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Regional Differences in Blood-Brain Barrier Permeability Changes and Inflammation in the Apathogenic Clearance of Virus from the Central Nervous System

Timothy W. Phares, Rhonda B. Kean, Tatiana Mikheeva, and D. Craig Hooper

The loss of blood-brain barrier (BBB) integrity in CNS inflammatory responses triggered by infection and autoimmunity has generally been associated with the development of neurological signs. In the present study, we demonstrate that the clearance of the attenuated rabies virus CVS-F3 from the CNS is an exception; increased BBB permeability and CNS inflammation occurs in the absence of neurological sequelae. We speculate that regionalization of the CNS inflammatory response contributes to its lack of pathogenicity. Despite virus replication and the expression of several chemokines and IL-6 in both regions being similar, the up-regulation of MIP-1β, TNF-α, IFN-γ, and ICAM-1 and the loss of BBB integrity was more extensive in the cerebellum than in the cerebral cortex. The accumulation of CD4- and CD19-positive cells was higher in the cerebellum than the cerebral cortex. Elevated CD19 levels were paralleled by κ-L chain expression levels. The timing of BBB permeability changes, κ-L chain expression in CNS tissues, and Ab production in the periphery suggest that the in situ production of virus-neutralizing Ab may be more important in virus clearance than the infiltration of circulating Ab. The data indicate that, with the possible exception of CD8 T cells, the effectors of rabies virus clearance are more commonly targeted to the cerebellum. This is likely the result of differences in the capacity of the tissues of the cerebellum and cerebral cortex to mediate the events required for BBB permeability changes and cell invasion during virus infection. The Journal of Immunology, 2006, 176: 7666–7675.

The outcome of virus infection of the CNS is dependent upon the pathogenicity of the virus and the nature of the antiviral immune response. In general, immune mechanisms efficiently clear most virus infections from the CNS, the only signs of the process being those associated with mild encephalitis. When the immune response is either inadequate or inappropriate, a CNS virus infection can cause severe encephalitis leading to significant neuropathology, neurological sequelae, and even death. In these situations, it is often difficult to determine whether disease pathogenesis is predominantly due to the direct consequences of virus infection or immunopathology. Most viruses that can spread to the CNS are pathogenic in the absence of a protective immune response, as is the case for rabies virus (1–4). Nevertheless, the immune response to a virus can be more pathogenic than the virus infection itself. For example, the often lethal acute CNS inflammatory response that occurs in normal rats infected with the neurotrophic Borna disease virus does not develop in immunocompromised rats, which survive despite persistent infection (5–7). Even where virus is cleared from the CNS without overt signs of pathology, either the virus infection or the antiviral immune response may have caused immune recognition of normally sequestered CNS Ags. This is believed to occasionally trigger persisting CNS autoimmune processes such as in acute postinfectious measles encephalitis (8) and possibly multiple sclerosis (9). Undoubtedly, it is important to control CNS immunity not only to protect neural function but also to prevent autoimmune sensitization.

Contact between CNS tissues and circulating elements of the immune system is regulated by the blood-brain barrier (BBB), a specialization of the vasculature in the CNS that renders it less permeable to cells and soluble factors than the vasculature of the periphery (10). An increase in BBB permeability, which can readily be detected by the leakage of markers from the circulation into the CNS tissues, has generally been associated with CNS inflammation in multiple sclerosis (11), experimental allergic encephalomyelitis (EAE) (12, 13), Borna disease (14), lymphocytic choriomeningitis virus infection (15), and West Nile virus encephalitis (16). Studies in models of CNS autoimmunity and virus-induced neuroinflammation have provided evidence linking enhanced BBB permeability with the development of a CNS inflammatory response (12–15). In these models, CNS inflammation invariably causes CNS tissue damage and disease (12–15). As demonstrated in EAE and Borna disease, CNS inflammation and its clinical sequelae can be prevented without suppressing the causative immune responses by interfering with the loss of BBB integrity (12–14). These studies confirm that the BBB plays an important role in protecting the CNS from immune-mediated pathology.

