American Journal of Pathology, Vol. 143, No. 1, July 1993

Viral Infection and Dissemination Through the Olfactory Pathway and the Limbic System by Theiler's Virus

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Theiler's murine encephalomyelitis virus (TMEV) infection of mice can produce a biphasic disease of the central nervous system (CNS). Most susceptible strains of mice survive the acute infection and develop a chronic demyelinating disease. In this report, we analyzed the routes of spread of TMEV within the CNS of nude mice and target sites eventually infected in the CNS. Compared to the immunocompetent mouse, in which an antiviral immune response is mounted but virus persists, the nude mouse develops a severe encephalomyelitis due to the lack of functional T lymphocytes and provides a useful model for the study of viral dissemination. We demonstrated, by immunohistochemistry, the presence of viral antigen in defined regions of the CNS, corresponding to various structures of the limbic system. In addition, we found a different time course for viral spread using two different sites of intracerebral inoculation, ie, via the olfactory bulb or the cortex. Limbic structures were rapidly infected following olfactory bulb infection and then showed a decrease in viral load, presumably due to loss of target neurons. Using either route of infection, the virus was able to disseminate to similar regions. These results indicate that limbic structures and their connections are very important for the spread of TMEV in the brain. In the spinal cord, not only neuronal but hematogenous pathways were suspected to be involved in the dissemination of Theiler's virus. (Am J Pathol 1993, 143: 221–229)

An important feature of virus infection of a host is that specific routes used for dissemination depend upon the particular tropism of the virus for defined cells and tissues. To appreciate the molecular basis of these interactions, we have initiated a concerted effort to study the spread of virus and the distribution of infected cells during Theiler's murine encephalomyelitis virus (TMEV) infection of mice. There are two important reasons to study TMEV. First, TMEV, a positive-sense, single-stranded RNA virus, is of considerable importance as a model for human diseases, such as poliomyelitis, postpolio syndrome, and multiple sclerosis.[1–4] The mouse is the natural host for this picornavirus and offers the advantage of using an entirely homologous system, ie, a murine virus in the mouse versus a heterologous or mixed system. Intracerebral (i.c.) infection of susceptible strains of mice with the Daniels (DA) strain of TMEV produces a biphasic disease of the central nervous system (CNS).[2] The acute phase of the disease consists of an encephalomyelitis with lesions in the spinal cord resembling poliomyelitis in humans. The susceptible SJL/j mouse survives the acute infection and progresses to develop a chronic demyelinating disease that mimics some of the features of multiple sclerosis.

A second reason for studying TMEV is that the mechanism by which TMEV infection results in demyelination is still unknown. Various hypotheses consider an immune-mediated mechanism[5–8] and/or a persistent infection to be the cause.[9,10] CD4+ and CD8+ T cells have been reported to play a role in the pathogenesis of disease.[8,11–14] CD4+ delayed-type hypersensitivity T cells are thought to initiate demyelination through a bystander-mediated event.[8] For example, the delayed-type hypersensitivity T cells may recognize TMEV-infected cells in the CNS releasing lymphokines that have the capacity to lyse or modulate the ability of the oligodendrocyte to maintain and synthesize myelin. Macrophages become

Supported by NIH grant NS23162, Multiple Sclerosis Society grant RG2356-A-4, and Cancer Center grant 5 P30-CA42014 (University of Utah Medical School). RSF is a Javits Neuroscience Scholar.

Accepted for publication February 3, 1993.

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activated and initiate attacks on myelinated structures. Similarly, CD8+ T cells have recently been demonstrated in the CNS of infected mice, suggesting that these cells play a role in demyelination through their ability to lyse TMEV-infected glial cells.[12]

In addition to immune cells, antibody may play a role in demyelinating disease. An anti-TMEV neutralizing monoclonal antibody can aid in the survival of TMEV-infected nude mice as well as allow remyelination to occur.[15] Furthermore, a monoclonal antibody with dual specificity for TMEV and galactocerebroside can enhance the demyelination in autoimmune CNS disease.[16] Thus, antibody-mediated events have been proposed to play a critical role in TMEV clearance and autoimmune injury.

