Early Loss of Astrocytes in Herpes Simplex Virus–induced Central Nervous System Demyelination

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Immunohistochernistry was used to study herpes simplex virus type 1–induced central nervous system demyelination in the trigeminal root entry zone of mice inoculated with herpes simplex virus type 1 by the corneal route. There was no change in peripheral nervous system myelin as shown by immunostaining for Po glycoprotein. Double immunoperoxidase staining for herpes simplex virus type 1 antigens and glial fibrillary acidic protein showed that most of the infected cells were astrocytes. Glial fibrillary acidic protein immunostaining was completely lost in the inferior medial portion of the trigeminal root entry zone at 6 days after herpes simplex virus type 1 inoculation, a time when central nervous system myelin was preserved as indicated by immunostaining for myelin basic protein. The pattern of glial fibrillary acidic protein staining did not change and herpes simplex virus type 1 antigens were no longer detected after day 8. There was a progressive loss of myelin basic protein staining within the area unstained by glial fibrillary acidic protein antisera on days 8 to 14. This pattern of astrocyte loss before central nervous system demyelination is strikingly different from the reactive astrocytosis seen in other demyelinating lesions, such as acute experimental allergic encephalomyelitis, progressive multifocal leukoencephalopathy, or acute multiple sclerosis. Herpes simplex virus type 1 infection in mice provides an unusual model of acute central nervous system demyelination preceded by a loss of astrocytes.

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Herpes simplex virus (HSV) infection induces a restricted central nervous system (CNS) demyelination under certain experimental conditions. Corneal inoculation of experimental animals with HSV type 1 (HSV-1) results in centripetal viral spread in axons and subsequent CNS myelin destruction near the trigeminal root entry zone (TREZ) [1, 2]. No myelin destruction has been observed in the peripheral nervous system (PNS) portion of the TREZ just adjacent to the demyelinating CNS lesions. The mechanism of this restricted CNS demyelination is not clear. We report here immunopathological findings in this model. HSV-1–infected astrocytes undergo active degeneration and are lost before CNS demyelination occurs, and there is a conformity of the areas lacking glial fibrillary acidic protein (GFAP) staining to the areas of subsequent CNS demyelination. These results suggest the possibility of a unique mechanism of demyelination whereby the abrupt destruction of astrocytes induces a HSV-associated demyelination.

Materials and Methods

Experimental HSV Demyelination

We inoculated the F strain of HSV-1 on the corneas of twenty-two 6 to 8-week-old female BALB/c mice by placing a drop of stock virus containing 10^8 plaque forming unit on both corneas and scarifying the cornea with a 19-gauge needle [3]. These inoculated mice were killed on 2, 4, 6, 8, 10, and 14 days postinoculation (PI). They were anesthetized with ether and perfused for 10 minutes through the heart with a fixative mixture of mercuric chloride and formalin. The pons, trigeminal roots, and ganglia were carefully removed, dissected, postfixed overnight in the same fixative, dehydrated in a graded series of ethanol and p-dioxane, and embedded in paraffin. Sections 6 μm thick were cut, mounted serially on numbered glass slides, and immunostained by using the peroxidase antiperoxidase (PAP) method [4]. Sections were treated sequentially with 3% normal sheep (or rabbit) serum, the primary antisera, sheep anti-rabbit (or goat) IgG diluted 1:40, rabbit (or goat) PAP diluted 1:80, and 3,3'-diaminobenzidine HCl with hydrogen peroxide. All dilutions were made with 0.5 M Tris buffer.

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The following antibodies were used at the second step: anti–HSV-1 rabbit serum (diluted 1:500) for detection of HSV-1 virus antigens, anti–myelin basic protein (MBP) goat serum (diluted 1:500) for CNS and PNS myelin, anti–Po glycoprotein rabbit serum (diluted 1:500) for PNS myelin, anti–glial fibrillary acidic protein (GFAP) (diluted 1:1,000) rabbit serum for astrocytes. Anti–HSV antibody (500 neutralizing units) was obtained from a New Zealand White rabbit after multiple intravenous inoculations of 10⁸ PFU of HSV. Immunocytochemical controls for HSV antigen detection included trigeminal ganglion sections from uninfected mice incubated with the anti–HSV serum and also sections from infected mice incubated with nonimmune rabbit serum. Both of these controls were invariably negative for immunoperoxidase staining. Immunoreactivity and control studies of anti–MBP serum [5], anti–Po serum [6], or anti–GFAP serum [7] are described elsewhere.

