Cervical lymphoid tissue but not the central nervous system supports proliferation of virus-specific T lymphocytes during coronavirus-induced encephalitis in rats

H. Imrich [a,*], S. Schwender [b], A. Hein [a], R. Dörries [a]

[a] *Institut für Virologie und Immunbiologie der Universität Würzburg, Versbacherstr. 7, D-97078 Würzburg, Germany*
[b] *Zentrallabor der Medizinischen Klinik der Universität Würzburg, Würzburg, Germany*

Abstract

The CD4^{+} T lymphocyte response in the central nervous system (CNS) and cervical lymph nodes (CLNs) of rats with different susceptibility to coronavirus-induced encephalitis was investigated. The majority of CD4^{+} T lymphocytes entering the virus-infected CNS in the course of the infection are primed cells that neither proliferate ex vivo nor can be stimulated to proliferation by viral antigens or mitogen in vitro. In contrast, T lymphocytes taken from CLNs of the same animals revealed a strong proliferative response. Restimulation of CLN lymphocytes by viral antigens disclosed a striking difference between the disease-resistant rat strain Brown Norway (BN) and the susceptible Lewis (LEW) strain. Whereas BN lymphocytes responded as early as 5 days post infection, it took more than 11 days until a comparable proliferation was detectable in LEW lymphocytes. From these data we postulate that the majority of T lymphocytes entering the virus-infected brain after sensitisation and expansion in cervical lymph nodes is unresponsive to further proliferation signals and that the kinetics and magnitude of T lymphocyte stimulation in CLNs play an important role in the clinical course of the infection.

Key words: Viral encephalitis; T cells; Proliferation; Central nervous system; Cervical lymph nodes

1. Introduction

The unaffected central nervous system (CNS) is an immunologically sequestered organ. This is evidenced by low expression of MHC antigens on very few meningeal and perivascular cells, the lack of immunostimulatory dendritic cells, absence of draining lymphatic vessels and a tight blood–brain barrier that prevents extensive lymphoid cell traffic (Wekerle et al., 1986; Sedgwick and Dörries, 1991). The immunologically privileged state is rapidly changed when the CNS becomes a target for viral infections. Inflammatory infiltrates in the CNS tissue clearly indicate the engagement of the immune system, but to date neither the afferent nor the efferent arm of a CNS-localised virus-specific immune response are completely understood. In particular, the upregulation of MHC class II

antigens in the inflamed virus-infected CNS (Sedgwick and Dörries, 1991; Sedgwick et al., 1991) raises many questions with respect to infiltrating CD4^{+} T lymphocytes. It has been shown in vitro that expression of MHC class II antigens can be induced by γ-interferon on CNS-resident glia cells (Wong et al., 1985). Microglia cells have been identified as the major cell type expressing class II antigens in the CNS (Matsumoto et al., 1986; Hayes et al., 1987) and perivascular microglia cells can function as antigen-presenting cells for CD4^{+} T lymphocytes (Hickey and Kimura, 1988). Hence, microglia cells might support expansion and differentiation of immunoregulatory important CD4^{+} T lymphocytes inside the CNS.

A suitable animal model to study these questions in vivo is the intracerebral infection of rats with the murine coronavirus strain JHM (JHMV). Dependent on the age and the genetic background of the animals different clinical courses have been reported (Watanabe et al., 1987; Dörries et al., 1991). Whereas in 3-week-old BN rats the infection remains subclinical, LEW rats

* Corresponding author. Phone (0931) 201 5954; Fax (0931) 201 3934.