Since immune-mediated responses to virus infection can play a marked role in disease and/or viral clearance, we have selected to use nude mice to initiate studies on viral spread and *in vivo* tropism in the absence of an effective immune response. The nude mouse infected with DA virus fails to produce viral antibodies or virus-specific T lymphocytes.[9,10] However, it is able to develop a demyelinating disease after i.c. infection with DA virus.[9] It has been reported that infection of nude mice with the DA virus leads to the involvement of the brain, particularly the hippocampus, brainstem, and spinal cord.[17,18] Specific structures involved in the early stages of infection and the routes of viral dissemination within the CNS have not been fully explored.

In this report, we present evidence that the limbic system plays a major role in viral dissemination during the early stages of infection. Depending on the route of i.c. DA virus infection, the time course of spread to specific regions in the CNS is different. With time, the virus localizes in very distinct areas of the brain and spinal cord. This distinct localization of virus provides target sites for immune attack leading to immunopathology.

Materials and Methods

Virus and Animals

The DA strain of TMEV is a tissue culture-attenuated virus and is maintained in our laboratory in BHK-21 cells.[18] A working stock was prepared and used for these experiments.

For this study, 4–6-week-old female nude mice were used (Jackson Laboratories, Bar Harbor, ME). To evaluate the route of spread of TMEV within the CNS, we designed two different i.c. routes of infection, via cortex or olfactory bulb. Two groups (21 mice in each group) were infected with 3×10^5 PFU (10 μl) of the DA strain of virus in the right side of the brain. For the cortical infection, a point two-thirds of the distance between the posterior edge of the right eye and right ear was selected for the injection site. For the olfactory bulb infection, mice were injected between the central line and right eye edge to a depth of 2 mm. Generally, nude mice developed clinical symptoms (paralysis) after 10 days, and 80% died within 3 weeks.[18] Animals from each group were perfused via the heart at 2, 4, 7, and 11–14 days postinfection (p.i.) with 4% paraformaldehyde-phosphate-buffered saline. Brains and spinal cords were removed and processed for paraffin embedding. Four-μm sections of both sagittal and coronal sections were cut for histology and immunohistochemistry.

Immunohistochemistry

To detect DA viral antigens, a hyperimmune rabbit antiserum to DA virus was used.[18] Deparaffinized brain and spinal cord sections were incubated with 5% heat inactivated goat serum for 30 minutes to inhibit nonspecific binding. The anti-DA rabbit sera was added at a dilution of 1:500 in 1% goat serum-phosphate buffer solution for 3 hours at room temperature, followed by a 1:50 dilution of a goat anti-rabbit IgG conjugated to horseradish peroxidase (TAGO, Burlingame, CA) for 30 minutes at room temperature. The slides were then incubated with a diaminobenzidine/H_2O_2 solution. The involved CNS structures were also visualized by staining adjacent sections with hematoxylin-eosin or with luxol fast blue. The sites of involvement were confirmed with a mouse CNS atlas.[19]

In Situ *Hybridization*

To detect viral RNA in the tissue, *in situ* hybridization was performed as follows. For the probe preparation, the TMEV clone pDA8, which is approximately 1600 bases long and complementary to the structural proteins VP1 and VP3 of the DA strain, was used. The specificity of pDA8 was confirmed by Northern blot and by sequencing.[16] The probes were radiolabeled to 10^8 cpm/μg DNA with a random primer method[20] using deoxycytidine 5′-[α-*thio*-^{35}S]triphosphate and deoxyadenosine 5′-[α-*thio*-^{35}S]triphosphate (DuPont, Boston, MA).

