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Activation of microglia cells is dispensable for the induction of rat retroviral spongiform encephalopathy

Regine Hansen,[1] Christian Sauder,[2] Stefanie Czub,[3] Eva Bachmann,[3] Simone Schimmer,[1] Annette Hegyi, and Markus Czub[1]

[1]Institut für Virologie und Immunbiologie, Universität Würzburg, Germany; [2]Abteilung Virologie, Institut für Medizinische Mikrobiologie und Hygiene der Albert-Ludwigs-Universität Freiburg, Germany; and [3]Pathologisches Institut, Universität Würzburg, Germany

In the course of retroviral CNS infections, microglia activation has been observed frequently, and it has been hypothesized that activated microglia produce and secrete neurotoxic products like proinflammatory cytokines, by this promoting brain damage. We challenged this hypothesis in a rat model for neurodegeneration. In a kinetic study, we found that microglia cells of rats neonatally inoculated with neurovirulent murine leukemia virus (MuLV) NT40 became infected *in vivo* to maximal levels within 9–13 days postinoculation (d.p.i.). Beginning from 13 d.p.i., degenerative alterations, i.e., vacuolization of neurons and neuropil were found in cerebellar and other brain-stem nuclei. Elevated numbers of activated microglia cells—as revealed by immunohistochemical staining with monoclonal antibody ED1—were first detected at 19 d.p.i. and were always locally associated with degenerated areas but not with nonaltered, yet infected, brain regions. Both neuropathological changes and activated microglia cells increased in intensity and numbers, respectively, with ongoing infection but did not spread to other than initially affected brain regions. By ribonuclease protection assays, we were unable to detect differences in the expression levels of tumor-necrosis-factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) in microglia cells nor in total brains from infected *versus* uninfected rats. Our results suggest that the activation of microglia in the course of MuLV neurodegeneration is rather a reaction to, and not the cause of, neuronal damage. Furthermore, overt expression of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 within the CNS is not required for the induction of retroviral associated neurodegeneration in rats. *Journal of NeuroVirology* (2001) **7,** 501–510.

Keywords: spongiform encephalopathy; microglia; activation; retrovirus; neurodegeneration; cytokines

Introduction

Microglia cells are ubiquitous within the central nervous system (CNS) parenchyma and comprise 5 to 20% of all glia cells (Lawson *et al*, 1991). Although the function of resting microglia is still unknown, microglia cells transform rapidly from a resting to an activated state under patho-physiological conditions. This activation-associated transformation, is characterized by overt morphological and immunophenotypical changes (reviewed by Streit 1999). Activated microglia cells are the central cellular element in the brain to initiate defense mechanisms against exogenous and endogenous noxae and to facilitate regenerative processes (Lotan and Schwartz, 1994; Rabchevsky and Streit, 1997). However, activated microglia cells are also able to damage neighboring cells, especially neurons, by secreting potentially cytotoxic molecules including

Address correspondence to Markus Czub, Canadian Science Centre for Human and Animal Health, Pathogenesis of biosafety level 4 agents, 1015 Arlington Street, Winnipeg, MB, R3E 3M4, Canada. E-mail: markus_czub@hc-sc.gc.ca

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free radicals, nitrogen oxides (Boje and Arora, 1992; Chao *et al*, 1992; Merrill *et al*, 1993), proteases, arachidonic acid, platelet-activating factor, quinolinate, cysteine, proinflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor-necrosis-factor-α (TNF-α). Microglia-derived neurotoxicity was demonstrated in a variety of experimental and natural encephalopathies (Benveniste, 1997; Oleszak *et al*, 1997; Zujovic *et al*, 2000).

Microglia cells are the major target cells for human (Dickson *et al*, 1991; Brinkmann *et al*, 1992; Dickson *et al*, 1994) and simian immunodeficiency virus (HIV, SIV) (Brinkmann *et al*, 1993; Czub *et al*, 1996) in the brain. However, the mechanistic link between retroviral CNS infection and neurological deterioration, like the AIDS dementia complex, is unclear. A widely regarded hypothesis is that microglial activation plays a significant role in mediating retrovirus-induced disease. This hypothesis is mainly based on *in vitro* studies showing increased levels of proinflammatory cytokines released from microglia cell cultures (Sopper *et al*, 1996), probably induced by retroviral gene products (Dawson *et al*, 1993; Toggas *et al*, 1994; Kong *et al*, 1996). Recent investigations on brain material from HIV-infected patients demonstrated increased levels of TNF-α gene products in the CNS of patients with HIV dementia in comparison to HIV-infected patients without neurological impairment (Tyor *et al*, 1992; Seilhean *et al*, 1997; Wesselingh *et al*, 1997). This suggests that TNF-α may participate in the development of neurodegenerative disease. However, formal proof that activated microglia cells really cause the development of neurodegenerative lesions and/or clinical signs in patients with HIV dementia or other neurodegenerative diseases such as Alzheimer's Disease or multiple sclerosis, is lacking.

