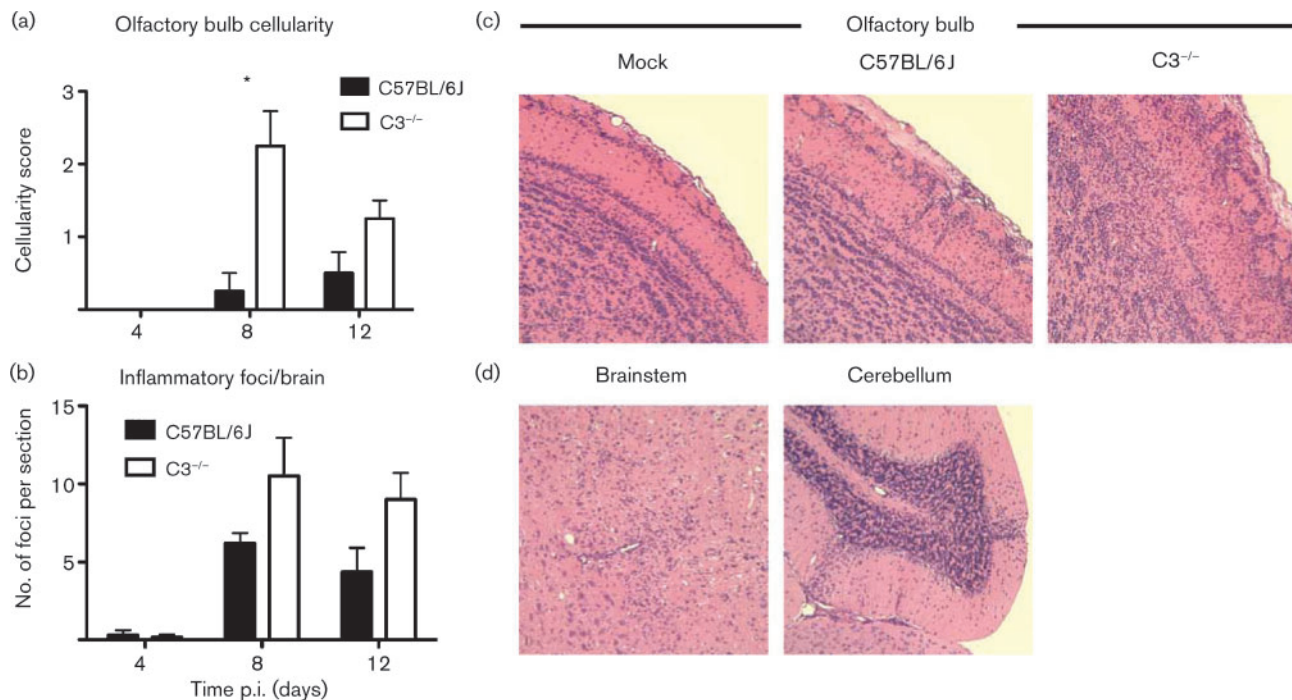


Fig. 2. V3533-induced brain inflammation in C57BL/6J and C3^{-/-} mice. C57BL/6J or C3^{-/-} mice were injected s.c. with 10⁶ p.f.u. V3533 or diluent alone in the left rear footpad. At different times p.i., three to five mice per group were sacrificed and 5 µm paraffin-embedded sagittal sections were generated and stained with H&E. Sections (two per mouse) were blinded and evaluated by an outside investigator. (a) Inflammatory-cell infiltration into the olfactory bulb was scored as described in Methods. Each data point represents the arithmetic mean ± SEM. **P* < 0.05 by two-tailed *t*-test. (b) Total number of foci of inflammatory-cell nuclei per brain section. Each data point represents the arithmetic mean ± SEM. (c) Representative images of inflammation and associated pathology at day 8 p.i. in the olfactory bulbs of C57BL/6J and C3^{-/-} mice. (d) Representative images of inflammation and associated pathology at day 8 p.i. in the brainstem and cerebellum of C3^{-/-} mice. Magnification, ×20.