Tissue sections were processed as described earlier. After rehydration, tissue sections were rinsed twice in distilled water, incubated for 15 minutes with 0.2 mol/L HCl, rinsed twice in distilled wa-

ter, and immersed in 1% Triton X-100 (EM Science, Gibbstown, NJ) for 90 seconds. After rinsing with phosphate-buffered saline, the slides were treated for 20 minutes at 37 C with proteinase K (BRL, Gaithersburg, MD) at 2 μg/ml in 20 mmol/L Tris-HCl and 2 mmol/L $CaCl_2$. The slides were dehydrated through ascending ethanol, air dried, and hybridized with 0.5 μg probe/ml in 50% formamide, 5× hybridization salts, 5× Denhardt's, 500 μg/ml salmon sperm DNA, 250 μg/ml HeLa cell RNA, 0.1% Triton X-100, and 10 mmol/L dithiothreitol.

Hybridization was carried out overnight at 37 C. Excess probe was removed by several changes of 2× and 0.1× standard saline citrate. Washed slides were dehydrated, air dried, and immersed in NTB-2 emulsion (Kodak, Rochester, NY). After 4 days of exposure, the slides were developed in Kodak D19 and fixed with Kodak standard fixer. The slides were counterstained with hematoxylin, coverslipped, and examined by light microscopy.

Results

Selected Sites Targeted by the DA Virus

To determine what sites or structures contained DA virus following infection, serial sections of brain and spinal cord were stained with hematoxylin-eosin or luxol fast blue or analyzed by immunohistochemistry to detect viral antigen or by *in situ* hybridization to detect viral RNA. The results of the *in situ* hybridization study were similar to the results obtained by the immunohistochemical experiments. The regions affected after DA virus infection of nude mice are depicted in Figure 1.

In the brain, the hippocampus (mainly CA3 region) (Figure 2A), amygdaloid nuclei, entorhinal cortex, and olfactory nuclei contained viral antigen-positive cells by 2 weeks p.i. In addition, the thalamus (especially anteroventral nucleus) frequently contained numerous viral antigen-positive cells (neurons). Furthermore, DA virus antigen could be detected in several areas of midbrain (interpeduncular nuclei, substantia nigra, and ventral tegmentum nuclei) at this time. Similarly, the cingulate cortex, nucleus tractus diagonalis Brocae, subthalamic nuclei, nucleus raphe dorsalis, hypothalamus, basal ganglia, and mamillary body often contained infected neurons. Besides the structures mentioned above, glial cells in the pons and/or medulla corresponding to the corticospinal tracts were positive for viral antigens (Figure 2B). In contrast, no positive cells were observed in the cerebellum, and the trigeminal nuclei were rarely infected.

Pathologically, areas within the brain containing viral antigens demonstrated microglial proliferation. Occasionally, macrophages were observed in these areas; however, other obvious cellular infiltrates were not present.

In contrast to the brain, the spinal cord contained many glial cells positive for DA viral antigen in the white matter. Glial cell proliferation associated with virus-positive cells in the white matter was usually restricted to the nerve root entry zones (Figure 3). In the gray matter, both the cell body and processes of anterior horn cells contained viral antigen and RNA (Figure 4). The nucleus was spared, and neuronophagia was often observed.

These results suggest that TMEV infects selected structures and disseminates to specific areas in the CNS. Most of the above structures are related to each other with connecting fibers and consist of the limbic system.

Viral Dissemination and Spread

Based on the observations that the DA virus mainly involves the limbic system, we predicted that the connections (pathways) among these structures should play an important role in viral spread (Table 1). To further define the route of dissemination in the CNS, we chronologically studied the sites of viral involvement by two routes of infection. One was via the olfactory bulb, which is a direct route for infection of the limbic system, and another was via the (parieto-occipital) cortex, which is an indirect pathway to the limbic system. Mice were infected by one or the other route of infection, and the brain and spinal cord were processed for visualization at various times up to 2 weeks (Table 1).

Spread Within the Brain

Spread of DA virus was compared using two different i.c. routes of injection (via the olfactory bulb or the cortex). The presence of DA virus in the brain of nude mice was chronologically evaluated by immunohistochemistry.

At 2 days p.i., DA virus antigen-positive cells were detected near the injection sites by either route of infection. Infection via the olfactory bulb led to infection of neurons in the olfactory nuclei, anteroventral nucleus, and hippocampus.