Microglia cells are the major target cells for neurovirulent murine leukemia viruses (MuLV) in the brain (Baszler and Zachary, 1990; Lynch *et al*, 1991; Czub *et al*, 1995). The infection of susceptible mice and rats with neurovirulent MuLV serves as a simple and well-defined animal model for analyzing the molecular causes leading to the development of retroviral induced neurodegeneration *in vivo* (reviewed in Portis and Lynch, 1998). MuLV-NT40 belongs to a group of neurovirulent ecotropic C-type leukemia viruses that cause a progressive, noninflammatory spongiform encephalopathy predominantly of brain-stem areas when inoculated into susceptible neonatal rats. The disease is not accompanied by immunopathological phenomenons and proceeds regularly with regard to the incidence of neurological disease, the incubation period and neurological signs. Neurological signs are manifested as ataxia and paresis, which progress to hindlimb paralyses accompanied by the atrophy of the skeleton muscles (Czub *et al*, 1995). The aim of our investigation on retroviral-induced neurodegeneration was to elucidate whether microglial activation and the release of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) were involved in the induction of retrovirus-associated neurodegeneration.

Results

The role of microglia activation in the pathogenesis of MuLV-NT40-induced neurodegeneration was investigated by a kinetic analysis on the chronological course of retroviral CNS infection, microglia activation, histopathological CNS lesions, and of clinical signs.

Dynamics of viral envelope protein expression in the CNS By means of viral envelope-specific immunohistochemistry (IHC), immunopositive cells were first detected at 8 days postinfection (p.i.) in the CNS. Immunopositive cells had an elongated nucleus and were associated with blood vessels, indicating infection of endothelial cells. At later stages, ramified (beginning at 11 days p.i.) and activated (beginning at 19 days p.i.) microglia cells as defined by their morphology (Figure 2A–C, G–H) and by double-labeling (Figure 2M) were also found to be infected. Immunopositive cells increased in number between day 9 p.i. and day 19 p.i., reaching maximum levels at 19 days p.i. (379 cells/0.8 mm^2, $n=2$). The number of immunopositive cells persisted at this level until the last day of quantification was performed (36 day p.i.: 356 cells/0.8 mm^2, $n=2$) (Figure 1).

Time course of lesion development Neuropathology induced by MuLV-NT40 is characterized by extensive vacuolar degeneration within the neuropil and in neurons, predominantly of brain-stem areas (Czub *et al*, 1995) including the deep cerebellar nuclei, the vestibulares nuclei, the cochleares nuclei, the reticular formation of the mesencephalon and metencephalon, and parts of the corpus callosum. There were some notable regions that were spared by degenerative changes. These included the cerebellar cortex, the hippocampus, the olfactory bulb, and the cortex parietalis and perirhinalis of the cerebrum. Inflammatory changes were never detected.

Clear evidence of spongiform degeneration was first noticed in one of five rats at 13 days p.i. Even at this early timepoint, evidence of spongiform degeneration was observed in virtually all brain regions that were also affected in advanced stages of disease. In general, the vacuoles were heterogenous in dimension and varied from round to ovoid. Often, vacuoles were divided by thin membranes (Figure 2). In brain regions where spongiform lesions had occurred early, the intensity of spongiform lesions (see Materials and methods) increased in parallel with time. Maximal intensity of brain lesions was reached at 25 days p.i. (Figure 1).

Microglia activation Activated microglia cells as assessed by ED1 immunostaining were rarely found in noninfected control rats at any time point (data