clearance kinetics were quite different. Serum titres in C57BL/6J were reduced about tenfold between 12 and 24 h p.i., and by day 2 the virus was undetectable in all but one animal tested. In contrast, C3^{-/-} mice sustained significantly higher serum titres on days 1 and 2 compared with C57BL/6J mice, and virus was still detectable in three of six animals on day 4. In the draining lymph node, virus titres in C3^{-/-} mice were higher than those in C57BL/6J up to day 2, although by day 4 they were equivalent. In the spleen, the virus titres were actually lower in C3^{-/-} mice over the first 24 h of infection; however, between days 1 and 6 clearance was more rapid in the C57BL/6J mice, mirroring the results observed in the serum.

Host complement plays no significant protective role following intracranial (i.c.) introduction of V3533

Given that C3^{-/-} mice were deficient in their ability to control V3533 in both the periphery and the CNS, it was possible that the effect of host complement that resulted in reduced neuropathology could be occurring prior or subsequent to virus invasion of the CNS. In order to address where in the infection process host complement

was acting to limit pathology, we introduced 10³ p.f.u. V3533 directly into the CNS of C57BL/6J and C3^{-/-} mice by i.c. injection. By circumventing peripheral infection, we were able to assess directly the importance of host complement within the CNS. Following i.c. infection, both C57BL/6J and C3^{-/-} mice showed similar outcomes (Fig. 4). Both groups began losing weight within 24 h of infection, and rapidly began exhibiting clinical signs of ascending encephalomyelitis. Peak weight loss and disease scores were observed on days 7–8 in both groups and were followed by protracted recovery. C57BL/6J and C3^{-/-} mice were indistinguishable in the kinetics of both disease onset and recovery, as well as the magnitude of peak weight loss and disease score, indicating that once V3533 has entered the CNS, the host complement system no longer plays a significant role in influencing disease outcome.

Anti-VEEV antibody responses are intact in C3^{-/-} mice

Complement activation is a requirement for the induction of antiviral antibody responses following infection with several different viruses. Whilst the results following i.c. infection of C57BL/6J and C3^{-/-} mice suggested that the

