CORONAVIRUS INFECTION AND DEMYELINATION

Development of Inflammatory Lesions in Lewis Rats

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1. ABSTRACT

Coronavirus infections of rodents can cause diseases of the central nervous system characterised by inflammatory demyelination. The lesions mimic in many aspects the pathology of multiple sclerosis in humans and of other neurological diseases. As an animal model for demyelination, we studied the MHV-JHM induced encephalomyelitis of Lewis rats. The pathomorphological analysis revealed patterns of lesions which developed in stages. Infected oligodendrocytes were first destroyed by necrosis. Later stages were characterized by demyelinated plaques. In the center of plaques, no virus antigen was found and oligodendrocytes were mainly destroyed by apoptosis. At the edge of plaques, virus antigen was expressed in parallel to infiltrations consisting of lymphocytes and macrophages. The prevailing mechanisms leading to demyelination may change individually and during defined stages of the disease. The transcriptional expression of chemoattractants and other mediators of inflammation was studied by semiquantitative RT-PCR. Virus induced inflammatory demyelination was accompanied by high expression of a relatively novel cytokine, the endothelial monocyte activating polypeptide II (EMAP II). By immunocytochemistry, EMAP II was detected in parenchymal microglia located both within the lesions and in unaffected areas. Furthermore, the level of transcriptional expression of the regulatory calcium binding S100 proteins MRP8, MRP14 and CP10 was associated with inflammatory demyelination and expression of IFN γ , IL-2, TNF α , and iNOS.

2. INTRODUCTION

Inflammatory demyelination is an important feature characteristic for a number of chronic central nervous system (CNS) diseases in both animal and human for which the etiology and/or pathogenesis is not yet clear. The most enigmatic example is multiple sclerosis (MS) which is driven by an immune pathological process leading to selective destruction of myelin. Similar processes are induced by virus infections such as Visna in sheep, canine distemper or Theilers virus in mice (Wege *et al.*, 1995; Fazakerley *et al.*, 1993; Kyuwa *et al.*, 1990). To analyse mechanisms of virus induced demyelination, the encephalomyelitis caused by murine coronaviruses in rodents has received much attention. Here we describe results of studies obtained by intracerebral infection of Lewis rats with MHV-JHM. The outcome is largely dependent on host factors and the virus variant employed for infection. Of major interest is the subacute demyelinating encephalomyelitis (SDE), which develops after intracerebral infection of Lewis-rats with MHV-JHM (Barac-Latas *et al.*, 1997; Flory *et al.*, 1995; Zimprich *et al.*, 1991; Wege *et al.*, 1984; Watanabe *et al.*, 1983). Clinical signs such as ataxic gait and paresis develop several weeks to months *p. i.*, animals may recover completely, or progress to a fatal outcome. The neuropathological hallmark of disease are lesions of primary demyelination in the white matter of the CNS. The pathogenic mechanisms leading to the histological changes are controversially discussed. In this context, in both autoimmune and virus induced experimental models the pathogenetic mechanisms driving inflammatory demyelination may be very similar. Increasing interest is focused to the role calcium binding S-100 proteins, chemoattractants and cytokines, which initiate and control inflammatory responses. As a first stage to analyse these parameters in more detail, we studied the transcriptional expression of some immune modulatory molecules which play an important role during inflammatory processes in the CNS.

3. MATERIALS AND METHODS

Specific pathogen free Lewis-rats between 3 to 8 weeks of age were intracerebrally inoculated with MHV-JHM. For histology, animals were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. Parts of brain and spinal cord were embedded in paraffin. To obtain specimens for RT-PCR, animals were dissected and left half of the brain frozen in liquid nitrogen before storage at -80 °C. The right half of the brain and spinal cord was further processed for histology. Combinations of histological stainings, immunocytochemistry and *in situ* hybridisation were employed as described previously (Barac-Latas *et al.*, 1997; Gold *et al.*, 1994; Breitschopf *et al.*, 1992; Zimprich *et al.*, 1991). Processing of tissue for RT-PCR was performed according to Stühler *et al.* (1997). Semiquantitative RT-PCR was evaluated by parallel processing of probes sampled from consecutive cycle numbers. The specific primer pairs were derived from gene banks and literature. The PCR was optimised for each primer pair and the identity of the amplification product was proven by Southern blot hybridisation.

4. RESULTS

The following analysis was obtained with CNS-tissue of rats, which displayed 3 to 5 weeks *p. i.* clinical signs and neuropathological changes of SDE. The specimens were selected out of a large number of rats characterised in detail during our previous studies (Barac-Latas *et al.*, 1997; Stühler *et al.*, 1997; Flory *et al.*, 1995; Zimprich *et al.*, 1991).

4.1. Pathomorphological Changes and Development of Lesions

By quantitative determination of virus antigen expressing cells, macrophages and T-lymphocytes three patterns of the demyelinating disease were differentiated (Barac-Latas et al., 1997). The first pattern was characterised by intensive virus antigen expression, although only mild inflammations were detected. Cells and myelin appeared normal, but the mRNA-expression of proteolipid-protein (PLP), a major constituent of myelin, was reduced. By immunocytochemistry, both PLP, MBP and other myelin specific marker proteins remained at the same level as in uninfected white matter.