By 4 days p.i., many neurons in the olfactory nuclei, anteroventral nuclei, and hippocampus contained viral antigen, having been infected by the olfactory bulb route. In contrast, only a few neurons in

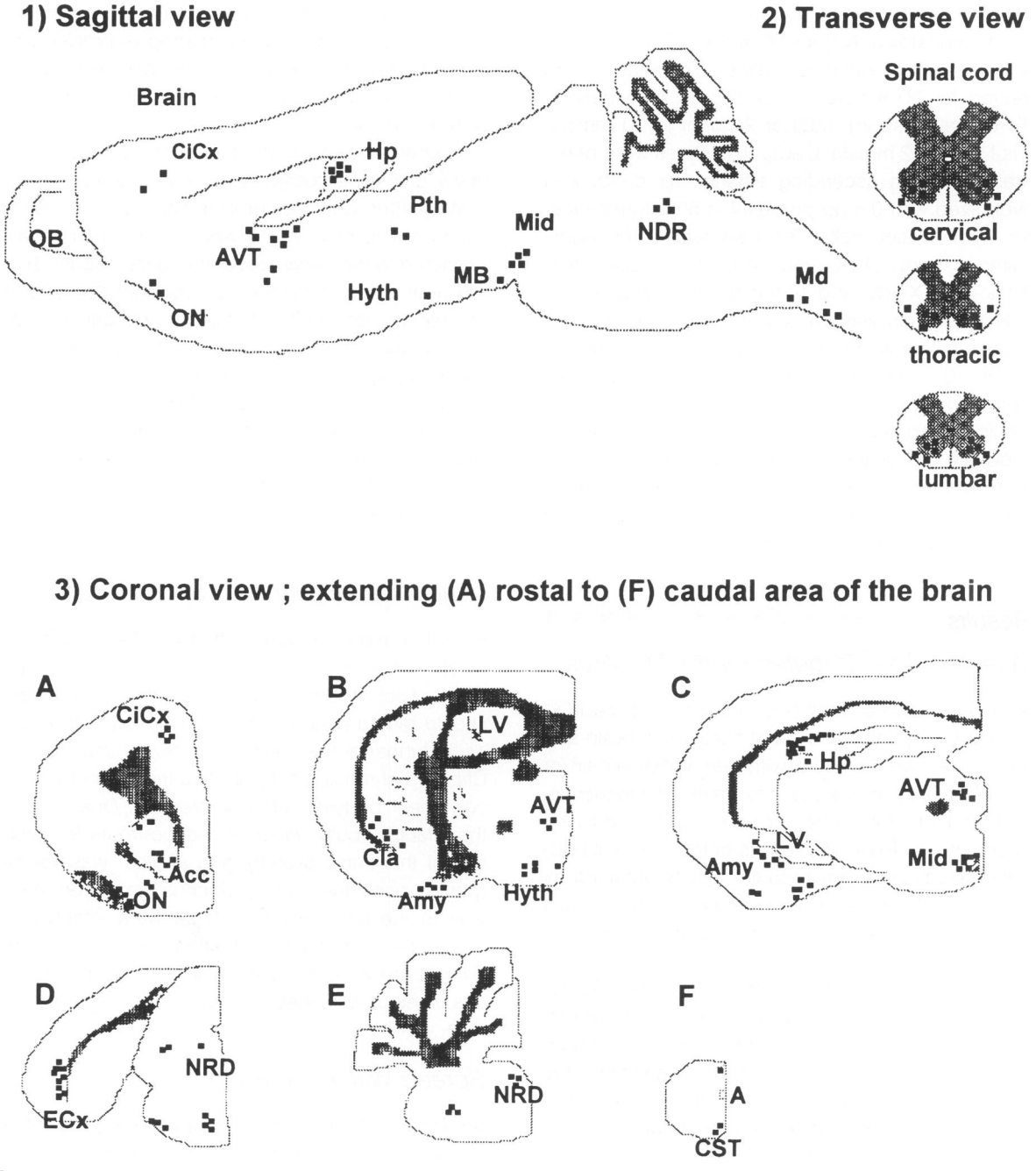

Figure 1. *The distribution of viral antigens in the CNS by immunohistochemistry. A, aqueductus; Acc, nucleus accumbens septi; Amy, amygdaloid nuclei; AVT, anteroventral nuclei of thalamus; CiCx, cingulate cortex; CST, corticospinal tract; Cla, claustrum; ECx, entorbinal cortex; Hyth, hypothalamus; Hp, hippocampus; LV, lateral ventricle; MB, mamillary body; Md, medulla oblongata; Mid, midbrain (substantia nigra, interpeduncular nuclei); NDR, nucleus dorsalis raphe; OB, olfactory bulb; ON, olfactory nuclei.*

the hippocampus or anteroventral nuclei or near the substantia nigra were infected with the DA virus by the cortical injection route.

Interestingly, by 7 days p.i. it appeared that the number of DA virus-positive cells in mice infected by the olfactory bulb route of infection had somewhat decreased in the CNS, since only a few cells were found to be positive. In contrast, mice infected by the cortical route had numerous neurons containing DA virus antigen in the hippocampus, and by this time DA virus had spread to the brainstem.