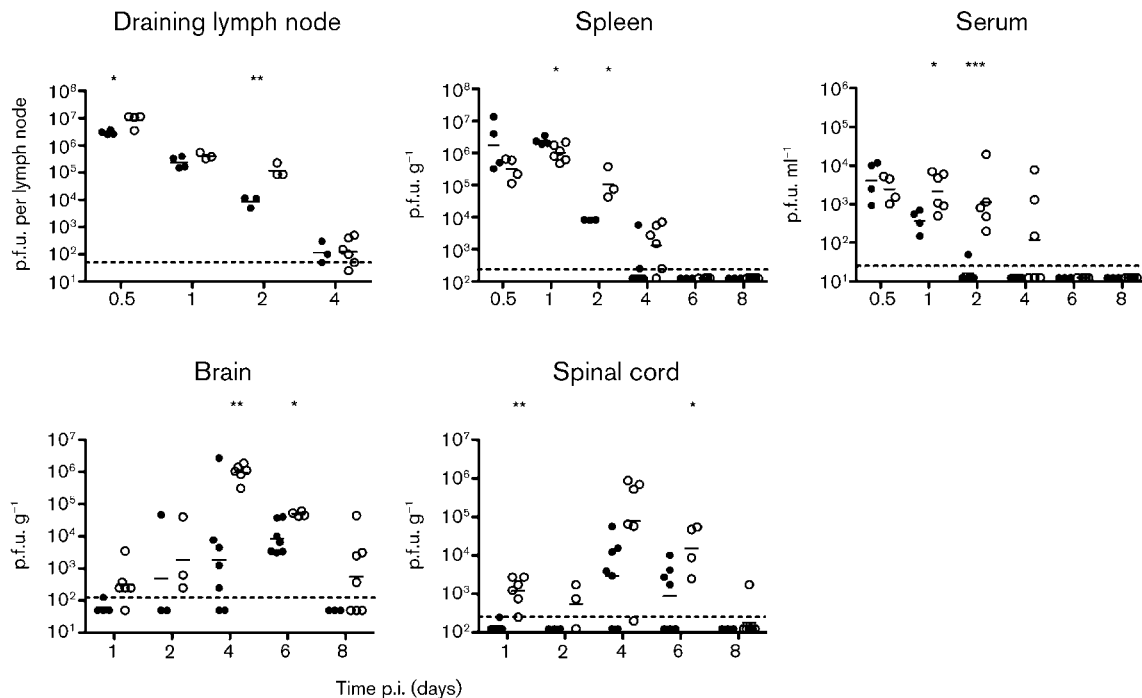


Fig. 3. V3533 tissue titres in C57BL/6J and C3^{-/-} mice. C57BL/6J (●) or C3^{-/-} (○) mice were infected s.c. with 10⁶ p.f.u. V3533 in the left rear footpad. At the indicated times p.i., serum, spleen, draining popliteal lymph node, brain and spinal cord were collected and homogenized. The viral burden in each tissue was determined by plaque assay. Data points represent individual titres pooled from three independent experiments, and horizontal bars indicate the geometric mean. **P*<0.05; ***P*<0.01; ****P*<0.001 by two-tailed *t*-test.

protective effect of complement was not related to antibody induction, we wanted to confirm this directly. C57BL/6J and C3^{-/-} mice were infected s.c. in the footpad with 10⁶ p.f.u. V3533, and serum was collected on days 2, 4, 8 and 12 p.i. to assay anti-VEEV binding and neutralization activity.

In both C57BL/6J and C3^{-/-} mice, VEEV-specific IgM was induced between days 2 and 4 p.i., whilst VEEV-specific IgG appeared between days 4 and 8 p.i. (Fig. 5a). At all time points examined, VEEV-specific IgM titres were similar between C57BL/6J and C3^{-/-} mice, with the exception of day 12 p.i. where titres were higher in the C3^{-/-} mice. IgG induction in C3^{-/-} mice appeared to be slightly delayed, as titres were lower on day 8 compared with those in C57BL/6J mice. Whilst this difference was statistically significant (*P*=0.0064, two-tailed *t*-test), titres in both groups were quite high. By day 12, IgG levels in both groups were equivalent. In addition to ELISA, which measured the amount of anti-VEEV antibody but not its biological activity, we assessed the anti-VEEV neutralizing activity of serum collected on day 4 p.i., the time point when viral burdens within the CNS were most divergent (Fig. 5b). Similar to the ELISA results, no difference was seen in neutralizing activity between C57BL/6J and C3^{-/-} mice (*P*=0.4851, two-tailed *t*-test). Together, these results demonstrated that complement is not required for the development of an anti-VEEV antibody response.

C5 activation has no protective effect during V3533 infection

Following cleavage of C3, C3b mediates assemblage of the C5 convertases C4bC2aC3b and C3bBbC3b, which act in turn to cleave C5 into C5a and C5b. These cleavage products can exert antiviral activity by at least two distinct mechanisms. C5a acts by recruiting innate effector cells to early sites of infection, whilst C5b initiates formation of the membrane attack complex, a pore-forming complex that can directly lyse virus particles or virus-infected cells (Zipfel & Skerka, 2009). Because either of these mechanisms could act early to limit virus replication and spread, we asked whether the protective effects of C3 that we observed were dependent on C5.

