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Early Infection of the Central Nervous System by the GDVII and DA Strains of Theiler's Virus

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The DA strain of Theiler's virus, a murine picornavirus, causes a persistent infection of glial cells of the white matter of the spinal cord, associated with chronic inflammation and primary demyelination. The GDVII strain causes an acute fatal grey matter encephalomyelitis. We characterized the target cells of GDVII and DA viruses 4 days following intracerebral inoculation, and we compared the levels of viral RNA within these cells. GDVII virus infected approximately 10 times more cells than DA virus. Whereas GDVII virus infected neurons exclusively, DA virus infected also astrocytes and possible macrophage-microglial cells. The levels of viral RNA in neurons infected with GDVII and DA viruses were of the same order. These results show that DA virus infects glial cells already at the beginning of the disease and that the more efficient spread of GDVII virus is probably not due to a higher level of RNA replication per cell.

The DA strain of Theiler's virus, a murine picornavirus, causes a persistent infection of the central nervous system (CNS) associated with chronic inflammation of the white matter of the spinal cord and primary demyelination (9). This disease is studied chiefly as a model for multiple sclerosis. Following intracranial inoculation, the virus infects the grey matter, first in the brain and then in the spinal cord, but the mice survive. It then migrates from the grey to the white matter of the spinal cord and persists there, in macrophage-microglial cells, oligodendrocytes, and astrocytes, if the mice are genetically susceptible (1, 5–7, 10). The GDVII strain, on the other hand, infects the grey matter only and kills the host from acute encephalomyelitis. The fact that mice inoculated with DA virus survive the early, grey matter phase of the infection suggests that the replication of this virus is more restricted than that of GDVII virus. The eventual migration of DA virus to the white matter suggests a specific tropism of the virus for glial cells. To examine these points, we identified the target cells of GDVII and DA viruses during the first few days following intracerebral inoculation and we compared the levels of viral RNA within these cells.

The plaque-purified GDVII and DA viruses were grown on BHK-21 cells. SJL/J mice, 3 to 4 weeks old, were purchased from the Institut Pasteur animal facility. Mice were inoculated intracranially with 40 μl of phosphate-buffered saline (PBS) containing 10^4 PFU of virus. They were sacrificed 4 or 5 days postinfection, depending on the experiment. The animals were perfused, under anesthesia, with PBS followed by 4% paraformaldehyde dissolved in PBS as described elsewhere (4). The brain and the spinal cord were dissected out, refixed by immersion in the same fixative, cut frontally into three and four blocks for, respectively, the brain and the spinal cord, dehydrated, and embedded in paraffin as described elsewhere (4). The tissue blocks were oriented to obtain frontal sections of the brain and longitudinal sections of the spinal cord. Eight-micrometer-thick sections were cut and picked on treated glass slides as described elsewhere (4).

We used the double in situ hybridization-immunocytochemistry assay which we described previously (3, 4) to examine whether glial cells were infected 4 days postinoculation. Astrocytes were identified with a hyperimmune anti-GFAP rabbit serum (Dakopatts) diluted 1/450. The secondary reagent was

TABLE 1. Identification of CNS cells infected by the GDVII and DA strains of Theiler's virus

Mouse	No. of slides studied/marker	Avg no. of infected cells/slide	No. of cells positive for indicated marker/total no. of infected cells (%)		
			GFAP[a]	PLP[b]	F4/80[c]
GDVII-1	4	230	5/517 (1)	5/1,351 (0.4)	30/891 (3.4)
GDVII-2	4	377	13/1,346 (1)	5/1,340 (0.4)	6/1,841 (0.3)
GDVII-3	4	164	3/513 (0.6)	0/679 (0)	0/781 (0)
DA-1	7	13	22/111 (19.8)	1/54 (1.8)	4/103 (3.9)
DA-2	7	20	43/241 (17.8)	0/66 (0)	4/109 (3.7)
DA-3	4	7	NA[d]	0/12 (0)	4/42 (9.5)