At 11–14 days p.i., DA virus had disseminated to many specific sites within the brain, including the nucleus accumbens septi and cingulate and en-

torhinal cortex. A few glial cells placed in corticospinal tracts also contained DA viral antigen. By this time, there were no large differences between the two injection routes except in the hippocampus and olfactory bulb. At this time, by the cortical route of infection, more virus-positive cells were present in the hippocampus as compared to the olfactory bulb route of infection. Furthermore, by the cortical route of infection, DA virus antigen-containing cells were never observed in the olfactory bulb.

The time course of DA virus dissemination displayed some differences between the olfactory and cortical routes of infection. The olfactory bulb route, which is a direct inoculation into the olfactory pathway, resulted in a more rapid spread of the virus to regions in the limbic system versus the cortical route of infection.

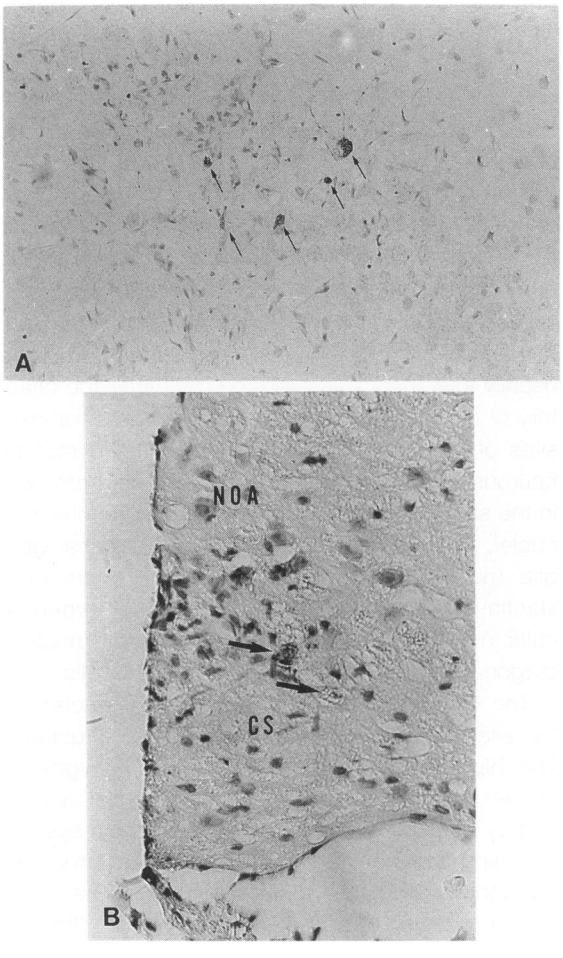

Figure 2. *Immunohistochemical study (antibody to DA virus).* **A**, *hippocampus. Many pyramidal neurons are antigen-positive (arrows), and dendrites are clearly stained. ×200.* **B**, *lower medulla. A few glial cells are viral antigen positive in the corticospinal tract. NOA, nucleus olivaris accessorius; CS, corticospinal tract. ×400.*