While BBB permeability changes and CNS inflammation have been studied in a variety of conditions where there is a pathological outcome, less is known about situations where CNS immunity successfully clears an infection without incident. Understanding how these differ is fundamental to providing the most appropriate therapy for CNS infection. For instance, it is unknown whether clearance of a virus from CNS tissues can occur in the absence of...
BBB permeability changes. It may be expected that enhanced BBB permeability is required to provide circulating immune cells contact with CNS tissue-derived chemokines, as well as allow immune effectors to infiltrate into CNS tissues. We have previously shown that the virus neutralizing Ab (VNA)-dependent clearance of the attenuated rabies virus CVS-F3 from CNS tissues involves CNS inflammation without neurological signs of disease (3). This suggests that either clearance of the virus occurs in the absence of significant BBB permeability changes or that the BBB becomes compromised, and the characteristics of the CNS inflammation in CVS-F3-infected animals are such that neuropathology is avoided. The results of this investigation demonstrate that the clearance of CVS-F3 from the infected CNS is associated with enhanced BBB permeability, which is primarily limited to the cerebellum and likely due to differences in the innate antiviral response between the tissues of the cerebellum and cerebral cortex.

Materials and Methods

Mice

The 8- to 10-wk-old male and female 129/SvEv mice used throughout the study were purchased from Taconic Farms and housed under pathogen-free conditions. Equivalent numbers of males and females were used in the experiments. All procedures were conducted in accordance with federal guidelines under animal protocols approved by the Thomas Jefferson University Institutional Animal Care and Use Committee.

Table II. Primer sequences used to synthesize synthetic cDNA standards for real-time quantitative RT-PCR

<table>
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<tr>
<th>Gene</th>
<th>5' Primer</th>
<th>3' Primer</th>
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<tr>
<td>L13</td>
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<td>TGCTCGGAGTTCCCAAGGAT</td>
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<td>Rabies N-protein</td>
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<td>ICAM-1</td>
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<td>IFN-γ</td>
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<td>CD11b</td>
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<td>CD19</td>
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<tr>
<td>κ-L chain</td>
<td>GCTACGCTTTGCGTCTT</td>
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Virus infection

129/SvEv mice were infected intranasally (i.n.) with 10⁵ focus-forming units of the CVS-F3 strain of rabies virus in 20 μl of PBS. CVS-F3 is an attenuated rabies virus that is nonpathogenic for immunocompetent adult mice regardless of the route of infection (3, 17, 18). When administered i.n., CVS-F3 causes a transient weight loss in normal mice (3); therefore, weight was assessed on a daily basis to provide insight into the progress of the infection.

BBB permeability

Sodium fluorescein, a low molecular mass molecule (376 Da), was used to detect the fluid phase shifts between the circulation and CNS tissue that occur when BBB permeability becomes enhanced (12). Briefly, mice received 100 μl of 10% sodium fluorescein in PBS i.p. Ten minutes later, cardiac blood was collected, and mice were transcardially perfused with PBS/heparin and then PBS alone. Brains were removed, separated into cerebral cortex and cerebellum, and snap-frozen in liquid nitrogen. Each tissue was then weighed, homogenized in 1/10 dilution in PBS and centrifuged at 14,000 × g for 2 min. Five-hundred microliters of the clarified supernatant was transferred into 500 μl of 15% trichloroacetic acid and centrifuged at 10,000 × g for 10 min while the pellet was retained for RNA isolation. One-hundred twenty-five microliters of 5 N NaOH was added to 500 μl of the supernatant, and the amount of fluorescein for each sample was determined using standards ranging from 125 to 4000 μg on a Cytolux II fluorometer (PerSeptive Biosystems). Levels of sodium fluorescein were assessed as previously described so that signals in CNS tissue samples could be normalized against the amount present in the circulation (12). Normalized sodium fluorescein uptake into brain tissue was
Core Reagent kit (Applied Biosystems). Primers and probes were designed cDNA using specific primer and probe sets (Table I) and the TaqMan PCR megaseq. Real-time quantitative RT-PCR was conducted on equal volumes of Microlab microscope. UV light source and photographed using a Nikon digital camera on a Leitz Microlab microscope.

In our analyses, we observed significant differences between the two tissues at a given time point established by the t test are denoted by * (p < 0.05) and ** (p < 0.005).