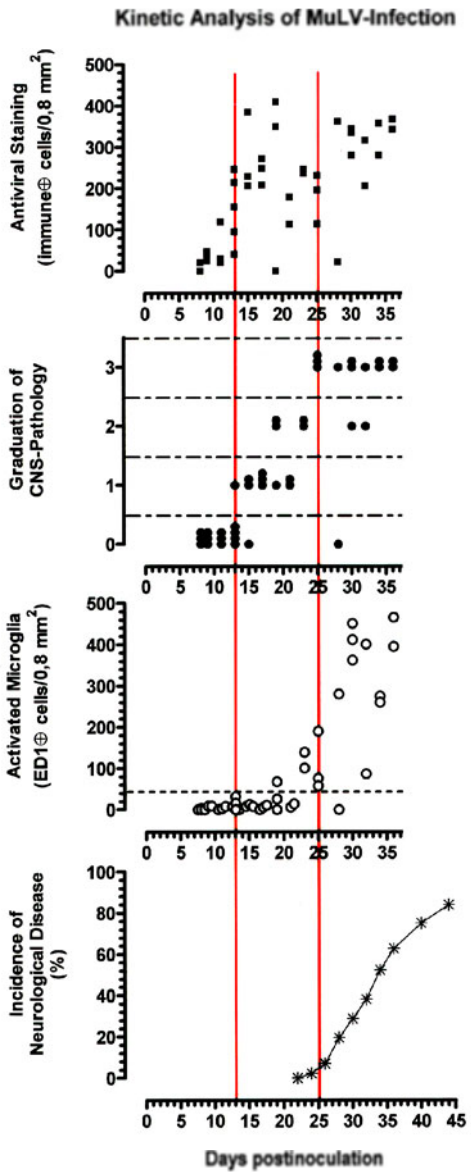

Figure 1 Kinetic analysis of MuLV-NT40 infection. Retroviral CNS infection (upper panel), induction of histopathological lesions (second upper panel), activation of microglia cells (second lower panel), and inauguration of neurological signs (lower panel), were analyzed on individual rats in a time-course manner. Important time points in the pathogenesis of MuLV-induced neurodegeneration are represented by vertical lines in red: at 13 days p.i. (left red line), we noticed a widespread expression of retroviral gene products in defined brain regions, such as deep nuclei cerebellares, nuclei cochleares, nuclei vestibulares, nucleus facialis, and cortex cerebri (upper panel). At this time point, we observed histopathological lesions in only one of five animals (second upper panel) and no increased numbers of activated microglia cells (second lower panel; horizontal dotted line indicates cutoff between normal and elevated numbers of acivated microglia cells). Also, clinical signs were not observed (lower panel). Histopathological lesions reached maximum levels at 25 days p.i. (right red line), which was accompanied by few activated microglia cells. At this time point, we recognized neurological signs in some MuLV-NT40-infected rats. Until the end of the observation period (36 days p.i.), numbers of activated microglia cells and the extent of neurological signs increased continously. Each symbol represents data from an individual animal.

not shown). In brains of MuLV-NT40-infected rats, we also noticed a few ED-1-positive cells between day 8 and 17 p.i., however, there was no difference in the numbers of activated microglia cells in noninfected *versus* infected rat brains up to 18 days p.i. Beginning at day 19 p.i., we found a progressive increase of activated microglia cells until the last time point analysed (Figure 1, Figure 2D–F, I–K).

No activation of microglia cells in the cortex cerebri Microglia activation seemed to be an event rather in response to the histopathological brain lesions but not a direct consequence of retroviral infection. To further elucidate this point, we examined the parietal and perirhinal cortex cerebri, i.e., brain regions that are spared from spongiform lesions, but not from retroviral infection of endothelial and microglia cells (Figure 2H). Activation of microglia cells was not observed in these areas (Figure 2K). Thus, to activate microglia cells retroviral infection alone was not sufficient but needed to be accompanied by obvious brain damage.

Clinical signs and incidence of neurological disease The incidence of neurological disease was determined in 64 rats for a time period between 22 and 45 days p.i. First clinical signs were reflex abnormalities of the hind- and forelimbs, followed by ataxia and undersized growth. Neurological signs were first observed day 24 p.i., at this time point the incidence was 2.3%, and continously increasing to 83.9% until the last point of investigation (45 day p.i.) (Figure 1).

Expression of proinflammatory cytokines in microglia cells One possible mechanism for the induction of retroviral-induced neurodegeneration is a release of potentially neurotoxic substances like proinflammatory cytokines IL-1β, IL-6, and TNF-α from activated microglia (Sopper *et al*, 1996). These cytokines may initiate and/or promote the development of neurodegenerative lesions. Therefore, we investigated whether the proinflammatory cytokines IL-1β, IL-6, and TNF-α were expressed in the course of MuLV-NT40-induced neurodegeneration. This was accomplished by examining the expression of mRNA for these proinflammatory cytokines in microglia cells isolated *ex vivo* (MG_{exvi}) at three different time points. Day 14 p.i. was selected because retroviral infection had spread to defined brain regions, however without microglia activation. The second time point (day 25 p.i.—data not shown) was chosen because retroviral infection persisted on maximum levels and was accompanied by some activated microglia cells. Finally, we analyzed the expression of proinflammatory cytokines at 74 days p.i.

Basal expression of the proinflammatory cytokine mRNAs IL-1β, IL-6, and TNF-α was detected in microglia cells isolated from all animals examined (Figure 3). Expression levels of cytokine mRNA from MuLV-NT40-infected rats were similar in comparison to those of noninfected control rats. Upregulation of cytokine gene expression was never

observed, at any time point. Quantitative comparison of mRNA from LPS-stimulated microglia cells to mRNA from nonstimulated microglia cells showed an 150-fold increase of IL-1β, IL-6, and TNF-α mRNA (Figure 3). This result indicates that microglia had not lost their functions with respect to their capability to secrete proinflammatory cytokines. From our results, we conclude that activated microglia cells do not release enhanced levels of proinflammatory cytokines, under the given circumstances. Moreover, MuLV-NT40 infection alone is not sufficient to induce cytokine gene expression in microglia cells, at the time points tested.

In addition to microglia cells, astrocytes, and other brain cells are also potential candidates for releasing cytokines in the brain (Schöbitz *et al*, 1994).