infected at the same age die either from an acute encephalitis within 5–6 days or suffer from a subacute monophasic disease, characterised by ataxic gait, hind leg paresis or tetraplegia. The neurological disease in LEW rats is accompanied by a strong inflammatory response, dominated by CD4$^+$, α/β T cell antigen receptor (TCR)$^+$ T lymphocytes, CD8$^+$, α/β TCR$^+$ T lymphocytes and ED1$^+$ macrophages, all of which home to virus-infected areas in the CNS (Dörries et al., 1991). Healthy BN rats recruit similar lymphocyte subsets into the CNS but usually fewer than in LEW rats (Dörries et al., 1991). Concomitant with the increase of lymphoid cells in the brain parenchyma of JHMV-infected rats, MHC class II antigen expression is upregulated on CNS-resident microglia cells and peripheral MHC class II$^+$ cells pass the blood–brain barrier (Sedgwick et al., 1991). In this context, presence of viral antigens might allow antigen-driven proliferation of CD4$^+$ T lymphocytes resulting in intracerebral increase of this lymphocyte subset. We addressed this question by examination of T lymphocytes from the CNS and CLNs of JHMV-infected BN and LEW rats at different times post infection. Here, we demonstrate that T lymphocytes from CLNs but not from CNS of JHMV-infected BN and LEW rats proliferate ex vivo and can be stimulated to proliferate by JHMV-antigens in vitro. This suggests that priming and clonal expansion of virus-specific T lymphocytes during JHMV-induced encephalitis occur in CLNs and that increase of this lymphocyte population in the brain parenchyma reflects accumulation rather than local expansion of virus-specific T lymphocyte clones.

2. Materials and methods

2.1. Virus

JHM virus for infection of animals was propagated as described earlier (Schwender et al., 1991). Virus particles used as antigen for stimulation of T lymphocytes were purified according to Wege et al. (1978). Purified viral particles were denatured for 30 min at 100°C before addition to the rat lymphocyte cultures. The same virus batch was used throughout all assays for antigen-specific proliferation. The stimulating capacity of this virus batch on T lymphocyte proliferation was verified on a JHMV-specific rat T cell line by [^{3}H]thymidine incorporation (data not shown).

2.2. Animals

LEW and BN rats were obtained from the Zentralinstitut für Versuchstierzucht, Hannover, Germany. They were JHMV seronegative and kept under SPF conditions until inoculation with virus. At the age of 3 weeks, the animals were intracerebrally infected with

80 μl of JHM virus containing 1.0×10^3 plaque-forming units. Their clinical state was recorded daily using the following score (Dörries et al., 1991): (0) no overt disease; (1) uncommon social behaviour, such as separation from other rats in the cage; (2) paresis of 1 or 2 legs; (3) paresis of more than 2 legs; (4) paralysis and (5) moribund.

2.3. Monoclonal antibodies

To stain lymphocytes for flow-cytometric analysis the following monoclonal antibodies (mAbs) were used: W3/25 specific for the rat CD4 (Williams et al., 1977), OX22 specific for the rat CD45RC (Spickett et al., 1983), OX39 specific for the rat IL-2 receptor (IL-2R) (Paterson et al., 1987), R73 specific for the α/β chains of the rat TCR (Hünig et al., 1989) and as a negative control 4B4 specific for the β chain of the human VLA4 (CD29) (Morimoto et al., 1985).

2.4. Isolation of lymphocytes from the CNS and cervical lymph nodes

Inflammatory lymphocytes were isolated from the brain according to a procedure published by our laboratory earlier (Schwender et al., 1991). Briefly, the killed animal was perfused with PBS. The CNS was removed and lymphoid cells were recovered by mechanical disruption and enzymatic digestion of the CNS followed by Percoll step gradient centrifugation. Lymphocytes were collected from interfaces of the appropriate density and sedimented in Hank's buffer (170 × g, 10 min, 4°C).

CLNs were taken from the killed animal and minced through a steel sieve in Hank's buffer. Connective tissue fragments were allowed to settle for 10 min at 4°C and lymphocytes were sedimented from the supernatant (170 × g, 10 min, 4°C).

2.5. Quantitation and phenotypic characterisation of isolated CNS lymphocytes

CNS isolated lymphocytes were quantitated and phenotypically characterised by two-color immunofluorescence and four-parameter (forward/side scatter, red/green fluorescence) flow cytometry as described earlier (Dörries et al., 1991). Briefly, lymphocytes were stained indirectly using a primary mouse mAb and a phycoerythrin (PE)-labelled secondary goat anti-mouse IgG, followed by another primary mouse mAb labelled with FITC.