To address this question, we infected C5^{+/+} and C5^{-/-} mice with 10⁶ p.f.u. V3533 s.c. in the footpad and followed weight loss and the development of outward signs of disease over time (Fig. 6a). Both mouse strains responded similarly, with mild weight loss and short-lived signs of febrile illness. At no time were any statistical differences observed between C5^{+/+} and C5^{-/-} mice. We next asked whether the loss of C5 resulted in a reduced ability to clear virus from the serum or in more rapid neuroinvasion. We infected C5^{+/+} and C5^{-/-} mice with 10⁶ p.f.u. V3533 s.c. in the footpad and on days 1 and 4 p.i. compared viral burdens in the serum and brain (Fig. 6b). In the serum,

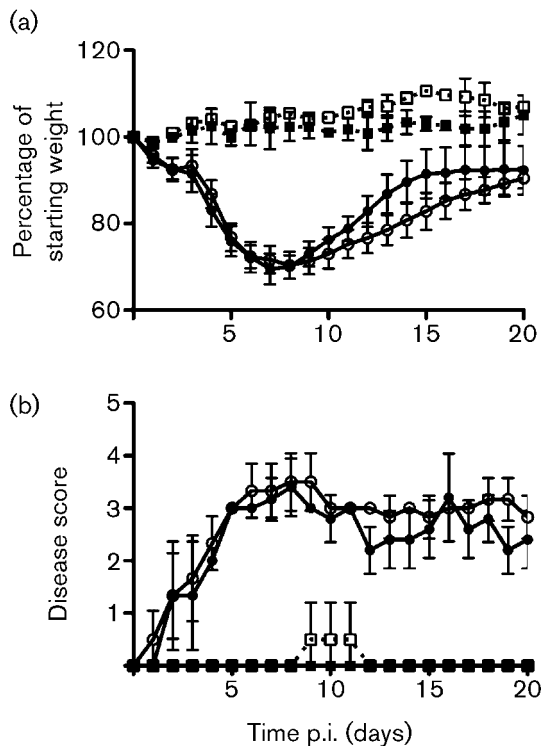


Fig. 4. Disease outcome following i.c. inoculation of V3533. C57BL/6J (■, ●) or C3^{-/-} (□, ○) mice (eight mice per group) were mock-infected (squares) or infected i.c. with 10³ p.f.u. V3533 (circles). Weight loss (a) and disease score (b) were assessed following infection. Mice were scored according to the scale described in Methods. Each data point represents the arithmetic mean \pm SEM. Differences between C57BL/6J and C3^{-/-} mice were not statistically significant based on repeated-measures ANOVA testing.

titres in both mouse strains were indistinguishable on day 1 p.i. and were undetectable by day 4, indicating that C5 played no significant role in mediating clearance of V3533 from the serum. Similarly, C5^{+/+} and C5^{-/-} mice had similar virus titres in the brain on days 1 and 4 p.i., suggesting that the efficiency of neuroinvasion was not enhanced in the absence of C5. Together, these results argue that the C5-dependent elements of the complement system do not exert a significant protective effect during VEEV infection.

DISCUSSION

The complement system has been reported to play a key protective role in the host response to a number of viral pathogens. For many viruses, including West Nile virus, influenza A virus and vesicular stomatitis virus, complement acts by enhancing B- and T-cell responses, thus facilitating adaptive control of the infection and clearance (Kopf *et al.*, 2002; Mehlhop *et al.*, 2005; Moulton *et al.*, 2008; Ochsenbein *et al.*, 1999). In the cases of Sindbis

virus and ectromelia virus, complement appears to act within the first hours of infection, limiting virus dissemination and thus downstream pathology (Hirsch *et al.*, 1980; Moulton *et al.*, 2008). In this study, we assessed the role of the host complement system in a model of recovery from acute VEEV infection. Overall, our results demonstrated that the complement system plays an important role in limiting neuropathology following VEEV infection. This protective effect resulted from complement acting in the periphery during the first 24 h of infection to limit the efficiency of neuroinvasion, and appeared to be independent of anti-VEEV antibody induction or C5-dependent effector mechanisms.

The results of this study clearly demonstrated that complement activity in the periphery, but not the CNS, had a profound effect on the extent of virus-induced neuropathology. In the absence of complement, clearance of VEEV from the serum was less efficient and virus appeared earlier within the CNS. The earlier arrival of VEEV within the CNS of C3^{-/-} mice, compared with wild-type mice, would allow the virus to replicate and spread to a greater degree prior to the generation of VEEV-specific B- and T-cell responses. A larger viral burden would most likely trigger a more robust T-cell response within the CNS, potentially resulting in more severe immunopathology. We feel that this is a probable explanation for the more severe encephalomyelitis and neuropathology observed in C3^{-/-} mice relative to that in C57BL/6J mice.