[a] The slides were reacted with anti-GFAP serum followed by in situ hybridization for viral RNA. The exposure times were 5.5 h for GDVII virus and 48 h for DA virus.
[b] The slides were reacted with anti-Theiler's virus serum followed by in situ hybridization for PLP mRNA. The exposure time was 24 h.
[c] The slides were reacted with monoclonal antibody F4/80 followed by in situ hybridization for viral RNA. The exposure times were 5.5 h for GDVII virus and 48 h for DA virus.
[d] Not available.

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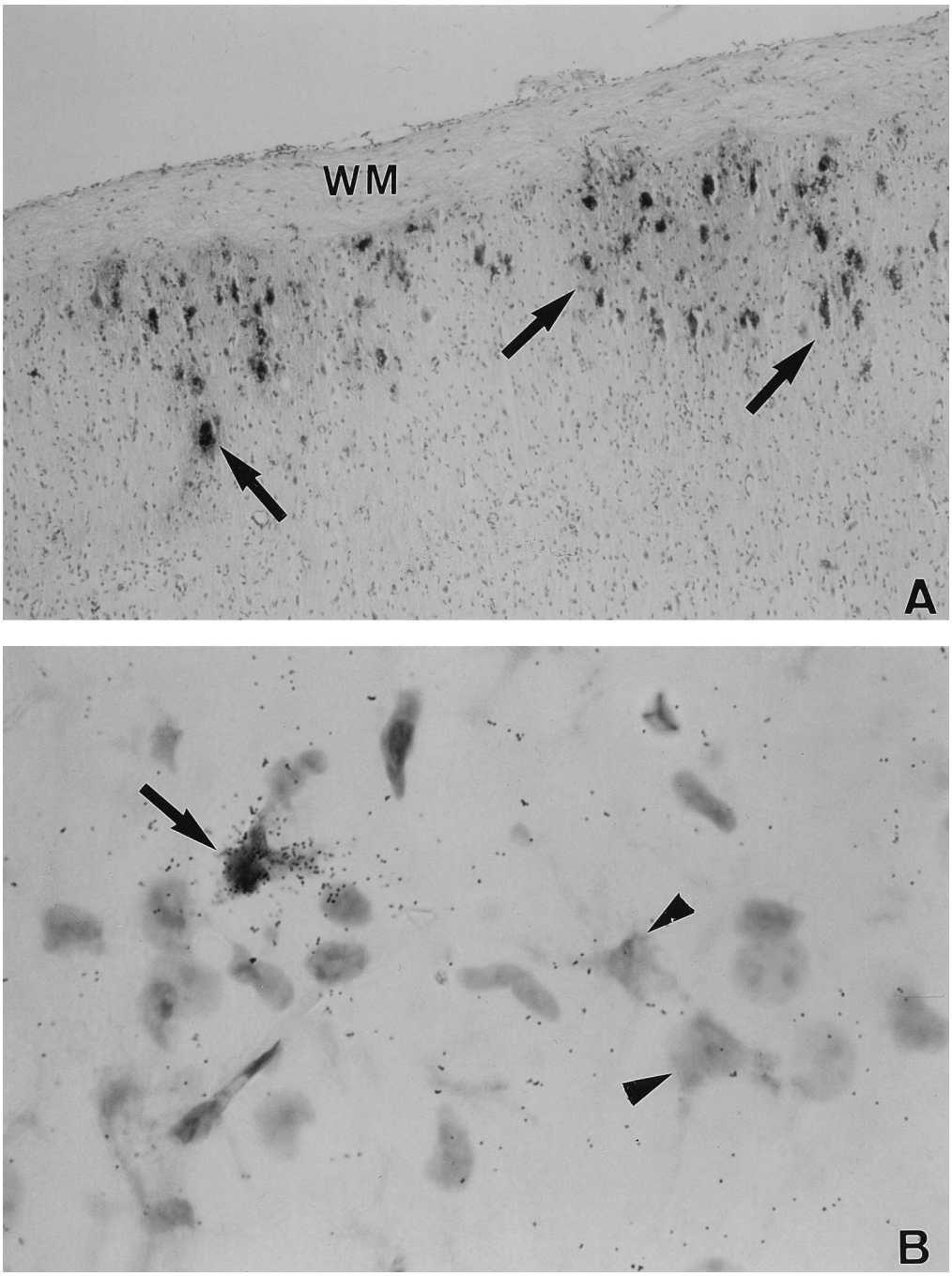