The second pattern represents the typical, sharply demarcated demyelinating plaques. These areas were surrounded by virus infected tissue which appeared otherwise unchanged. Within the plaques, only a few cells displayed viral antigen, PLP mRNA expression was reduced. Severe inflammatory infiltrations consisting of lymphocytes and high numbers of macrophages indicated the active demyelination process. The relation between necrotic versus apoptotic cells was analysed by morphological criteria and displaying the degree of DNA fragmentation by in situ tailing methods. Many T-cells appeared to be eliminated by apoptosis, whereas oligodendrocytes were affected by both cell destruction pathways. Necrosis of oligodendrocytes were in general associated with virus infection. Within the plaques, remyelination was associated with an increased number of oligodendrocytes displaying enhanced expression of PLP mRNA, virus antigen was not longer detectable in these lesions.

In the third type of lesions, virus antigen expression was very rarely, despite of ongoing demyelination and inflammation mainly characterised by macrophages containing myelin degradation products and cells dying from apoptosis. These lesions were surrounded by astroglial scar tissue.

4.2. Transcriptional Expression of Immunomodulatory Molecules in CNS Tissue

The study is based on rats for which neuropathological changes had been studied in detail. In total, we analysed CNS tissue from 30 rats with SDE. Furthermore, six uninfected controls, rats with EAE, rats at early times before clinical signs developed and animals, which recovered from SDE were included. A representative collection of specimens from rats with SDE was then further analysed by semiquantitative RT-PCR. These results are summarized in Table 1.

As a background to evaluate the degree of inflammation in CNS-tissue we monitored for the transcriptional expression of the cytokines IFN γ , TNF- α , IL-2 and for the nitric oxid synthetases iNOS and cNOS. Previous studies have shown, that these immunomodulatory substances are associated with diseased CNS infiltrated by immune cells. All rats which were diseased with SDE displayed relatively to the uninfected controls significant to strong upregulation of IFN γ , TNF- α , IL-2 and of the inducible nitric oxid synthetase iNOS. The constitutive expressed cNOS was detected in all CNS-samples at similar levels. Our major interest was to identify major calcium binding S 100 molecules, in particular the myeloid related proteins MRP8 and MRP14, the chemotactic S 100 protein CP-10 and the cytokine endothelial-monocyte activating protein EMAP II. The expression of MRP8, MRP14 and CP-10 varied to some extent among CNS tissue among individual rats. Considering all specimens investigated and taking into account the histological results a strong correlation between their expression and the inflammatory response was observed.

Table 1. Transcriptional expression of cytokines and immunomodulatory substances in CNS tissue of Lewis rats demonstrated by semiquantitative RT-PCR

Case No.	Organ	CP-10	MRP8	MRP 14	EMAP II	IFN- γ	TNF- α	IL-2	iNOS	cNOS
1 contr.	spleen	+++	++++	++++	++	+	$\pm$	-	-	-
2 contr.	brain	+	+	$\pm$	++	-	-	+	-	+++
3 EAE ¹	brain	++	++++	++	++++	++++	++	++	+	+++
4 SDE	brain	+	+	+	++++	+++	+	++++	+	+++
5 SDE	brain	++	+	+	++++	++++	+++	+++	++++	+++
6 SDE	brain	+++	++++	-	++++	+++	++++	+++	+++	++++
7 SDE	brain	+++	++++	+	++++	+++	+++	+	++	++++
8 SDE	brain	+	$\pm$	$\pm$	++++	++	+	++	$\pm$	+++
9 SDE	brain	+	++	$\pm$	+++	+	+	++	$\pm$	+++
10 SDE	brain	++++	+++	++	++++	+++	++	+++	+	++++

¹EAE 11 days post sensitisation with MBP

Furthermore, the transcriptional expression of EMAP II was strong in most cases. Recently, a monoclonal antibody suitable for immunocytochemical stainings had been established and was employed to locate EMAP II protein in tissue sections (Schluesener *et al.*, 1997). The results are illustrated by Figure 1. In uninfected, healthy rats the expression of EMAP II is restricted to monocytes, macrophages and perivascular microglia. Parenchymal microglia of control rats is free of EMAP II by immunostaining. By contrast, parenchymal microglial cells of SDE lesions displayed EMAP II. The localisation of EMAP II expressing microglial cells is not limited to perivascular cuffs. EMAP II expressing microglial cells are only weakly positive for MHC class II antigens and not positive for ED1, which represents a marker of phagocytotic macrophages.