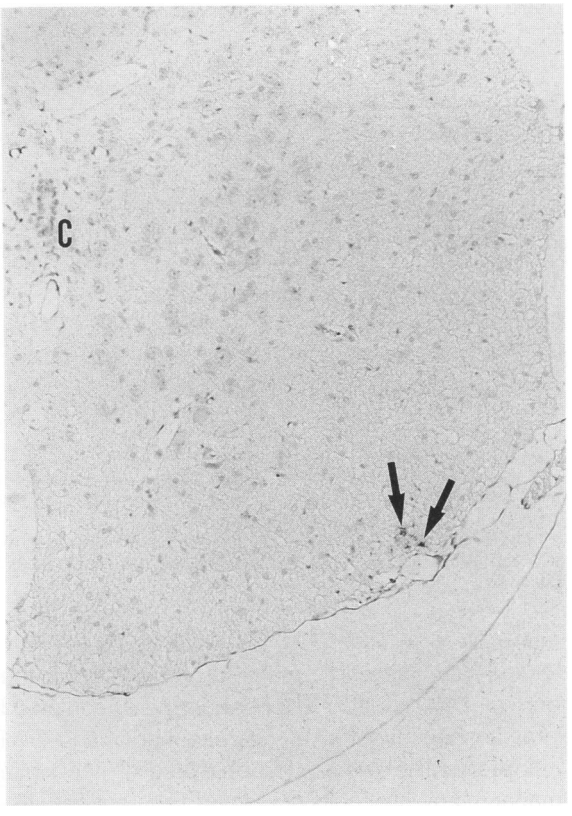

Figure 3. *Transverse section of spinal cord (thoracic cord). Few glial cells (arrows), which locate at root entry zone, are positive for viral antigens. Immunohistochemistry (antibody to DA virus). ×100.*

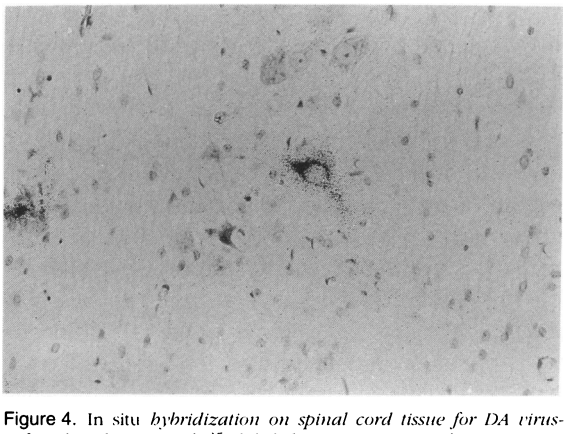

Figure 4. In situ *hybridization on spinal cord tissue for DA virus-infected nude mice with ^{35}S-labeled DA virus DNA probe. Large neurons (probably motor neurons) in the gray matter show positive hybridization. ×400.*

Dissemination of DA Virus in the Spinal Cord

The presence of DA virus in the spinal cord of nude mice injected by two i.c. routes was also evaluated by immunohistochemistry.

At 2 days p.i. a small number of glial cells in the white matter of the spinal cord were positive by ei-

Table 1. *Viral Spread and Distribution via Two Different Routes of Infection*

	Olfactory ncl.	Hippocampus	Ant. thalamus	Post. thalamus	Midbrain	Spinal cord g.m.	Spinal cord w.m.
2 days p.i.							
OB	+	++	++	–	–	–	+
CO	–	+	–	–	–	–	+
4 days p.i.							
OB	++	+++	+++	+	+	+++	+++
CO	–	+	+	–	+	+	+++
7 days p.i.							
OB	+	–	+	–	+	++	++
CO	–	++	+	+	+	+++	+++
11–14 days p.i.							
OB	++	+	+++	+++	++	++	++
CO	+	+++	+++	+++	++	+++	+++

The severity of involved areas was scored – to +++ depending on the average number of virus antigen-positive cells in each region; –, no virus antigen-positive cells detected; +, a few antigen-positive cells comprising the lesion; ++, a moderate number of antigen-positive cells; +++, over 20 antigen-positive cells in the lesion.