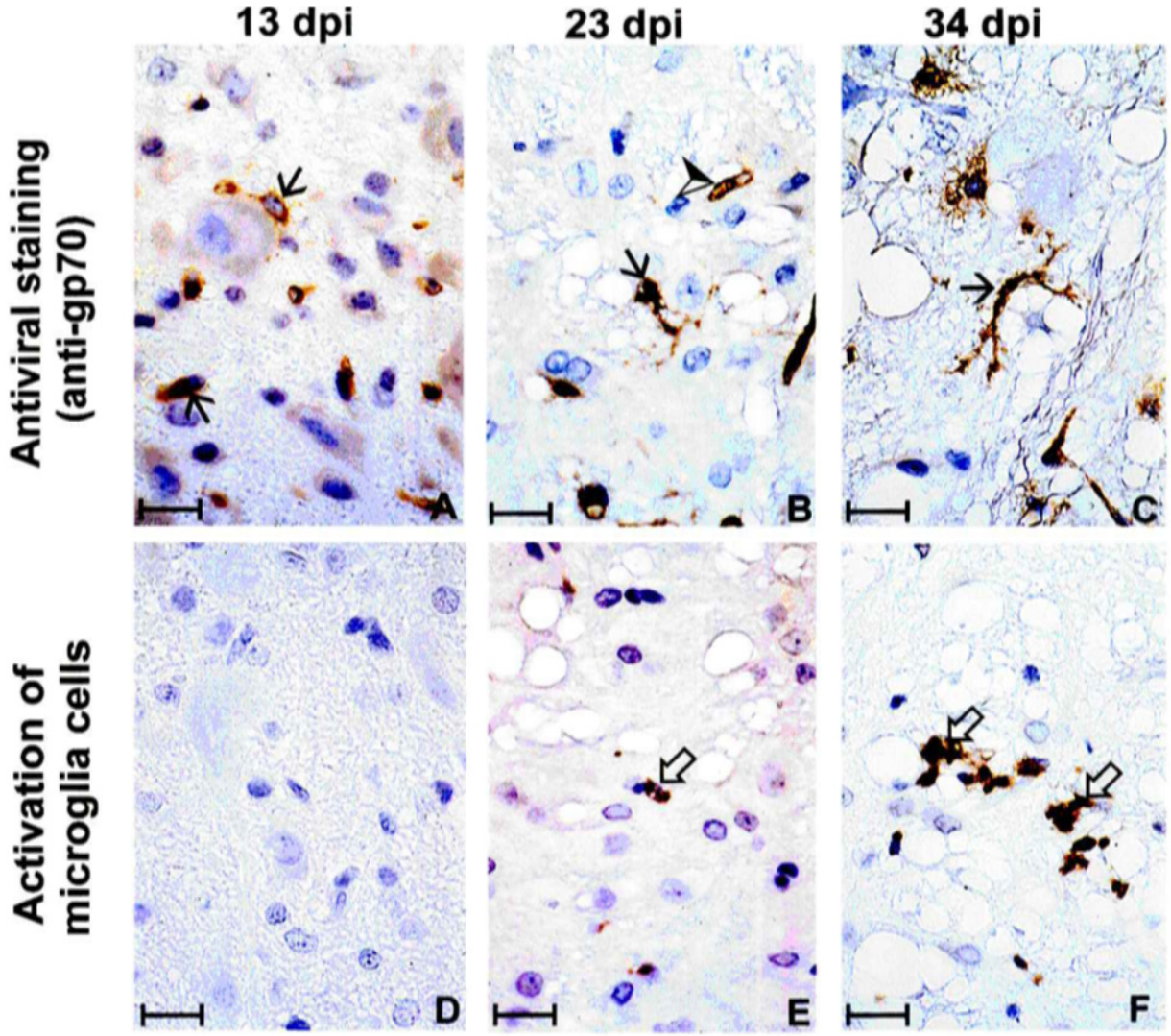

Figure 2 Immunohistochemical analyses of rat brains. Rat brain tissues were analyzed by immunohistochemistry at various time points after MuLV-NT40 inoculation. Antiviral staining was performed with a polyclonal goat antiserum, which recognizes the viral envelope protein (Figure 2: A–C, G, H, bar = 17 μm), and with monoclonal antibody ED1 (Dijkstra *et al*, 1985) to visualize activated microglia cells (D–F, I, bar = 17 μm; K, bar = 26 μm). The photomicrographs of each timepoint represent brain sections of one respective rat. Immunopositive cells were identified by their brown color. In the deep cerebellar nuclei, we found similar expression levels of retroviral gene products in a time interval ranging from 13 to 67 days p.i. (A–C, G). Microglia cells were the main target cells for MuLV infection (→), but infection of endothelial cells was also observed (➢). Maximal levels of retroviral infection were detected beginning from 13 days p.i. (A). However, activated microglia cells could not be observed (D). Activated microglia cells (⇨) were rarely found at 23 days p.i., a time point when spongiform lesions were clearly established (E). At later timepoints, numbers of activated microglia cells had increased (F, I), thus following the extent of spongiform lesions (D–F, I). Retroviral infection alone was not sufficient to induce activation of microglia cells as shown by comparison of the deep cerebellar nuclei (G, I) and the cerebral cortex (H, K). In both the deep cerebellar nuclei (G) and in the cerebral cortex (H), similar numbers of microglia cells were infected. Whereas in the deep cerebellar nuclei extensive spongiform lesions were accompanied by numerous activated microglia cells (I), the cortex cerebri failed to exhibit spongiform lesions and activated microglia cells (K). A negative control for antiviral staining is shown in L (bar = 26 μm). Double staining with antiviral antibody gp70 (light brown) and the monoclonal antibody ED-1 (dark brown) clearly showed that microglia cells are the main infected target cell in the brain (M). (*Continued*)