The double staining method was used for the simultaneous identification and localization of two antigens (GFAP and HSV antigens) by using a modification of procedures described by Erlandsen and colleagues [8]. To obtain two different colors, the following substrates of peroxidase were used: 3,3'-diaminobenzidine for brown reaction products and 4-Chloro-1-naphthol tends to fade a few hours after immunostaining, so co-localization of GFAP and HSV antigens to the same cells was confirmed by serial examination of the slides and recording the loss of the blue color from the doubly stained cells.

Attempts were made to identify by immunohistochemistry oligodendroglia with antisera, but consistently satisfactory immunostaining could not be obtained in this formalin-fixed material. Also, attempts were made to do electronmicroscopy by removing the paraffin-embedded pons and trigeminal roots used for immunohistochemical staining and reembedding the tissue in epon resin for thin sectioning. Review of this material identified degenerating astrocytes containing viral particles. Poor morphological details due to mercuric chloride fixation, however, precluded definitive study and presentation of the electronmicrographs.

Other Conditions of Demyelination
To compare the immunocytochemical findings observed in HSV-induced demyelinated lesions with other cases of demyelination, we reexamined immunocytochemically lesions of experimental allergic encephalomyelitis (EAE), progressive multifocal leukoencephalopathy (PML), and acute multiple sclerosis (MS). We immunostained paraffin sections of pontine lesions of EAE rats killed 10 and 14 days after immunization [9], and sections of cerebral lesions of PML (Patient 4; 61-year-old man) [10] and pontine lesions of acute MS (Patient 1; 6-year-old girl) [11]. The antibodies used were anti–MBP goat serum and anti–GFAP rabbit serum at a dilution of 1:500 and 1:1,000, respectively.

Results
Experimental HSV Demyelination
The CNS-PNS transition in the trigeminal root was clearly demonstrated by GFAP-stained glial cells in the CNS portion and Po-stained PNS myelin in the PNS portion (Fig 1). MBP antiserum stained both the CNS and PNS myelin. HSV immunoreactivity was never observed in the normal trigeminal ganglia, roots, or pons.

HSV immunoreactivity first appeared in neurons within the trigeminal ganglion on day 2 PI. In the ganglion, the HSV-stained neurons and satellite cells rapidly increased in number on day 4 PI and decreased on day 6 PI, and stained cells were no longer detected on day 8 PI, as already reported [12]. In the trigeminal root, HSV immunoreactivity was observed in some glial cells in the CNS part of the TREQ on day 4 PI (Fig 2). Most of the HSV-stained glial cells were morphologically normal. There was no inflammatory cell infiltration around these HSV-stained glial cells. Immunostaining of GFAP, MBP, and Po was generally normal. On day 6 PI, the HSV-stained glial cells increased in number. The distribution of infected cells was restricted to the medial aspect of the CNS part of the root extending from the PNS-CNS transitional zone to the junction of the pons (Fig 3). In the lesions, many HSV-stained glial cells were destroyed to small fragments or debris. With double immunostaining, most HSV-stained cells or their fragments were also stained with anti–GFAP serum (Fig 4). Most of the astrocytes, doubly stained with GFAP and HSV antiserum, were degenerating within such a restricted area. Therefore, the lesion was observed as an area lacking in GFAP immunoreactivity (Fig 5). In contrast to the remarkable changes in the astrocytes, the CNS and PNS myelinated fibers were immunocytochemically or morphologically normal, except for a few CNS fibers with an altered immunoreactivity near the infiltrating mononuclear cells (see Fig 5). On day 8 PI, CNS myelinated fibers began to change, both immunocytochemically and morphologically in the area where GFAP staining was completely lacking. Here, the HSV-stained glial cells were no longer observed in the lesion. On day 10 PI, an active destruction of CNS myelin was apparent and an area lacking in MBP immunostaining was evident. Interestingly, the extent of the demyelinated lesions was similar to the area where the GFAP immunostaining was lacking and the demyelination never extended beyond the area lacking GFAP (Fig 6). Although there was active demyelination and many macrophages containing MBP-stained materials in the lesions, no more active degeneration was observed in the remaining GFAP-stained astrocytes. With Bodian staining, most axons in the lesions were well preserved. In contrast to the CNS-myelinated fibers, Po-stained PNS myelin was apparently normal, even at areas near the transitional zone of the root. On day 14 PI, loss of MBP staining and MBP-stained debris was evident over almost the entire area where the GFAP immunostaining was absent. Destructive changes in CNS myelin were apparent on the edge of

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Fig 1. Paraffin-embedded sections of peripheral nervous system—central nervous system (PNS-CNS) transitional zone of the trigeminal root from control mouse immunostained with Po antiserum (A), myelin basic protein (MBP) antiserum (B), or glial fibrillary acidic protein (GFAP) antiserum (C). In A, Po antiserum stains peripheral myelin sheaths in the root. CNS myelin sheaths in the root are unstained. In B, myelin sheaths in both PNS and CNS parts of the roots are intensely stained with MBP antiserum. In C, immunostaining with GFAP antiserum is confined to the CNS part of the root, and it is not present in the PNS. (Original magnification for A–C, × 104.)