Real-time quantitative RT-PCR

Tissue pellets prepared as detailed above were used to quantify the expression levels of specific mRNAs in CNS tissues so that BBB permeability and gene expression were assessed in the same samples. RNA was isolated from the pellet using the Qiagen RNeasy kit (Qiagen) and then treated with DNase I (Qiagen). cDNA was synthesized from 2 μg of total RNA using dT15 primers and Moloney murine leukemia reverse transcriptase (Promega). Real-time quantitative RT-PCR was conducted on equal volumes of cDNA using specific primer and probe sets (Table I) and the TaqMan PCR Core Reagent kit (Applied Biosystems). Primers and probes were designed using the Web Primer 3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Primers were synthesized by our in-house Nucleic Acid Facility (Kimmel Cancer Institute, Thomas Jefferson University), and probes were purchased from Integrated DNA Technologies. The probes are double-labeled with black hole quencher-1 at the 3′ ends and the reporter dyes Hex or 6-Fam at the 5′ ends. Real-time quantitative PCR was performed using a Bio-Rad iCycler iQ Real-Time Detection System. Data were calculated based on a threshold cycle (Ct) determined as the PCR cycle at which the fluorescent signal becomes higher than that of the background (cycles 2–10) plus 10 times the SD of the background. To determine the number of copies of specific mRNAs in each sample, synthetic cDNA standards ranging from 400 to 500 bp in length were made for each gene and standard curves constructed with Ct data obtained using dilutions of these standards. Gene-specific primer sets for the standards are listed in Table II. Data are expressed as the number of copies of specific mRNA per copy of the housekeeping mRNA L13 in a particular sample. With respect to the CD4 primer-probe set, it has been reported that mRNA transcripts resembling CD4 (L3T4) are expressed in the cortex of the mouse brain (19). The CD4 primer-probe set used here may detect these transcripts as suggested by the relatively high CD4 mRNA levels seen in normal cortex. Isolation of mononuclear cells from the CNS and flow cytometry

Mononuclear cells were isolated from the brains of groups of 15 CVS-F3-infected mice using a modification of a previously described technique (20). Briefly, perfused brains were separated into cerebral cortex and cerebellum and homogenized in 10% PBS by mincing the tissues with a scalpel and then passing through an 18-gauge needle. Homogenates were transferred to a 15-mL conical tube and underlaid with 35% Percoll. The gradients were centrifuged at 2000 rpm for 20 min at 4°C. The supernatant was aspirated off, and the cell pellet was washed twice. Live cells were counted by trypsin blue exclusion. Mononuclear cells were resuspended in FACSVersus buffer, aliquoted, and stained with Abs specific for the following cell phenotype markers: FITC-anti-CD8, FITC-anti-CD19, PE-anti-CD4, and PE-anti-CD11b (BD Pharmingen). Two-color fluorescence was assessed on a FACSCalibur fluorometer (BD Biosciences). Yields of mononuclear cells from the CNS tissues of equal numbers of uninfected mice were insufficient to perform flow cytometric analysis.

Immunohistochemistry

Brains were snap frozen in Tissue-Tek OCT Compound (Sakura Finetex) for immunohistochemical analysis, and sectioned at 10 μm using a Thermo Shandon cryostat. Sections were fixed in cold 80% acetone and then stained with FITC-anti-CD19 (catalog no. 557398; BD Pharmingen) or PE-anti-CD4 Ab (catalog no. 01065; BD Pharmingen) for 1 h. Photographs were taken with a Nikon digital camera on a Leitz Microlab microscope and are reproduced at a final magnification of ×220. Cells bearing CD4 and CD19 were rare in sections of both the cerebral cortex and cerebellum of uninfected mice (data not shown).

Virus-specific Abs

Sera from mice infected with CVS-F3 were assessed for rabies-specific Abs in direct ELISA using UV-inactivated ERA rabies virus as the trapping Ag. Briefly, plates (Polsorb; Nalge Nunc International) were coated at room temperature with 100 μL of a 5 μg/mL ERA stock solution and incubated overnight in a humidified chamber. The plates were washed with PBS containing 0.05% Tween 20 and blocked with 1% BSA in PBS for

FIGURE 2. Localization of BBB permeability changes in the brain of CVS-F3-infected mice. Brains were removed from sodium fluorescine-treated mice either uninfected or infected with CVS-F3 8–10 days previously and photographed under UV illumination as described in Materials and Methods.
Results

BBB permeability is increased regionally as rabies CVS-F3 virus is cleared from CNS tissues