VEEV invasion of the CNS is thought to occur by diffusion of virus from the blood through fenestrated capillary endothelium to nearby unmyelinated peripheral nerve endings, followed by centripetal spread to the brain (Charles *et al.*, 1995). One consequence of this route of neuroinvasion is that the efficiency of invasion is a direct function of infectious virus concentration within the serum. This hypothesis is supported by the observation that there was a minimum virus concentration in the serum of VEEV-infected mice (approx. $\geq 10^4$ p.f.u. ml⁻¹) that was required for invasion of the brain (K. Bernard, unpublished results). Thus, any host function that limits either the magnitude or duration of serum viraemia would also reduce the efficiency of neuroinvasion. Whilst this connection has not been tested directly, a number of studies with VEEV, as well as with Sindbis virus and Western and Eastern equine encephalitis viruses, have correlated the duration of serum viraemia with virulence (Byrnes & Griffin, 2000; Jahrling, 1976; Jahrling & Gorelkin, 1975; Jahrling & Scherer, 1973; Marker & Jahrling, 1979).

In C57BL/6J mice, virus titres in the serum were significantly reduced from their peak by 24 h p.i., and were nearly absent by 48 h p.i., prior to the development of a detectable VEEV-specific antibody response. Thus, it is possible that natural antibodies play a significant role in clearing VEEV from circulation. Natural antibodies are produced constitutively, independently of internal or external stimuli, and have a fairly wide range of binding

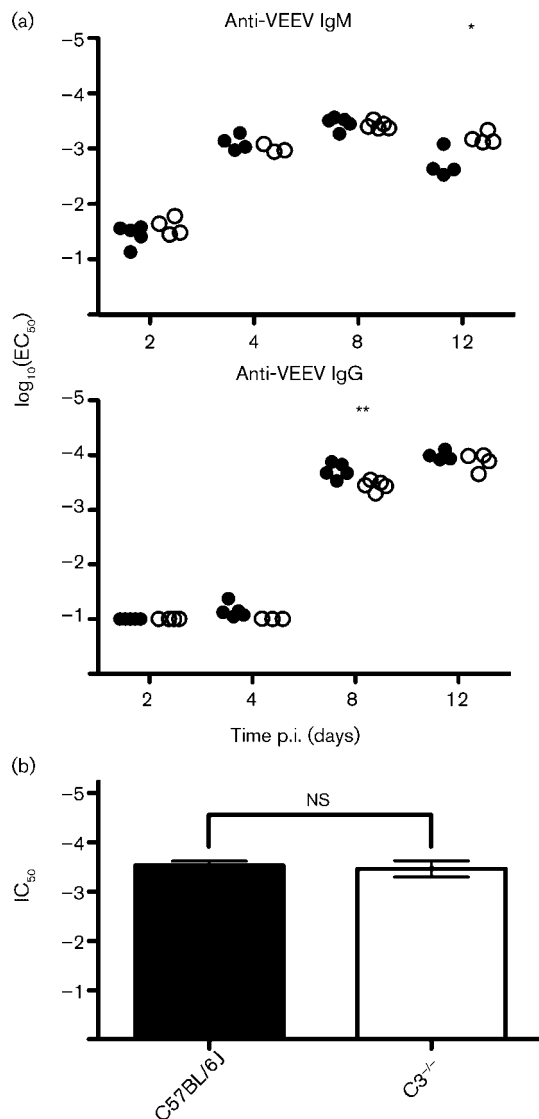


Fig. 5. The anti-VEEV antibody response is intact in C3^{-/-} mice. C57BL/6J (●) or C3^{-/-} (○) mice were infected s.c. with 10⁶ p.f.u. V3533 in the left rear footpad. (a) At the indicated times p.i., mice were bled and the amount of VEEV-specific IgM and IgG was determined by anti-VEEV ELISA. Data are presented as the log₁₀ reciprocal dilution at which 50% of the maximal absorbance value was reached (EC₅₀). Individual data points represent single animals and are pooled from two independent experiments. (b) Anti-VEEV neutralizing activity of serum collected from C57BL/6J and C3^{-/-} mice on day 4 p.i. Data are presented as the log₁₀ reciprocal dilution at which 50% of infectivity was inhibited and bars represent the arithmetic means ± SEM of three to five animals per group. **P*<0.05; ***P*<0.01 by two-tailed *t*-test. NS, Not significant.