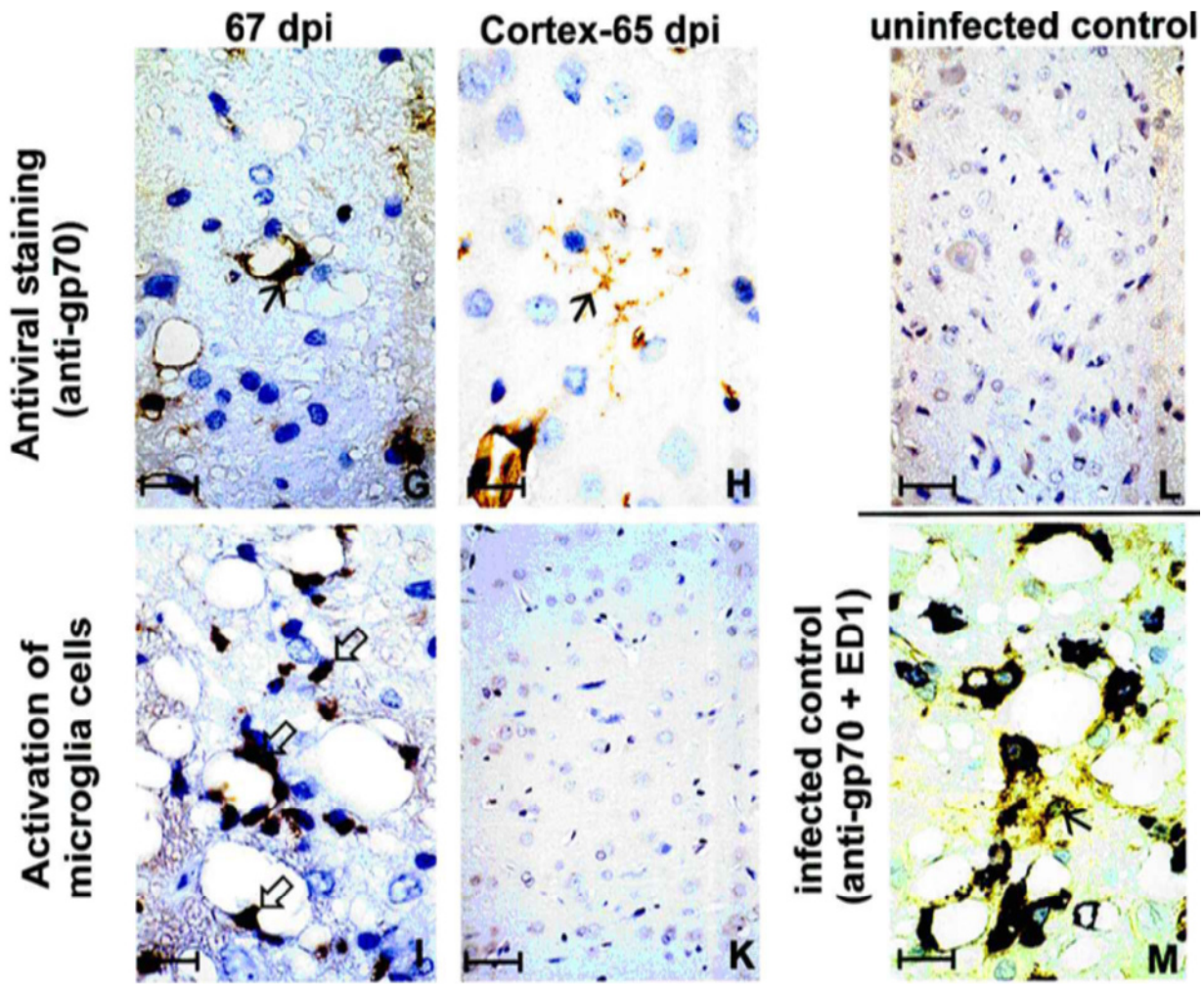

Figure 2 (Continued).