FIG. 1. Identification of CNS cells infected by the GDVII and DA strains of Theiler's virus. (A) Longitudinal section of the spinal cord of an SJL/J mouse infected with the GDVII strain. The animal was sacrificed 4 days postinfection. Viral capsid antigens were detected by immunocytochemistry. The section was counterstained with hematoxylin. The section shows several infected cells with the characteristic morphology of neurons (arrows) and the absence of viral antigens in the adjacent white matter (WM). Original magnification, ×630. (B) Frontal section of the brain of an SJL/J mouse infected with the DA strain. The animal was sacrificed 4 days postinfection. The section was reacted with an antiserum against GFAP, a specific astrocyte marker, and then hybridized in situ with a ^{35}S-labelled virus-specific cRNA probe. The section was counterstained with hematoxylin. The arrow points to an infected astrocyte; the arrowheads point to uninfected ones. Original magnification, ×1,000.

biotinylated anti-rabbit immunoglobulins (Vector Laboratories) diluted 1/200. Bound secondary antibody was detected with an avidin-biotin-peroxidase complex (ABC; Vector Laboratories). Macrophage-microglial cells were identified with the F4/80 rat monoclonal antibody (Serotec) diluted 1/3 (8). Detection of this antibody was performed with biotinylated anti-rat immunoglobulins (Vector Laboratories) diluted 1/200 and the ABC reagent. Viral RNA was detected in the same sections by in situ hybridization. The cRNA probe was labelled with ^{35}S-UTP (1,200 Ci/mmol) by in vitro transcription of linearized plasmid pBS-GD7, which contains nucleotides 1 to 280 of the genome of virus GDVII inserted into the Bluescript

vector. In situ hybridization was performed as described elsewhere, including RNase treatment after hybridization to reduce background (4). To study the infection of oligodendrocytes, viral antigens were detected first, with a 1/300 dilution of a rabbit hyperimmune anticapsid serum which has been described previously (3). Detection was done with biotinylated anti-rabbit immunoglobulins (Vector Laboratories) diluted 1/200 and the ABC complex. The same slides were then hybridized in situ to detect proteolipid protein (PLP) mRNA. This is a specific and convenient marker of oligodendrocytes (11). The cRNA probe was transcribed from plasmid pLH-116, which consists of the entire coding region of mouse PLP cDNA cloned in the pGEM3 vector (a gift of L. Hudson). The sections were scanned systematically under the microscope with a 63× objective. Every infected cell encountered was scored as positive or negative for the corresponding glial cell marker. The vast majority (98%) of the infected cells could be unambiguously categorized.

The data from this study are presented in Table 1. On average, there were 200 infected cells per slide for virus GDVII and only 25 for virus DA. As already described by several authors, the majority of these cells could be identified as neurons by morphological criteria. Table 1 shows that virus GDVII did not infect significant numbers of glial cells. Furthermore, GDVII virus was never found in white matter, even adjacent to intensely infected areas of grey matter (Fig. 1A). On the other hand, 18% of the CNS cells infected by the DA virus were astrocytes. An example of an infected astrocyte is shown in Fig. 1B. The difference between the percentages of GFAP-positive infected cells for both viruses was highly significant (two-group *t*-test; $P < 0.0002$). Table 1 also shows that a small fraction of the cells infected by DA virus might have been macrophage-microglial cells. Neither the DA nor the GDVII virus infected the satellite oligodendrocytes present in the grey matter to a significant extent.