5. DISCUSSION

Destruction of myelin can involve a number of mechanisms (Fazakerley *et al.*, 1993). Demyelination can be due to a cytolytic virus infection, promoted by immune reactions against virus infected cells or involve virus induced autoimmunity. In the model of the experimental allergic encephalomyelitis (EAE) the disease is mainly triggered by CD4⁺ - T-cells specific for CNS-antigens. The extent of demyelination and the intensity of inflammation depends from the balance between specific antibodies and cellular immune mechanisms (Linington *et al.*, 1988). The pathomorphological changes observed in MS, EAE-models and virus induced demyelination are remarkably similar suggesting that identical mechanisms may operate in the evolution of demyelinating lesions (Barac-Latas *et al.*, 1997; Ozawa *et al.*, 1994). In vivo the mechanisms of oligodendrocyte damage appear to be related to the stage of the disease process. The virus infection during the early stage of SDE was cleared in many areas. It is conceivable, that the antiviral effect of certain cytokines and T-cell responses controls the spread of virus without destruction of infected glial cells (Stohlman *et al.*, 1995; Pearce *et al.*, 1994; Körner *et al.*, 1991). Before any tissue damage was visible, the synthesis of PLP was downregulated. Therefore, the virus infection of oligodendrocytes may impair their function of producing and maintaining myelin proteins. However, this mechanism alone does not explain the inflammation process. In general, oligodendrocyte destruction by T-cell mediated cytotoxicity mediators released from macrophages is thought to result in apoptosis. Necrosis is the dominant

Figure 1. Demyelinating lesion in a Lewis rat with SDE and expression of EMAP II. The expression of EMAP II is not limited to macrophages within the demyelinated lesions. The cytokine is also expressed on microglia cells within the myelinated areas. a) Demyelinated area in the white matter of the spinal cord. b) Consecutive section, EMAP II expression represented by dark dots adjacent to the weakly stained nuclei. EMAP II is also found in ramified cells within still myelinated areas (upper part of the figure). c) Demyelinated area in the hippocampal region. Small bundle of myelinated fibers adjacent to the demyelinated area on the left side of the figure. d) Consecutive section, in both the demyelinated and myelinated areas phagocytes and microglia express EMAP II. A small bundle of myelinated fibers. Bar = 100 μ m; a, c staining for myelin with luxol fast blue; b, d immunostaining with an anti-EMAP II mAb.

pattern of cell death in antibody—and complement mediated cytotoxicity. Many SDE-lesions displayed IgG and binding of complement C9. In these areas such complement fixing antibodies may promote demyelination (Piddlesen *et al.*, 1993; Zimprich *et al.*, 1991). In late stages virus antigen was detectable only in a few glial cells, in particular in astrocytes. However, destruction of myelin sheaths and oligodendrocytes continued. These lesions may involve a "bystander demyelination" maintained by macrophages. Individual patterns of demyelination may prevail at defined stages of the disease process. In brain tissue from MS patients, a similar variability in the pattern of demyelination and oligodendrocyte death has been observed (Ozawa *et al.*, 1994).

Chemokines and cytokines involved in chemoattraction play a pivotal role for the state of the blood-brain barrier, attraction of macrophages and the regulation of inflammation. As a first step to study this network, we employed RT-PCR to monitor the transcriptional expression of the calcium binding S 100 molecules such as the myeloid related proteins MRP8, MRP14, CP-10 and the cytokine EMAP II. Further studies by immunocytochemistry and *in situ* hybridisation are indicated in order to localise and identify specific cell types within the morphological context. At present, for the rat system suitable antibodies for immunocytochemistry of CP-10, MRP8 and MRP14 are not yet available.

CP-10 is a potent chemotactic factor for murine and human myeloid cells (Lackman *et al.*, 1993). *In vivo* CP-10 was described to locate at endothelial cells of the small blood vessels thus promoting the recruitment of monocytes and adhesion to the vessel walls. MRP8 and MRP14 are two calcium-binding proteins of the S-100 family which are expressed at distinct stages of monocytic differentiation (Imamichi *et al.*, 1993). Their biologically active form involve heterodimeric complexes which had been associated with some human inflammatory disorders. In adult mice, MRP8/14 proteins were identified primarily in immature myeloid cells of the bone marrow, in the splenic red pulp and marginal zone. These proteins are highly expressed in recruited neutrophils and monocytes. Important chronic inflammatory reactions had been associated with an altered MRP expression (Bhardwaj *et al.*, 1992). These proteins may serve as useful parameters to characterise the local activity of inflammations in the CNS.

The endothelial-monocyte activating polypeptide II represents a relatively novel and potent cytokine, which had been localised previously in monocytes and macrophages (Kao *et al.*, 1994). Most pronounced expression was described for perivascular microglia, but it is normally not detectable in parenchymal microglia. In experimentally induced autoimmune diseases of rats, recently a widespread activation of EMAP II within the CNS was reported (Schluesener *et al.*, 1997). These data indicate, that this cytokine may be a regulator of microglial cell reactivity in autoimmune inflammation of the CNS. Our data show, that EMAP II is also associated with the development of virus induced demyelinating lesions. Microglial cells are of increasing interest as regulatory elements of immunosurveillance in the CNS. It remains to be established whether EMAP II expression is an early activation marker for rat parenchymal microglia. The role of chemokines and cytokines in virus induced or immune mediated demyelination remains an interesting topic for further research.

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