Astrocytes or other brain cells may release proinflammatory cytokines, consequently leading to an activation of microglia cells (Choe *et al*, 1998). Therefore, we performed RNase protection assays using whole brain mRNA from MuLV-NT40-infected rats in comparison to noninfected control rats. The same time points as before were investigated. Expression levels of the proinflammatory cytokine mRNAs IL-1β, IL-6, and TNF-α in brain material was much lower than in MG_{exvi}. Again, we found no differences in cytokine expression between MuLV-NT40-infected rats and noninfected controls, at days 14 and 25 p.i. (data not shown). From these results, we conclude that proinflammatory cytokines such as IL-1β, IL-6, and TNF-α are not initially involved in MuLV-NT40-associated neurodegeneration.

Discussion

In this study, we show that spongiform encephalopathy induced by MuLV-NT40 was followed by activation of microglia cells and not vice versa. Our results clearly demonstrate that microglia activation was not responsible for the induction of brain lesions. Furthermore, microglia activation was not accompanied by increased levels of the proinflammatory cytokines IL-1β, IL-6, and TNF-α indicating that these cytokines are dispensable for the induction of MuLV-associated neurodegeneration.

Activation of microglia cells is frequently observed in many neurodegenerative as well as inflammatory diseases such as Alzheimer's disease (Eikelenboom and Veerhuis, 1996), multiple sclerosis (Gonzalez-Scarano and Baltuch, 1999), experimental allergic encephalomyelitis (EAE) (Benveniste, 1997), prion diseases (Giese *et al*, 1998), and retroviral encephalopathies induced by HIV (Weis *et al*, 1994), SIV (Czub *et al*, 2000), and neurovirulent MuLV (Gravel *et al*, 1993; Robertson *et al*, 1997; Choe *et al*, 1998). At present, it is not clear how microglia cells are implicated in the pathogenic process. However, a number of *in vitro* studies have shown that microglia cells activated by different stimuli are directly or

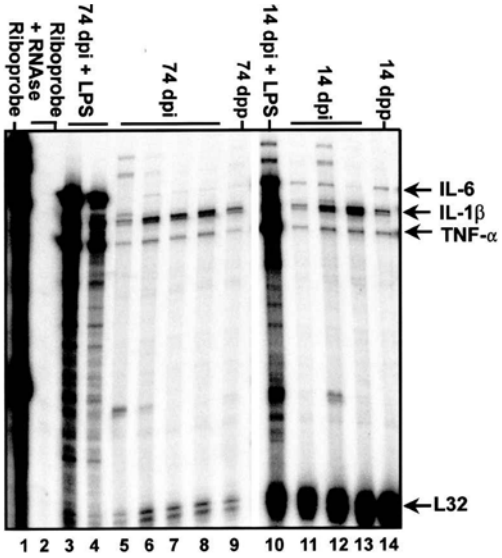

Figure 3 Expression of proinflammatory cytokines in microglia cells isolated *ex vivo*. We investigated the expression of the proinflammatory cytokines IL-1β, IL-6, and TNF-α in microglia cells isolated *ex vivo* from rats neonatally infected with MuLV-NT40 days pi (dpi) or non-infected rats days postpartum (dpp), by means of RPA. Each lane represents data from an individual animal. Three different time points were chosen for our analysis (days 14, 74 pi/pp, and day 25 pi/pp—data not shown). In all samples from microglia cells isolated *ex vivo* (MG_{exvi}) basal expression of mRNA for the proinflammatory cytokines IL-1β, IL-6, and TNF-α was detected (lanes 5–9, 11–14). An upregulation of cytokine mRNA expression in MuLV-NT40 infected rats (lanes 5–8, 11–13) versus noninfected control rats (lanes 9, 14) was not observed. Quantitative evaluation (on a Molecular Dynamics Phosphorimager using ImageQuant software) of levels of LPS-stimulated MG_{exvi} mRNA compared to levels of non-stimulated MG_{exvi} mRNA revealed an 150-fold increase of IL-1β, IL-6, and TNF-α mRNA (lane 4, 10).

indirectly neurotoxic to cultured neurons. Mechanistically, nitric oxide, glutamate, cytokines, and nondefined microglia-derived neurotoxins, respectively, might be responsible for neuronal cell death (Boje and Arora, 1992; Chao *et al*, 1992; Piani *et al*, 1992). Such *in vitro* studies are helpful to analyze phenotypical characteristics and possible functions of microglia cells, but it is quite clear that these studies cannot really reflect the *in vivo* situation.

Therefore, we performed a kinetic study on Fisher rats neonatally infected with neurovirulent MuLV-NT40. We simultaneously analyzed four parameters per individuum, i.e., retroviral CNS infection, induction of histopathological lesions, activation of microglia cells, and inauguration of neurological signs, in a time-course manner. Activation of microglia cells was determined by staining with antibody ED1, which is a highly sensitive marker for microglia activation (Dijkstra *et al*, 1985). It is possible though, that different forms of activated microglia exist and that not all of these different forms would be equally well detected by ED1 immunostaining. Increased numbers of activated microglia cells in rats neonatally infected with MuLV were only found in brain regions where neurodegenerative lesions had already occurred, but increased numbers of activated microglia cells were never detected before morphological damage had been established. Furthermore, we did neither observe increased numbers of activated microglia cells in cortical areas where microglia infection but no histopathological alterations occur. These results clearly indicate that microglia activation was rather a consequence than the cause of retroviral-induced neurodegeneration.