avidities, depending on the antigen (Ochsenbein & Zinkernagel, 2000). One possible explanation for the delay in serum clearance observed in the C3^{-/-} mice is that complement activation is required for the maximal antiviral activity of natural antibodies.

In addition to enhancing natural antibody-mediated neutralization of circulating virus particles, complement activation might be facilitating serum clearance of VEEV by antibody-independent mechanisms. Seven distinct complement receptors (CR1, CR2, CR3, CR4, SIGN-R1, CR1g and C1qR) have been identified as binding and facilitating clearance of complement-bound pathogens from the circulation (Ghiran *et al.*, 2000; Helmy *et al.*, 2006; Holers *et al.*, 1992; Kang *et al.*, 2006; Kemper & Atkinson, 2007; Klickstein *et al.*, 1997). These receptors are expressed by phagocytic cells, such as marginal zone macrophages in the spleen and Kupffer cells in the liver, and could potentially act to eliminate complement-bound VEEV particles from the serum. This mechanism potentially explains the higher virus burdens observed in the spleens of wild-type mice over the first 24 h of infection compared with C3^{-/-} mice.

The finding that anti-VEEV IgM and IgG responses were intact in the absence of complement was surprising given the complement dependence of antibody responses to other virus infections. Complement activation can enhance virus-specific antibody induction through numerous mechanisms. Recognition of complement-coated antigen by CR1 and/or CR2 expressed on B cells can lower the signalling threshold required for activation, whilst CR1 and CR2 expressed by follicular dendritic cells can act to retain complement-coated antigen within B-cell follicles, enhancing presentation to B cells (Barrington *et al.*, 2002; Carter & Fearon, 1992). CR1 and CR2 have been demonstrated to be essential for the humoral response to both West Nile virus and herpes simplex virus (Mehlhop *et al.*, 2005; Verschoor *et al.*, 2003). It is possible that the replication of VEEV to such high titres (10⁶–10⁷ p.f.u. per draining lymph node) in secondary lymphoid organs may provide sufficiently concentrated viral antigen and inflammatory stimulus to render CR1/CR2 function unnecessary. Type I interferon, which is produced in large amounts during VEEV infection, can also act directly on B cells to promote antibody production and thus may compensate for the lack of CR1/CR2 signalling in C3^{-/-} mice (Coro *et al.*, 2006; Fink *et al.*, 2006; White *et al.*, 2001).

Complement activation can play a major role in the recruitment and activation of natural killer cells, neutrophils, monocyte/macrophages, dendritic cells and other inflammatory-cell populations (Gutzmer *et al.*, 2006; Li *et al.*, 2006; McWilliam *et al.*, 1996; Mócsai *et al.*, 2002; Norgauer *et al.*, 1993; Ross, 2000; Walport, 2001). Thus, it is possible that the early inflammatory-cell response to VEEV is at least partially complement dependent, acting through regulation of the recruitment, activation status and/or antiviral effectiveness of these inflammatory-cell populations following recruitment to sites of infection.