Abbreviations: ncl., nucleus; ant., anterior; post., posterior; g.m., gray matter; w.m., white matter; OB, olfactory bulb injection; CO, cortical injection; Ant. thalamus include anteroventriclar nuclei and other anterior nuclei of thalamus. Post. thalamus includes all posterior nuclei of thalamus.

ther route of injection. By the olfactory route of infection, at 4 days p.i. many large neurons in the gray matter (probably anterior horn cells) contained viral antigen. Figures of neuronophagia and glial cell proliferation were often observed. In the white matter, many glial cells contained virus antigen, especially those cells in the ventral nerve entry zone of the thoracic cord. By the cortical route of infection, only a few anterior horn cells were positive for viral antigen, and the virus-positive cells were mainly in the ventral root entry zone of the white matter.

At 7 days p.i., by either route of infection, the gray and the white matter was extensively involved. However, mice infected by the cortical route had slightly more virus antigen-positive cells than those infected by the olfactory bulb route.

Many positive cells were detected at 11–14 days p.i. in both the gray and the white matter by either route of infection. At this time, no large differences were observed with regard to the areas infected, numbers of cells, or distribution of virus. In the white matter, both the ventral and dorsal regions were often affected at this stage.

Thus, in the spinal cord, the olfactory bulb route of infection leads to an earlier involvement of gray matter when compared to the cortical route of infection. However, with time, similar areas were infected.

Discussion

This report analyzes DA virus dissemination and tropism in the CNS. We have demonstrated several new aspects of Theiler's virus infection concerning: 1) specific neurotropism of the virus in the CNS at the initial stage of infection; 2) the chronological changes between two different i.c. routes of infection; and 3) the dissemination of virus to the spinal cord.

First, we document specifically infected regions in the CNS of nude mice and extend in more detail the results of Love et al[17] and Zurbriggen and Fujinami.[18] The hippocampus, amygdaloid nuclei, entorhinal cortex, cingulate cortex, thalamus (anteroventral nuclei), midbrain, and spinal cord were usually infected by 2 weeks p.i. by either the olfactory or cortical route of infection. The most common sites of DA virus replication were in the pyramidal neurons of the hippocampus and anterior horn cells in the spinal cord. In addition to these, the olfactory nuclei, mamillary body, hypothalamus, basal ganglia (nucleus caudatus/putamen), midbrain (substantia nigra, ventral tegmentum area, interpeduncular nuclei), nucleus raphe dorsalis, and nucleus diagonalis Brocae all contained infected cells.

The distribution of these lesions is restricted by the anatomical relationship among these structures. The hippocampus, entorhinal cortex, amygdaloid nuclei, mamillary body, anteroventral nuclei, and olfactory nuclei together make up the limbic system, including the olfactory pathway. These structures are tightly associated with each other.[21] The limbic system was first described by Broca[22] as a "limbus" around the medial part of the telencephalon. Later, this concept was extended to all areas that include the afferents and efferents of the limbic lobe (parahippocampal and cingulate lobe). This system is

especially important for emotion and memory. The limbic system also has a connection to the basal ganglia, such as the nucleus accumbens septi and the substantia nigra, which is called the mesolimbic structure. Axons from the nucleus raphe dorsalis and nucleus diagonalis Brocae terminate in the olfactory bulb.[23,24] Thus, the regions in the brain containing lesions are anatomically related and the virus disseminates quickly within those structures. In the acute stage of DA virus infection, limbic structures and their connections are the main routes of dissemination in the CNS and display a figure of "limbic encephalitis."