Intranasal infection of immunocompetent adult mice with CVS-F3 causes a transient reduction in body weight beginning at 7 days postinfection (p.i.) and becoming maximal at 11 days p.i. (Fig. 1A), with no other signs of disease. When administered via this route, CVS-F3 spreads throughout the cerebral cortex and cerebellum with peak levels of viral nucleoprotein mRNA appearing in both tissues between 8 and 12 days p.i. (Fig. 1B). Nucleoprotein mRNA levels then drop >10-fold from days 12 to 18 and are down to a few residual copies by day 24 p.i. (Fig. 1B). As measured by the leakage of sodium fluorescein from the circulation into CNS tissues, BBB permeability becomes enhanced in the cerebral cortex and cerebellum by 8 days p.i. (Fig. 1C). However, despite the fact that virus nucleoprotein mRNA levels in the cerebral cortex and cerebellum do not significantly differ between days 8 and 12 p.i., BBB permeability is more extensive and prolonged in the cerebellum than in the cerebral cortex over this time period (Fig. 1C). Evidence of this regionalization can also be seen when intact brains from CVS-F3-infected mice that have received sodium fluorescein are examined under UV light (Fig. 2).

Chemokine responses in the CNS during CVS-F3 infection

The substantial differences in BBB permeability between the cerebral cortex and cerebellum in the CVS-F3-infected brain suggest that the CNS resident cells of these areas must somehow differ in their capacity to respond to the infection. Based on the participation of the CNS inflammatory response in CVS-F3 clearance (3), we speculated that some aspect of the proinflammatory response to the virus may differ between the cerebral cortex and cerebellum. Chemokine production by cells in the area of an infection plays an

Statistical analyses

Results are expressed as the mean ± SEM for each group of mice. Evaluation of the significance of differences between the means of parameters at individual time points was performed using the Mann-Whitney U test or the unpaired t test. The Wilcoxon signed rank test was used to compare overall differences in the response patterns in different tissues. In all cases, p < 0.05 was considered significant. Graphs were plotted and statistics assessed using GraphPad Prism 3.0 software (GraphPad).

1.5 h before for the addition of the samples. Samples were diluted 1/4 in PBS and titrated 4-fold. After a 2-h incubation at room temperature, plates were washed with PBS containing 0.05% Tween 20. Bound Ab was detected using peroxidase-conjugated anti-mouse IgG whole molecule (1/1000 dilution; Sigma-Aldrich) with 3,3’,5,5’-tetramethylbenzidine (Sigma-Aldrich) in phosphate-citrate buffer as a substrate. Absorbance was read at 450 nm in a microplate spectrophotometer (Biotek). Data are calculated as the last dilution of serum in ELISA, which gave an OD greater than half the maximal titer.

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important role in clearing a pathogen by facilitating accumulation of the appropriate immune effectors. We therefore assessed the levels of mRNAs specific for the chemokines IFN-γ-inducible protein-10 (IP-10), MCP-1, MIP-1α, MIP-1β, and RANTES in the cerebral cortex and cerebellum through the course of CVS-F3 infection (Fig. 3). At 6 days p.i., before BBB permeability becomes significantly enhanced, IP-10, MCP-1, MIP-1α, and RANTES are all elevated to significantly higher levels in the cerebral cortex than the cerebellum, as are rabies nucleoprotein mRNA levels. From 8 through 24 days after infection, the expression of most of the tested chemokines, although differing between the tissues at certain time points, are up-regulated to a more or less comparable extent in the cerebral cortex and cerebellum with RANTES and MIP-1α being marginally higher in the cortex, whereas IP-10 and MCP-1 tend to be slightly higher in the cerebellum. The only exception to this generalization is MIP-1β, which is expressed at significantly higher levels in the cerebellum throughout the infection.

Regional differences in IL-6, TNF-α, IFN-γ, and ICAM-1 responses in the CNS during CVS-F3 infection

The similar chemokine responses of the CVS-F3-infected cerebral cortex and cerebellum indicate that some feature of the proinflammatory response that more directly facilitates the loss of BBB integrity may either be preferentially activated in the cerebellum or down-regulated in the cerebral cortex. TNF-α and IL-6 are of particular interest in this regard because of their postulated contribution to BBB permeability changes (21–26), proinflammatory effects (27–29), and the fact that rabies virus infection up-regulates their expression in the CNS (30–33). We therefore compared TNF-α and IL-6 mRNA levels in cerebral and cerebral cortex over the course of CVS-F3 infection (Fig. 4, A and B). At the same time, we assessed ICAM-1 and IFN-γ levels because of the potential impact of these molecules on BBB function and the inflammatory response (34–39) (Fig. 4, C and D). Considerably more copies of TNF-α and ICAM-1 mRNAs are expressed in the cerebellum during CVS-F3 infection (Fig. 4). Elevations of both mRNAs were significant as early as 6 days p.i., became maximal between days 10 and 12, and began to decline by day 18 p.i. The up-regulation of IFN-γ expression, which also becomes significant at 6 days p.i., is ~2-fold greater in the cerebellum than the cerebral cortex throughout most of the infection (Fig. 4). On the other hand, IL-6 mRNA levels are higher in the cortex than the cerebellum at day 6 of infection, but peak levels in both tissues are comparable (Fig. 4B). Notably, levels of all of these mRNAs begin to rise before BBB permeability becomes significantly enhanced (compare Figs. 1C and 4). Nevertheless, the greatest increases in the expression of TNF-α and IFN-γ coincide with the onset of enhanced BBB permeability.