Fig 2. Paraffin section of the transitional zone of the trigeminal root from herpes simplex virus (HSV)-infected mouse on day 4 after inoculation immunostained with HSV antiserum. HSV-stained glial cells (arrows) are seen in the central nervous system part of the root. (Original magnification, × 411.)

Fig 3. Sections of the pons and trigeminal root from herpes simplex virus (HSV)-infected mouse on day 6 after inoculation immunostained with HSV antiserum. HSV-stained glial cells are restricted within the inferior medial portion of the trigeminal root entry zone. (Original magnification, × 42.)
the lesions. Po-stained thin myelin sheaths were observed in the center of the lesions and showed a continuity to the PNS myelin sheaths. Figure 7 summarizes the time course of the number of HSV-stained glial cells within the lesion, change in the GFAP-stained glial cells, and MBP-stained CNS myelin sheaths.

**Other Conditions of Demyelination**

In demyelinating lesions of EAE (10 and 14 days PI), PML, and acute MS, actively demyelinating changes were observed, as described elsewhere [9–11] (Fig 8). Alteration or loss of MBP immunoreactivity was observed in the degenerating myelin sheaths. MBP-stained myelin debris or macrophages containing MBP-stained materials were numerous, yet astrocytes were intact immunocytochemically and morphologically, even near infiltrating mononuclear cells or macrophages. On the contrary, the GFAP-stained astrocytes were increased in number and immunoreactivity in the surrounding lesions compared with findings in the control rat CNS or nonneurological control brains. In these lesions, there were hypertrophic astrocytes heavily stained with GFAP antiserum.

**Discussion**

Viral infection can result in CNS demyelination by a direct viral cytolysis of oligodendroglia as in PML [13] and coronavirus infection in mice [14, 15] or by immune-mediated mechanisms directed at CNS myelin or oligodendroglia as in postinfectious encephalomyelitis of measles and vaccinia [16, 17]. Moreover, both mechanisms appear to operate in some animal models of demyelination as in Theiler’s virus infection [18,
Fig 6. Sections of the trigeminal root from herpes simplex virus (HSV)-infected mouse on day 10 after inoculation immunostained with glial fibrillary acidic protein (GFAP) (A) or myelin basic protein (MBP) antiserum (B). Demyelinated lesion was observed as an area lacking in MBP immunostaining in the central nervous system part (left side of the picture) of the trigeminal root entry zone. The demyelinated area seems to be similar to the area lacking in GFAP immunostaining in the adjacent section. The demyelination does not extend over the area where GFAP immunostaining is deficient. (Original magnification for A and B, ×212.)

Fig 7. Time course of the number of herpes simplex virus (HSV)-stained glial cells (top), changes of glial fibrillary acidic protein (GFAP)-stained glial cells (middle), and changes of myelin basic protein (MBP)-stained myelinated fibers (bottom) demonstrated by a semiquantitative method. The degree of each change was evaluated as mild (+), moderate (++,), and severe (+++) in 4 to 8 trigeminal root entry zones (TREZ). Their mean and standard values are shown in this figure. On day 4 after inoculation, HSV-stained glial cells were observed in the medial central nervous system (CNS) part of the TREZ. A few GFAP-stained astrocytes have begun to degenerate. On day 6 after inoculation, HSV-stained cells were increased in number. More GFAP-stained astrocytes were degenerated and lysed out. A lack of GFAP immunoreactivity appeared to be evident. MBP-stained CNS myelin sheaths, however, were normal. On day 8 after inoculation, HSV-stained cells were no longer observed in the lesion where GFAP immunostaining was lacking, but the remaining GFAP-stained astrocytes no longer degenerated. MBP-stained CNS myelin began to degenerate. At 10 and 14 days, CNS myelin sheaths were actively degenerating within the area lacking in GFAP immunostaining.