Brain damage and neurological impairment in AIDS encephalopathy induced by the retroviruses HIV or SIV is thought to result at least partially from proinflammatory cytokines released by infected, activated microglia cells (Dickson *et al*, 1993). This hypothesis is supported by studies showing increased expression of proinflammatory cytokines such as IL-1, IL-6, and TNF-α in the cerebrospinal fluid (Grimaldi *et al*, 1991; Perrella *et al*, 1992; Laverda *et al*, 1994) as well as in brain autopsy material of patients infected with HIV (Tyor *et al*, 1993; An *et al*, 1996; Seilhean *et al*, 1997; Wesselingh *et al*, 1997). In contrast to these studies, we were unable to detect enhanced levels of IL-1β, IL-6, and TNF-α in MuLV-NT40-infected rats, neither in microglia cells isolated *ex vivo* nor in total brain material. Thus, we demonstrate both that the proinflammatory cytokines IL-1β, IL-6, and TNF-α were dispensable for the induction and sustenance of MuLV-associated neurodegeneration and that activated microglia cells do not necessarily express the proinflammatory cytokines IL-1β, IL-6, and TNF-α. The reason for the difference between cytokine involvement of MuLV and lentiviral induced encephalopathy might originate from the fact that lentiviral CNS infections are often accompanied by inflammatory changes. CNS-invading leukocytes might be a direct or an indirect source of proinflammatory cytokines.

Our results clearly contrast observations obtained on brains from mice infected with other neurovirulent MuLV (Nagra *et al*, 1994; Choe *et al*, 1998). Both TNF-α and Fas were detected in astrocytes and in some microglial cells (Choe *et al*, 1998). The study suggested that MuLV-induced neural cell death was likely due to Fas- and TNF-α-mediated cell death mechanisms (Choe *et al*, 1998). Similarly, Nagra *et al* (1994) found enhanced levels of TNF-α, IL-6, and quinolic acid in MuLV-degenerated mouse brains. Although virological, immunological, as well as histopathological data point to a uniform pathomechanism of MuLV neurodegeneration in both mice (Czub *et al*, 1991; Lynch *et al*, 1991) and rats (Kai and Furuta, 1984; Czub *et al*, 1995), differences in viral and/or host genetics could have accounted for the contrasting cytokine expression patterns observed in Choe's and Nagra's as compared to our study. Alternatively, technical aspects could be considered to explain the conflicting results: our study demonstrates basal expression of proinflammatory cytokines in microglia cells and brains of both infected and noninfected rats. However, there

might have been regional, probably minor, differences in the expression levels of proinflammatory cytokines as suggested by the other studies (Choe *et al*, 1998).

Although activated rat microglia cells were not involved in the initial formation of neurodegenerative MuLV alterations, it is striking that clinical, i.e., neurologic disease emerged simultanously with the activation of microglia cells. This observation raises the possibility that the clinical outcome of neuropathological diseases might be strongly influenced by the number of activated microglia cells. In consistency with this hypothesis are studies on the AIDS-encephalopathy, demonstrating a positive correlation between the number of activated microglia cells and the severity of neurological disease (Glass *et al*, 1995; Berman *et al*, 1999). Indirect support for the significance of activated microglia cells for the severity of neurological disease comes from observations on other murine MuLV models in which spongiosis was certainly induced but neither activation of microglia cells nor clinical disease (Askovic *et al*, 2000). However, in the fastest model for MuLV-induced neurodegeneration, i.e., infection of mice with FrCasE (Portis *et al*, 1990), there is neither microglia activation, in spite of severe neurological signs (Lynch *et al*, 1995). From this, it appears unlikely that microglia activation contributes to formation of clinical signs in MuLV encephalopathy.

Thus, in the course of MuLV-induced neurodegeneration, different stages of activation of microglia cells appear to be associated with various biological effects: during the early phase, morphological CNS damage is initiated. For the induction of this CNS alteration, retroviral infection of microglia cells was previously shown to be essential and sufficient (Lynch *et al*, 1995). Whether gain or rather loss of microglia functions brings on morphological damage remains to be elucidated. However, our results stand in favor of deprived microglial competence, at the beginning of MuLV-induced neurodegeneration, but not of microglial hyperfunction. In the second phase of MuLV neurodegeneration, microgliosis turns up, probably as a reaction to severe morphological damage or to other signals. Although being activated, microglia cells did not secrete proinflammatory cytokines. This makes us believe that rather regenerative than detrimental functions were executed at this time, e.g., phagocytosis of dead cells. Finally, neurological disease turns up, in parallel with still activated microglia cells. However, additional signs of microglial hyperfunctions are still missing at this time point, as we did not see elevated levels of proinflammatory cytokines, although release of other microglia-derived toxins cannot be excluded. It is also possible, at this final point of the disease, that the microglia cells are deadly exhausted, with the consequence of lacking protective and regenerative support to sustain normal brain functions. According to this concept, neurological disease arises upon a variety of microglial reactions, which are in no way stereotypic but vain to combat the effects of exogenous insult.