Regional differences in immune cell accumulation in the CNS of CVS-F3-infected mice

It may be expected that an elevated proinflammatory response together with increased BBB permeability would promote immune cell invasion into the CNS tissues. If this is the case, there should

**FIGURE 4.** Differential up-regulation of TNF-α, ICAM-1, and IFN-γ but not IL-6 expression in the cerebellum and cerebral cortex during CVS-F3 infection. Levels of mRNAs specific for TNF-α, IL-6, ICAM-1, and IFN-γ in the cerebral cortex and cerebellum of the CVS-F3-infected mice described in the legend to Fig. 1 were assessed as detailed in Materials and Methods. Data are expressed as the mean ± SEM copies of specific mRNA per copy of the housekeeping gene L13 mRNA in cerebellar and cortical tissues from infected mice minus the background copy number in similar tissues from uninfected mice. Background levels were as follows: TNF-α, cortex 0.0000014 ± 0.00000041, cerebellum 0.00013 ± 0.000026 (A); IL-6, cortex 0.0025 ± 0.00037, cerebellum 0.0017 ± 0.00012 (B); ICAM-1, cortex 0.00069 ± 0.00010, cerebellum 0.27 ± 0.0098 (C); and IFN-γ, cortex 0.00008 ± 0.000032, cerebellum 0.00016 ± 0.000089 (D). Statistically significant differences in expression levels between the same tissues at particular time points in infected and uninfected mice, determined by the Mann-Whitney U test, are denoted by * (p < 0.01) and ** (p < 0.005). Significant differences in the mean expression levels between the two tissues at a given time point established by the t test are denoted by † (p < 0.05) and †† (p < 0.005). Throughout the course of infection, the expression levels of both TNF-α and ICAM-1 mRNAs were statistically higher in the cerebellum than the cortex as determined by the Wilcoxon signed rank test (†, p < 0.05).
be greater cell infiltration into the cerebellum than the cerebral cortex of CVS-F3-infected mice. To test this hypothesis, we first measured the levels of cell-specific mRNAs in the cerebellum and cerebral cortex tissues of the CVS-F3-infected mice studied in Figs. 1, 3, and 4 as an estimate of the accumulation of immune/inflammatory cells (Fig. 5). Cerebellar CD4, CD8, and CD11b mRNA levels become significantly higher than those of normal uninfected animals by 8 days p.i. and continue to accumulate through day 12 p.i., remaining elevated for at least 24 days p.i. However, the pattern of changes in CD19 mRNA levels, a B cell marker, is unique. While becoming significantly elevated by day 8 p.i., CD19 mRNA levels in the cerebellum do not peak until 18–24 days p.i. In the cerebral cortex, the changes in cell-specific mRNA levels over time after infection are somewhat different (Fig. 5). Although the changes in CD4 and CD8 mRNA levels have similar kinetics in both regions, the elevations in CD4 mRNA are significant in the cerebellum but not in the cerebral cortex, whereas those of CD8 are significantly higher in the cerebral cortex. CD11b mRNA levels initially become up-regulated in the cerebral cortex in a comparable fashion to the cerebellum but do not reach the same levels and are more transient. On the other hand, while the significant elevations seen in cortical CD19 mRNA levels are lower than those of the cerebral cortex, they are also maintained for at least 24 days p.i. Flow cytometric analysis reveals that higher percentages of CD4-, CD8-, and CD19-positive lymphocytes can be recovered from the cerebellum than the cerebral cortex of mice infected with CVS-F3 12 days previously (Fig. 6). In repeat experiments, more CD4 T cells were consistently recovered from the cerebellum (5033 ± 698 cells per mouse) than the cerebral cortex (1092 ± 169 per mouse), whereas CD8 T cell recovery was more variable (2314 ± 1412 cerebellum; 731 ± 410 cerebral cortex).