and although there has never been documentation of viral particles crossing the axonal membrane, this pattern of spread probably occurs because there is productive HSV infection primarily in astrocytes at the entry zone area very soon after HSV inoculation. Townsend and Baringer [26, 27] have proposed immune-mediated inflammatory demyelination in this model, based on the finding of a decrease in the extent of demyelination in T-cell–deficient (nude) mice and cyclophosphamide-immunosuppressed mice inoculated with HSV by the corneal route. Even in immunocompetent mice, only rare macrophages were noted stripping myelin lamellae, and it was supposed that bystander rather than immune-specific demyelination was the main mechanism, with demyelination produced by proteases released from activated macrophages [20]. Obtaining the opposite result of more widespread and severe de-
Fig 8. Paraffin sections of the pons from the rat with experimental allergic encephalomyelitis on day 10 after inoculation immunostained with myelin basic proteins (MBP) antiserum (A) or glial fibrillary acidic protein (GFAP) antiserum (B), sections of the cerebral cortex from a subject with progressive multifocal leukoencephalopathy (PML) immunostained with MBP antiserum (C) or GFAP antiserum (D), or sections of the pons from a subject with acute multiple sclerosis (MS) stained with MBP antiserum (E) or GFAP antiserum (F). In A, MBP-stained myelin ovoids and fragment were observed around vessels (asterisk) infiltrated with mononuclear cells. In the adjacent section (B), GFAP-stained astrocytes are increased in number and immunoreactivity around the lesions. In C, there is a small focal PML demyelinated lesion in the cerebral cortex where there are JC virus-positive hypertrophic oligodendroglia (data not shown) and MBP immunostaining is lost. In the adjacent section (D), GFAP-stained astrocytes are increased in number and immunoreactivity and some are hypertrophic. In E, there is a perivascular demyelinated MS lesion where MBP immunoreactivity is decreased or lost. In the adjacent section (F), GFAP-stained astrocytes are increased in number and immunoreactivity. (Original magnification for A and B, × 164; for C and D, × 82; for E and F, × 82.)
myelination in cyclophosphamide-treated mice inoculated with HSV-1 on the snout, Kristensson and colleagues [28] suggested a direct viral cytolytic effect to explain at least the early stage of demyelination. The contradictory results could be related to differences in the dose and time of cyclophosphamide treatment; differences in virus strain, inoculum, and route of inoculation; and differences in the outbred strains of mice. Both inflammatory and direct viral cytolytic demyelination may be operative in this model.

The immunohistochemical techniques used in this study cannot settle the disagreement over the relative importance of inflammatory versus viral cytolytic demyelination in this model. Our study using MBP immunostaining confirmed the location and time course of CNS demyelination described by light and electron microscopy [1, 2]. Double label immunohistochemistry with anti-GAP and anti-HSV sera (see Fig 4) indicates that at the early stage, the HSV-1 infection is overwhelmingly in astrocytes. There is rapid lysis of astrocytes as shown by focal areas in which GAF immunostaining is completely lost by 6 days after HSV inoculation. Loss of MBP immunostaining indicating demyelination begins just after this extensive lysis of astrocytes, and the area of demyelination as defined by loss of MBP immunostaining is strictly confined to the area of astrocyte destruction as shown by loss of GAF immunostaining. In contrast, we have found in lesions of acute MS and in early lesions of PML and EAE that there are no degenerative changes in astrocytes, morphologically or immunocytochemically; but we did observe reactive astrocytes or astrocytosis in these lesions. Therefore, it is likely that the early loss of astrocytes is characteristic of HSV-induced demyelination and not other demyelinating lesions.

Astrocytes have long been thought to play a skeletal role in the CNS, separating various synapses and serving as a means of nutrient transport from blood to neurons; and astrocyte proliferation is a well-known response to nonspecific CNS damage. The astrocyte also plays an immunological role for presenting antigen to T lymphocytes [29]. They express major histocompatibility complex class I antigen on their surface and would be targets for class I-restricted cytolytic T lymphocytes [30]. In addition, recent experimental studies have suggested the requirement of astrocytes for regeneration of CNS myelin [31–33].

On the basis of the studies reported here, it is tempting to speculate that the rapid astrocyte destruction itself contributes to HSV-induced demyelination. Neither the temporal sequence of astrocyte loss preceding demyelination nor the correspondence in areas of astrocyte destruction and demyelination, however, prove a cause and effect relation. It has been shown that HSV infection in oligodendroglia follows a longer time course and there is relative preservation of oligodendroglia at the time of extensive astrocyte loss and onset of destruction of myelin lamellae [1, 25], but we cannot exclude oligodendroglia damage as a cause of the demyelination. More detailed study of HSV-1 infection in oligodendroglia is needed to help determine the significance of the early astrocyte loss in this model.

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