Material and methods

Virus MuLV-NT40 was isolated after serial passage of a molecular clone of Friend-MuLV, FB29 through Fisher rats (Czub *et al*, 1995). Viral stocks were prepared from confluently infected Fisher rat embryo cells. The titer of viral stock used varied between 1.0×10^5 and 5×10^5 FFU/ml. Viral titrations were performed using a focal immunoassay (Czub *et al*, 1995).

Experimental design Inbred F344-Rats were infected intraperitoneally at 24–48 h postnatally with MuLV-NT40 and were observed for signs of clinical neurologic disease (Czub *et al*, 1995) and/or were sacrificed at various times after inoculation for further analyses. A kinetic analysis (time interval ranging from 8 to 36 days after inoculation) was performed by means of histopathological and immunohistochemical examination on 41 brains of rats neonatally inoculated with MuLV-NT40.

Histopathological and immunohistochemical (IHC) analyses We investigated brains of rats neonatally inoculated with MuLV-NT40 at a time interval ranging from 8 to 36 days p.i., with a series of four coronal sections per animal. Rats were killed by CO_2 inhalation and perfused through the left ventricle with PBS until the effluent ran clear. Subsequently, a perfusion with 4% formaldehyde in PBS was performed. Brains were removed and the tissues were fixed for 48 h in 4% formaldehyde in PBS and embedded in paraffin. Sections (1 μm) were stained with hematoxylin and eosin. Vacuolization in specified areas (see Results) were evaluated at a magnification 200× using an ocular grid (per area: five fields/individuum). Results were recorded as percent vacuolated of total area and categorized into four groups: none (0), mild (1), moderate (2), and severe (3). Sections without vacuolization constituted the first group. Mild lesions consisted of few and small vacuoles. Moderate lesions consisted of occasional vacuoles, occupying 20% to 40% of an 200× visual field. Severe CNS lesions were characterized by extensive vacuolization, occupying more than 40% of the visual field.

For IHC, 1 μm thick sections from paraffin-embedded tissues were deparaffinated, rehydrated, and permeabilized in 0.5% Triton-X-100/phosphate-buffered-saline (PBS) with 1 mg/ml Proteinase K at 37°C for 5 min. Endogenous peroxidase activity was suppressed by incubation with 0.3% H_2O_2 in methanol for 2 min. After washing, slides were blocked in PBS with 10% normal serum (Merck Tissue Gnost Uni-Pak Kit) for 15 min. A goat polyclonal anti-MuLV-gp70 serum provided by Prof. R. Friedrich, Institute of Virology, Gießen, was used

in a dilution 1:2000. Activated microglia cells were stained by monoclonal antibody ED1 (Dijkstra *et al*, 1985) in a dilution of 1:100 (Serotec). Slides were washed twice and then incubated with a biotinylated secondary antibody. Bound antibodies were revealed with an avidin-biotin-peroxidase kit (Bio-Genex-Super Sensitive Immunodetection) at 37°C for 30 min and diaminobenzidine as a substrate. Sections were counterstained with hematoxylin.

Infected CNS cells and activated microglia cells, respectively, were quantified by counting immunopositive cells in five visual fields of investigated brain regions (deep cerebellares nuclei, nuclei cochleares, nuclei vestibulares, nucleus facialis, cortex cerebrum) using an ocular grid with an total area of 0.2 mm^2.

Isolation of microglia cells Microglia cell isolation was performed as described in detail before (Hansen *et al*, 2000). Briefly, brains were removed from perfused animals, stripped of meninges, enzymatically digested, and subjected to a density gradient. Microglia cells were collected from the 1.077/1.066 g/cm^3 interface and analyzed further.

Detection and quantitation of cytokine mRNAs by ribonuclease protection assay (RPA) mRNA was isolated from total brains and from microglia isolated *ex vivo*, respectively employing Oligo$(dT)_{25}$ Dynabeads from Dynal following the recommendations of the manufacturer. Cytokine mRNAs for IL-1β, IL-6, and TNF-α were measured by RPA as described in detail before (Sauder and de la Torre, 1999). Bands corresponding to protected cytokine mRNAs were quantitated on a Molecular Dynamics Phosphorimager using ImageQuant software (Becton Dickinson) and were normalized to the amounts of L32, a ribosomal house keeping gene, in each lane. At each time point, mRNAs from microglia cells from individual infected and noninfected rats were analysed. Microglia cells isolated *ex vivo* from rats neonatally infected with MuLV-NT40 were also stimulated *in vitro* with LPS (100 ng/10^5 cells, 5 h), and isolated mRNA was used subsequently as a positive control.

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