**Ab production during CVS-F3 infection**

VNA are known to be essential for the clearance of rabies virus from the CNS (3). It may be expected that BBB permeability changes are important for Ab infiltration from the circulation into CNS tissues. However, the BBB permeability changes in the cerebral cortex of CVS-F3-infected mice are relatively low and transient, becoming statistically insignificant before a drop in virus replication is seen (see Fig. 1). In fact, the greatest elevations in serum CVS-F3-specific Ab titers occur from days 12 to 24 of infection (Fig. 7A), after BBB integrity has been restored (Fig. 1). These data, together with evidence that cells expressing CD19 infiltrate into the CNS tissues, suggest that the production of VNA within the tissues may be important. To test this possibility further, we first determined whether Ab is being produced in the CNS tissues by assessing κ-L chain mRNA levels (Fig. 7B). Levels of κ-L chain mRNA in the CNS tissues increase (Fig. 7B) as rabies virus nucleoprotein mRNA levels decrease (Fig. 1). Also noteworthy is that, as is the case for CD19 mRNA (Fig. 5), κ-L chain mRNA reaches higher levels in the cerebellum than the cerebral cortex. We next used immunohistochemistry to confirm whether B cells and the CD4 T cells that may be required to provide helper function for Ab production are present in the CNS tissues as CVS-F3 is cleared. These analyses confirm that both CD4 T cells

**FIGURE 5.** Accumulation of immune/inflammatory cells in the brain following infection with CVS-F3. The appearance of cells expressing CD4 (A), CD8 (B), CD11b (C), and CD19 (D) in the cortex and cerebellar tissues of the CVS-F3-infected mice described in the legend to Fig. 1 was estimated by assessing the levels of marker-specific mRNAs over time after infection as detailed in Materials and Methods. Data are expressed as the mean ± SEM copies of specific cell marker mRNA per copy of the housekeeping gene L13 mRNA in infected tissues minus the background level in similar tissues from uninfected mice. Background levels were as follows: CD4, cortex 0.0038 ± 0.000047, cerebellum 0.00002 ± 0.0000031 (A); CD8, cortex 0.000002 ± 0.0000002, cerebellum 0.000002 ± 0.0000002 (B); CD11b, cortex 0.0013 ± 0.000023, cerebellum 0.00032 ± 0.000035 (C); and CD19, cortex 0.00042 ± 0.000012, cerebellum 0.000082 ± 0.000011 (D). Statistically significant differences between the expression levels in the cerebellum or cerebral cortex of infected and uninfected mice at particular time points, determined by the Mann-Whitney U test, are denoted by * (p < 0.01) and ** (p < 0.005). Significant differences in the mean expression levels between the two tissues at a given time point established by the t test are denoted by # (p < 0.05) and ## (p < 0.005).
proinflammatory cytokine IL-6, are expressed in these tissues over
the course of the infection, despite differences in immune cell accumulate. These findings also suggest that the capacity of the different regions to respond to the infection by elaborating mediators that contribute to immune cell recruitment is at least partly shared. Nevertheless, because of the similar expression of these chemokines in the infected cerebral cortex and cerebellum, we conclude that their activity has no direct bearing on the differential BBB permeability changes and cell infiltration between these tissues. Moreover, the expression of RANTES, MIP-1α, IP-10, MCP-1, and IL-6 in the absence of CNS pathological changes suggests that any postulated contributions of these factors to the pathogenesis of CNS inflammatory diseases are likely to be indirect.

Unlike the other chemokines tested, where there are no clear differences in expression between the cerebral cortex and cerebellum of CVS-F3-infected animals, MIP-1β mRNA levels are significantly higher in the latter. In both the levels in the cerebral cortex and cerebellum and the timing of expression during infection, MIP-1β expression closely parallels BBB permeability changes. Because MIP-1β can be elaborated by neurovascular endothelial cells (40) and these cells also can express its receptor, CCR5 (41), we speculate that the expression of this chemokine in CVS-F3 infection may either contribute to, or be induced in conjunction with BBB permeability changes. Another proinflammatory factor that is expressed at higher levels in the cerebellum than

and CD19 B cells accumulate in the CNS tissues by day 18 p.i. (Fig. 8). CD4 T cells were found scattered throughout the parenchyma of the cerebral cortex and cerebellum, as well as in focal accumulations that were generally larger and more common in the latter (Fig. 8, A and B). CD19 B cells also appeared in low numbers throughout the cerebral cortex and cerebellum (Fig. 8, C and D). However, focal accumulations of B cells were only observed in the cerebellum (Fig. 8D, inset). These data suggest that the production of Ab in the CNS tissues may be more relevant to virus clearance than the infiltration of circulating Abs across the BBB.