The figures presented in Table 1 cannot be used to calculate absolute percentages of infected cells belonging to each category of glial cells. This would require that each cell marker label every cell of the corresponding category, which is probably not the case. Since we did not find a marker that would label all types of neurons, it was impossible to determine the fraction of infected cells which were not identified by any of the markers. However, since the patterns of infection for both viruses were established under identical experimental conditions, our data allow a reliable comparison of the target cells for both viruses.

Table 1 shows that the number of infected cells per CNS section was approximately 10-fold higher for the GDVII virus than for the DA virus. This greater spread could mean that GDVII virus replicates to higher levels in individual cells. We examined this point by comparing the amount of viral RNA per cell for the two viruses, using in situ hybridization. This comparison is possible only if the number of autoradiographic grains per cell and per hour of exposure is proportional to the number of copies of viral RNA per cell. As described previously, this relationship can be studied by infecting BHK-21 cells, harvesting them at various times postinfection, and hybridizing them in situ (2, 3). The relationship was not linear when the ^{35}S-labelled cRNA probe described above was used (data not shown). Instead, it was more or less exponential, which resulted in an overestimation of the differences of RNA content between cells in simple comparisons of the grain densities under the microscope. On the other hand, the relationship was linear when the same cRNA was labelled with [^{3}H]UTP (93Ci/mmol), in accord with previous work (not shown) (2, 3). The nonlinearity observed with the ^{35}S-labelled probe could have been due both to the very high specific activity of the probe and to the high efficiency of grain formation characteristic of ^{35}S. Whatever the mechanism, we wish to caution against the use of ^{35}S-labelled probes in in situ hybridization to obtain quantitative information.

TABLE 2. Quantification of viral RNA in CNS cells infected by the GDVII and DA strains of Theiler's virus

Mouse	No. of slides studied	Exposure time (range in h)	No. of cells over which grains were counted	No. of grains/cell/h of exposure	
				Mean	SEM
GDVII-4	3	41–47	505	0.656	0.019
GDVII-5	3	29–47	315	0.812	0.038
GDVII-6	3	29–47	467	1.267	0.048
GDVII-7	4	39–47	289	0.864	0.037
DA-4	7	120–293	126	0.230	0.019
DA-5	8	96–293	944	0.275	0.007
DA-6	7	137–293	220	0.250	0.014
DA-7	8	137–293	45	0.218	0.024

CNS sections, obtained 4 days postinoculation with the GDVII or the DA virus, were hybridized in situ with the ^{3}H-labelled cRNA probe described above. After suitable exposure times, the slides were developed, counterstained with hematoxylin, and systematically scanned under the microscope with a 100× objective. The number of grains per cell was determined for every positive cell encountered. The results are shown in Table 2. On the average, CNS cells infected with virus GDVII contained three times more grains than cells infected with virus DA.

Glial cells are present both in the grey and white matter, whereas the cell bodies of neurons are found only in the grey matter. It has been known for some time that, during early grey matter infection, GDVII and DA viruses infect neurons. Our results show that DA virus also infects astrocytes and possibly macrophage-microglial cells, whereas GDVII virus does not. This difference of tropism may explain why DA virus is able to persist in glial cells of the white matter later on. Virus GDVII infects approximately 10 times more grey matter cells than virus DA (Table 1). This efficient spread could reflect a fast replication cycle, a large progeny, a high affinity for a cellular receptor, or any combination of the above. Although there was a clear difference in viral RNA content per infected cell for the two viruses, it was only threefold in favor of GDVII virus. This small difference may not be sufficient to explain the 10-fold more efficient spread of GDVII virus in grey matter.

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