Flow-cytometric analysis was done on a FACScan (Becton-Dickinson, Heidelberg, Germany). Collected data were processed by a Hewlett Packard 6000 computer and the Consort 32/Lysis II software package. The number of CD4$^+$, α/β TCR$^+$ cells per CNS was calculated by multiplying the percentage of positive

cells by the total number of lymphocytes recovered from the CNS of individual animals.

2.6. Determination of T lymphocyte proliferation

Lymphocytes were isolated each day post infection from the CNS of three animals and pooled in RPMI 1640 medium supplemented with 3% rat serum. In order to recover a comparable proportion of CD4$^+$ T lymphocytes at different days post infection, isolated lymphoid cells were passaged through a nylon wool column. A 10-ml syringe was loaded with 0.6 g of nylon wool (Type 200L, Du Pont, Boston, MA) and equilibrated with 10 ml of RPMI 1640 (5% FCS). After closing the outlet of the syringe, 3 ml of RPMI 1640 (5% FCS) were added followed by a pre-incubation for 1 h at 37°C (5% CO_2). Subsequently, 1×10^7 isolated lymphocytes in 3 ml RPMI 1640 (5% FCS) were added to the column followed by another incubation of 1 h at 37°C (5% CO_2). Non-adherent T lymphocytes were eluted from the column with 40 ml of RPMI 1640 (5% FCS). Subsequently, they were collected by low-speed centrifugation ($170 \times g$, 4°C, 10 min) and resuspended in RPMI 1640 supplemented with 3% rat serum. Dependent on cell recovery they were seeded at least in duplicates but usually in triplicates or quadruplicates into the wells of a microtiter plate (100 μl/well). Cell densities varied between 1×10^6/ml and 1×10^7/ml. The actual amount is stated in the context of experiments described in Results. Ex vivo proliferation of T lymphocytes was assessed after 48 h of culture at 37°C (5% CO_2), by adding 14.8 kBq of [^{3}H]thymidine (specific activity 1.59 TBq/mMol) per well for another 24 h. Incorporated radioactivity was determined by harvesting the lymphocytes on glass fiber filters, lysing of cells by hypotonic wash and subsequent count of radioactive decays in a β-plate counter. Mean values were calculated and plotted versus the time post infection. The same assay protocol was used to determine antigen- or mitogen-inducible proliferation. JHMV-antigens (20 μg/ml), control antigen (keyhole limpet hemocyanine (KLH), 20 μg/ml) or concanavalin A (2 μg/ml) were added immediately after taking the cells into culture. After 48 h of incubation, cells were radioactively labelled and further processed as described above.

CLN cells were processed in an identical way, except that they were not passaged through nylon wool.

3. Results

3.1. Course of the infection and dynamics of the inflammatory T lymphocyte response

The mean clinical score of infected LEW and BN rats during the first 3 weeks post infection is shown in
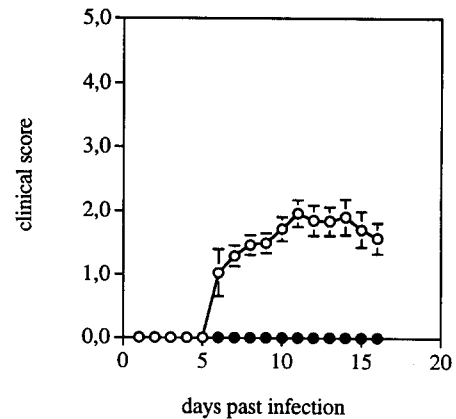

Fig. 1. Clinical course of the infection. The average of the clinical score of intracerebrally infected BN (●) and LEW (○) rats was calculated daily and plotted versus the time post infection. Because animals of each rat strain were sacrificed every day post infection, the number of observed animals per day dropped from initially 193 at 1 dpi to 24 at 18 dpi. Vertical bars indicate the SEM.

Fig. 1. Comparable to our earlier findings (Dörries et al., 1991; Schwender et al., 1991), clinical signs of the infection started to develop in LEW rats at day 6 post infection and reached a maximum at 11 days post infection (dpi). Thereafter, animals started to recover from disease. The infection remained subclinical in BN rats.