Discussion
To reach CNS tissues, circulating cells and factors must cross the BBB. However, in most experimental situations where integrity of the BBB is compromised by a CNS inflammatory response, pathological sequelae follow (12–15). This is not the case for rabies CVS-F3 infection, where clearance of the virus from the CNS is associated with enhanced BBB permeability and CNS inflammation but no overt neurological signs. We speculate that characteristics of the immune response to the infection, particularly regional differences in the loss of BBB integrity and inflammation, may minimize damage to CNS tissues.

Although there may be some disparity in the spread of CVS-F3 through the cerebellum and cortex in the first 6–8 days after intranasal infection, the extent of virus replication detected by nucleoprotein mRNA levels appears comparable at days 10–12 p.i. The likelihood that virus replication and spread does not substantially differ between the cerebellum and cortex is supported by the fact that similarly elevated levels of mRNAs specific for the chemokines RANTES, MIP-1α, IP-10, and MCP-1, as well as the proinflammatory cytokine IL-6, are expressed in these tissues over
the cerebral cortex of CVS-F3-infected mice is TNF-α. The contribution of TNF-α to the induction of enhanced BBB permeability is controversial with some investigations concluding that it plays a direct role (42–45), whereas others have concluded that this is not the case (46–48). Although our experiments here are not directed at this controversy, it should be noted that the levels of TNF-α expressed in the cerebellum at days 8 and 18 of CVS-F3 infection are comparable, whereas the extent of BBB permeability is disparate. This implies that TNF-α may not directly cause the loss of BBB integrity during virus infection but may contribute to its induction through a secondary mechanism, for example, by stimulating adhesion molecule expression by the neurovasculature.

The expression of TNF-α is closely paralleled by the expression of ICAM-1 in CVS-F3-infected CNS tissues with higher levels in the cerebellum than cerebral cortex. ICAM-1 is an adhesion molecule that has been implicated in CNS inflammation in a variety of models (34–36). In the context of a CNS virus infection, ICAM-1 and other adhesion molecules would serve to identify blood vessels in the infected area to circulating immune cells expressing its ligands, including LFA-1 and MAC-1. ICAM-1 expression is initially up-regulated in the CNS of CVS-F3-infected mice before the onset of BBB permeability changes. This suggests that targeting circulating immune cells to the neurovasculature may be a prerequisite to the development of enhanced BBB permeability. The fact that the extent of BBB permeability mirrors that of ICAM-1 expression tends to support this hypothesis.

If changes in BBB permeability are mediated by an interaction between immune and neurovascular endothelial cells, it may be expected that these immune cells may be the first to enter the CNS tissues when BBB integrity is lost, preferentially targeting the cerebellum. Immune effector cells that participate in clearing the virus may be within this initial population of invading cells or follow afterward. To gain insight into the possible contributions of different cell types to these processes, we examined the kinetics of the accumulation of mRNAs specific for CD4 T cells, CD8 T cells, monocytes, and B cells in the cerebellum and cerebral cortex of CVS-F3-infected mice. At 8 days after infection, when significant changes in BBB permeability were first detected, the only cell-specific marker that was significantly elevated in the cerebellum but not the cerebral cortex was CD4. These findings suggest that a CD4 T cell subset may make an important contribution to the loss of BBB integrity during CVS-F3 infection. The possibility that this may be mediated through the production of IFN-γ is supported by the close association between changes in BBB integrity and the appearance of IFN-γ mRNA in the cerebellum vs cerebral cortex over the course of the infection.