Due to the absence of an available C5 knockout in the C57BL/6 background, we had to assess the role of C5 expression in the B10.D2-H2^dH2-T18^c/SnJ background. This difference in genetic background probably explains the milder disease that we observed in V3533-infected

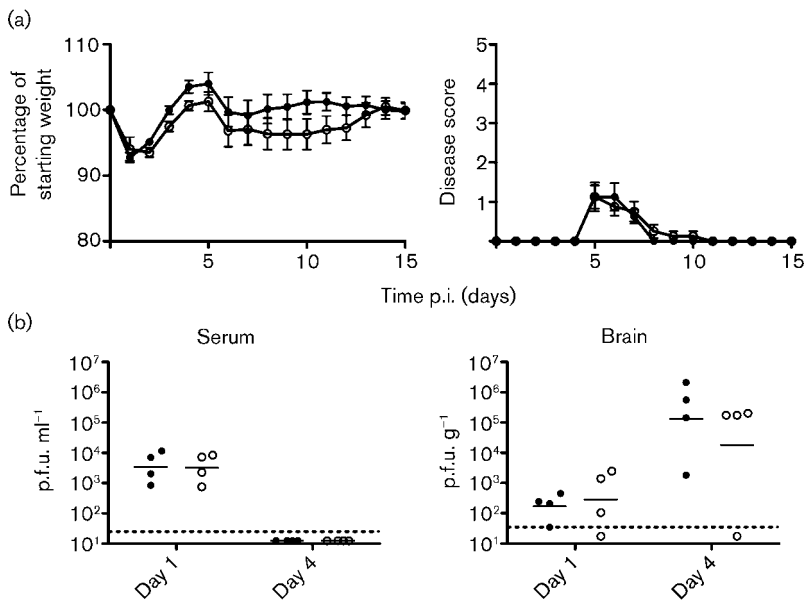


Fig. 6. Complement component C5 plays no significant role during VEEV infection. C5^{-/-} (○) and C5^{+/+} (●) mice were infected s.c. with 10⁶ p.f.u. V3533 in the left rear footpad. Weight loss and disease score were determined following infection (a). Mice were scored according to the scale described in Methods. Each data point represents the arithmetic mean ± SEM of data collected from eight mice per group. Differences in weight loss ($P=0.2561$) and disease score ($P=0.7521$) between C5^{-/-} mice and C5^{+/+} mice were not statistically significant, based on repeated-measures ANOVA testing. (b) At the indicated times p.i., serum and brain were collected and the viral burden was determined by plaque assay. Data points represent titres from individual mice, and horizontal bars indicate the geometric mean. Differences between C5^{-/-} mice and C5^{+/+} mice were not statistically significant (two-tailed *t*-test).

wild-type B10.D2-Hc¹H2^dH2-T18^c/nSnJ (C5^{+/+}) mice compared with C57BL/6 mice. The finding that C5 plays no protective role during VEEV infection was surprising, as it meant that two well-described effectors of the complement system – the anaphylatoxin C5a and the membrane attack complex – were not involved in controlling the infection. Instead, these results specifically implicate the C3 cleavage products C3a and C3b as key mediators of protection during VEEV infection.

Together, the results described here demonstrate that interactions between VEEV and the host complement system during the first hours of peripheral infection can have profound effects on downstream disease outcome. Specifically, we showed that complement activation limited the severity of VEEV-induced disease by reducing the efficiency of viral dissemination into the CNS. This effect appeared to be independent of anti-VEEV antibody induction or the C5-dependent functions of the complement system.

METHODS

Viruses. Isolation of the V3533 mutant of VEEV and generation of the pV3533 molecular clone have been described previously (Aronson *et al.*, 2000). Virus stocks of V3533 were generated by *in vitro* transcription from a linearized plasmid, pV3533, which encodes the full-length V3533 cDNA, using a T7-specific mMessage mMachine *in vitro* transcription kit (Ambion). *In vitro*-generated transcripts were then electroporated into BHK-21 cells using a Bio-Rad electroporator as described previously (Aronson *et al.*, 2000; Davis *et al.*, 1989). Culture supernatants were harvested 18 h after electroporation, clarified and stored as single-use aliquots at -80 °C.