Second, we investigated the chronological differences between two i.c. routes of infection and their relationship to disease. In the present study, we demonstrated that the DA virus has a particular tropism to special neurons in the limbic structure. However, neurotropism may also depend on both the sites of entry and pathways of spread.[25,26] Infection at a specific site has the potential of producing a unique pattern of viral distribution,[27,28] and for some viruses even the route of spread and tropism may vary depending on the site of inoculation.[29] Therefore, to test the hypothesis that the DA virus can be transmitted via the connections within the limbic system, we designated two routes of i.c. infection. One is a direct infection leading to the limbic system and the other is separate from limbic structures. In our study, following the time course of DA virus spread, we observed some differences between the olfactory bulb and cortical routes of infections. Interestingly, total viral titers as determined by viral plaque assay showed no significant difference between the two routes of infection at 4 and 7 days p.i., when whole brain and spinal cord were compared (data not shown). The olfactory bulb route of infection resulted in a more rapid spread within the brain than the cortical route of infection up to 11–14 days p.i. The delayed appearance of virus in the hippocampus and the thalamus by the cortical route may be due to a time lag during which the virus spreads from the cortex to the limbic system pathway. This route is considered to be from the cortex via the entorhinal region to the hippocampus. After invasion of the limbic system, the virus spreads to similar areas in the brain by both routes of infection. This suggests that once the virus enters the limbic pathway, the dissemination of DA virus is uniform due to the specific neurotropism within the limbic system.

After 7 days p.i. there was differential involvement of hippocampal neurons between the two routes of infection. By the olfactory bulb route of infection, the extent of hippocampus infection seemed to diminish as determined by immunohistochemistry. The decrease in viral antigen-positive cells in the hippocampus is most likely due to a decrease of target neurons due to virus-induced cell death. Most of these hippocampal neurons have been already infected and destroyed by the virus before 7 days p.i. via the olfactory bulb route. In contrast, by the cortical route of infection, the infection of the hippocampus was detected after 7 days p.i., probably due to the delay of spread and infection.

Third, we examined the chronological lesion development in the spinal cord. The dissemination to the spinal cord is not as simple or straightforward as in the brain. The routes from the brain to the spinal cord are still unclear. We were able to detect viral antigens in glial cells in the white matter of the spinal cord at 2 days p.i.; however, we were unable to find viral antigen-positive cells in the gray matter at this stage. Some pathways between the brain and spinal cord were used for the dissemination. For example, the neurons mainly affected at the early stage of infection are large neurons in the anterior horn (probably motor neurons). A major pathway from the brain to anterior horn cells is the corticospinal tract. In our study, we could detect a few infected glial cells, placed in the corticospinal tract at 11–14 days p.i. This indicates that the virus may use this tract as one of the routes for dissemination to the spinal cord. However, an early site for virus presence in the spinal cord is the ventral root entry zone[30] and not the corticospinal tract. Therefore, other routes for the dissemination of the DA virus in the spinal cord are used. The ventral root entry zone is known to have a "leaky" blood-brain barrier.[31,32] During viremia, this area is more vulnerable to viral infection than other areas in the CNS. We suggest that hematogenous spread is also important for dissemination to the spinal cord. The axons of motor neurons in the anterior horn consist mainly of ventral roots. Retrograde invasion from the ventral nerve entry zone is also consistent with early spread to the anterior horn cells. This is supported by the presence of the viral RNA in the endothelial cells.[18] Our data are consistent with dissemination of the virus to the spinal cord by several routes.

In conclusion, our results indicate that in the early stages of infection the DA virus infects the limbic system utilizing the connecting fibers of limbic structures for dissemination within the brain. For viral dissemination to the spinal cord, our data suggest two possible mechanisms for spread, ie, via the connecting fibers in the CNS but also through a hematogenic route. Both may play important roles.

Acknowledgments

We thank Ellen White, Katherine Kokoshka, Loan Thi Hong Nguyen, and Elena Searles for their dedication and excellent technical assistance. We are grateful to Dr. Caroline Kurtz and Dr. Lou Ann Barnett for helpful discussions and Jewelyn Jenson for preparation of the manuscript.

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