The greater permeability seen in the cerebellum by comparison with the cerebral cortex of CVS-F3-infected animals correlates with the fact that CD4, CD11b, and CD19 mRNA levels increased to higher levels in the cerebellum. On the other hand, CD8 mRNA levels were somewhat higher in the cerebral cortex than the cerebellum. Although analysis of mRNA levels provides insight into the kinetics of the CNS inflammatory response, there is a possibility that cells in the cerebellum and cerebral cortex express different mRNA levels. We therefore used flow cytometry to compare the accumulation of these cell subsets in the two areas of the CNS at day 12 of infection when CD4 mRNA was maximal and BBB permeability still elevated. In concert with the mRNA results, greater numbers of CD4- and CD19-positive cells were recovered from the cerebellum than the cerebral cortex. Immunohistochemical analysis of the tissues suggests that these increases may result from focal accumulations of these cell types in the cerebellum. On the other hand, the numbers of CD8-positive cells in the cerebellum tended to be higher than in the cerebral cortex, whereas equivalent levels of CD8 mRNA were seen in the two areas at day 12 p.i. Moreover, CD11b-positive cell recovery from the cerebral cortex was slightly higher than from the cerebellum, despite CD11b mRNA levels being ~2-fold higher in the latter. Based on immunohistochemistry (data not shown), we speculate that this disparity may be caused by variability in the expression of CD11b on macrophages and microglia in the two areas together with differences in the capacity to recover each of these cell types.

Mice lacking functional CD8 T cells can clear CVS-F3 from their CNS tissues, albeit more slowly than congenic normal animals (3). In contrast, CVS-F3 infection is lethal for mice that are unable to make Abs, regardless of whether they have T cells (3). If virus-specific Abs are major players in the clearance of CVS-F3 from infected brain tissues, the loss of BBB integrity in CVS-F3 infection may be expected to play an important role in the delivery of VNA to the infected CNS tissues. Although the BBB permeability changes seen in the vasculature of the cerebellum may be sufficient to provide circulating Abs access to those tissues, the

FIGURE 8. Patterns of lymphocyte accumulation in the CNS tissues of CVS-F3-infected mice. Sections from either the cerebral cortex (A and C) or cerebellum (B and D) of CVS-F3-infected mice were prepared 18 days p.i. and stained for CD4 (red fluorescence) or CD19 (green fluorescence) as detailed in Materials and Methods. The distribution patterns of CD4 (A and B) and CD19 (C and D)-positive cells generally found throughout the tissues are shown in the primary photographs. Where focal accumulations of these cells were observed, these are presented as insets.
BBB of the cerebral cortex develops considerably less permeability during infection with CVS-F3 compared to the cerebellum. We speculate that the immune cells expressing IFN-γ that target the ICAM-1-positive neurovascular endothelial cells have the capability to induce the loss of BBB integrity through mechanisms that are unknown whether sufficient levels of VNA to clear the virus from the cerebral cortex are produced in situ or there is a contribution from Ab produced in the cerebellum.

We believe that the capacity to control the infection by the production of VNA is an important element of how CVS-F3 can be cleared from the CNS tissues in the absence of significant pathology. However, as is the case for other more pathogenic CNS inflammatory reactions (e.g., Ref. 49), immunity to CVS-F3 is predominantly driven by a Th1 response (3). Moreover, like such pathological CNS inflammatory diseases as EAE (12, 13), Borna disease virus infection (14), and fatal lymphocytic choriomeningitis virus infection (15), there is a loss of BBB integrity during infection with CVS-F3. In these examples of CNS inflammation, there is a clear relationship between BBB dysfunction and disease (12–15). We speculate that limiting the loss of BBB integrity and inflammation in the cerebral cortex by preferentially targeting necessary permeability changes to the cerebellum may be critical to preventing neurological damage. For example, because of their structure, the tissues of the cerebellum may have greater tolerability than the cerebral cortex to edema resulting from the loss of BBB integrity.

To conclude, we find that a neurotrophic virus infection can be cleared without clinical sequelae if certain elements of the immune response, including BBB permeability changes, are predominantly directed to the cerebellum. We speculate that regional differences in the induction of TNF-α in the CVS-F3-infected CNS are the foundation for these disparate permeability changes. Through the up-regulation of ICAM-1 on neurovascular endothelial cells, TNF-α bears responsibility for attracting circulating immune cells. We speculate that the immune cells expressing IFN-γ that target the ICAM-1-positive neurovascular endothelial cells have the capacity to induce the loss of BBB integrity through mechanisms that are not yet fully resolved. MIP-1β, produced at higher levels in the cerebellum than the cerebral cortex, may also participate in this process. Understanding how such changes in BBB permeability may occur without neuropathological consequences, as is the case for the clearance of CVS-F3, has important implications for the development of methodologies to deliver therapeutics across the BBB.

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Disclosures

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