Mouse studies. C3^{-/-} mice (on the C57BL/6J background) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and bred in house under specific-pathogen-free conditions. C57BL/6J,

B10.D2-Hc⁰H2^dH2-T18^c/oSnJ (C5^{-/-}) and B10.D2-Hc¹H2^dH2-T18^c/nSnJ (C5^{+/+}) mice were purchased from The Jackson Laboratory as needed. All experimental manipulation of mice was performed in a Biosafety Level 3 animal facility following a 7-day acclimatization period. For infections, 6–10-week-old female mice were anaesthetized via intraperitoneal injection with a mixture of ketamine (50 mg kg⁻¹) and xylazine (15 mg kg⁻¹) and then inoculated either in the left rear footpad with 10⁶ p.f.u. virus in diluent (PBS with 1% donor calf serum and Ca²⁺ and Mg²⁺) for s.c. infections, or directly into the brain with 10³ p.f.u. virus in diluent for i.c. infections. Mock-infected mice received diluent alone. Weight loss and disease score were assessed daily in infected animals. The scale used for disease scoring was: 0, no signs; 1, hunched posture, ruffled fur; 2, mild motor dysfunction, altered gait; 3, moderate motor dysfunction, ataxia; 4, severe motor dysfunction, hind limb paresis/paralysis; 5, moribund. Mice that lost >35% of their starting weight or became moribund were euthanized according to UNC Institutional Animal Care and Use Committee guidelines.

Virus titres. To assess VEEV titres *in vivo*, infected mice were sacrificed, bled and then perfused through the heart with 10 ml PBS. Spleen, draining popliteal lymph node, brain and spinal cord were then removed, weighed and frozen at -80 °C in diluent. Tissues were thawed and homogenized and used to infect BHK-21 cells in a standard plaque assay (Simpson *et al.*, 1996).

Histological analysis. Mice were sacrificed at the times indicated by exsanguination and then perfused through the heart with 4% paraformaldehyde (pH 7.3). Brains were embedded in paraffin, cut into 5 μm sagittal sections and stained with H&E. Stained sections were blinded and scored by an outside investigator for the overall extent of inflammatory-cell infiltration, as well as the total number of inflammatory foci per section. The extent of inflammatory-cell infiltration was scored on an arbitrary numerical scale of 0–3, with a score of 0 representing no detectable infiltration and a score of 3 representing the maximal extent of infiltration observed within the experiment.

Antibody analysis. VEEV-specific serum IgG and IgM levels were assessed by a standard indirect ELISA. Purified, intact VEEV particles

(250 ng per well) were used to coat 96-well NUNC Immulon 4HBX plates (Thermo Scientific) overnight at 4 °C. After washing, the plates were incubated with serial dilutions of heat-inactivated mouse serum containing 10% blocking buffer (Sigma) overnight at 4 °C. Plates were washed again, incubated with HRP-conjugated goat anti-mouse IgM or IgG (Southern Biotech) for 2 h at 4 °C and then developed using *o*-phenylenediamine dihydrochloride tablets (Sigma) in equal volumes of 0.1 M citric acid and 0.1 M sodium citrate. Development was allowed to proceed for 30 min before the reaction was terminated with 0.1 M NaF. A_{450} was measured using a FLUOstar Omega microplate reader (BMG Labtech). Log₁₀ half-maximum ELISA titres were calculated using GraphPad Prism software v. 5.0 and represented the log of the reciprocal dilution at which the half-maximum absorbance values were achieved.

To assess anti-VEEV neutralizing activity, serum was collected and either left untreated or heat inactivated at 56 °C for 1 h. The serum was then serially diluted in diluent and co-incubated with non-propagating, GFP-expressing VEEV viral replicon particles (GFP-VRP, as described by Pushko *et al.*, 1997) for 1 h at 37 °C. GFP-VRP/serum mixtures were then used to infect BHK-21 cells at an m.o.i. of 0.05. At 18 h p.i., infected cells were harvested by trypsinization, washed, fixed with 2% paraformaldehyde in PBS and analysed on a CyAn flow cytometer using Summit 5.2 software (Dako). IC₅₀ titres were calculated using GraphPad Prism software v.5.0 and represented the log of the reciprocal dilution at which 50% inhibition of infectivity was achieved.

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