Contribution of CD4$^+$, α/β TCR$^+$ T lymphocytes to the inflammation was determined by 2-color immunofluorescence and flow cytometry using the pooled leukocyte fraction from the CNS of three animals every 2nd day post infection. In agreement with our earlier findings (Dörries et al., 1991), the amount of CD4$^+$, α/β TCR$^+$ T lymphocytes started to increase in the CNS of both rat strains at day 6 post infection (Fig. 2). Initially, LEW rats recruited CD4$^+$ T cells more rapid and to a higher extent compared to BN rats. The increase paralleled the sharp onset of clinical symptomatology in LEW rats (Fig. 2, upper panel; and Fig. 1).

3.2. Phenotypic characterisation of CD4$^+$ T lymphocytes from the CNS

CD4$^+$ T lymphocytes infiltrating the CNS were examined for their composition with respect to naive and primed memory cells, respectively. Fig. 2 demonstrates that the majority of CNS-infiltrating CD4$^+$ lymphocytes consisted of antigen-primed cells by virtue of absence of the CD45RC molecule. Early post infection (2 dpi), roughly 40% of detectable CD4$^+$ T lymphocytes were naive (CD45RC$^+$) whereas with proceeding infection this population contributed less than 4% to the recoverable CD4$^+$ subset of T lymphocytes. This holds true for healthy BN rats as well as for diseased Lewis rats. Interestingly, double staining of CNS iso-
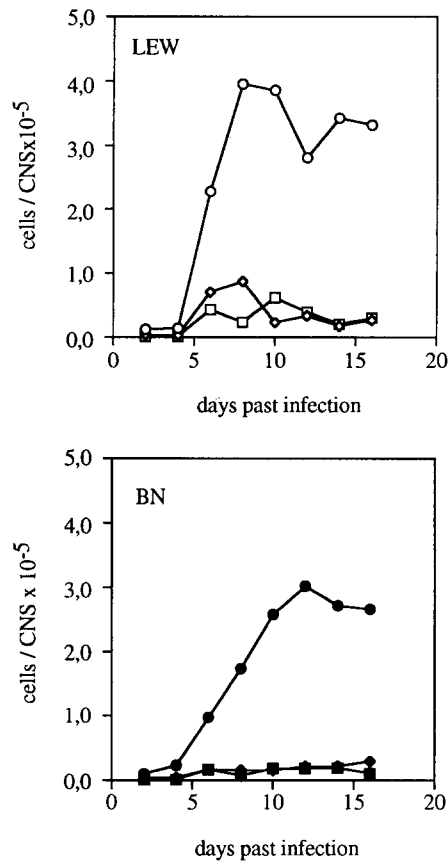
Fig. 2. Phenotype of CD4$^+$ T cells from the CNS. Leukocytes isolated from the CNS of JHM virus-infected LEW (open symbols) and BN (closed symbols) rats were double-labelled by mAbs specific for CD4 and α/β TCR (LEW o; BN •), CD4 and CD45 RC (LEW ◇; BN ◆) or CD4 and CD25 (LEW □; BN ■) and subjected to flow cytometry. Total events were gated for lymphocytes by criteria of forward and side scatter. Leukocytes isolated from the CNS of three animals of each rat strain were pooled.

lated leukocytes for CD4 and IL-2R disclosed a very low proportion of such cells amongst the inflammatory infiltrate (Fig. 2). Between 20 and 3% of infiltrating leukocytes were of the CD4$^+$, IL-2R$^+$ phenotype at 6 dpi and 18 dpi, respectively. This result predicted that only a minority of CNS-infiltrating CD4$^+$ cells were in a state to respond to IL-2 by proliferation. Thus, we enriched T lymphocytes from CNS-isolated leukocytes and assayed them for proliferation.

3.3. Enrichment of T lymphocytes from CNS-isolated leukocytes

From previous experiments, the contribution of T lymphocytes to the CNS-extractable leukocyte fraction varies considerably in the course of the infection. Especially, early post infection or in the uninfected CNS the vast majority of CNS extracted cells expressing the leukocyte common antigen (LCA) are microglia cells (Sedgwick et al. 1991). To allow comparison of data

from proliferation assays at different times post infection we enriched T lymphocytes from the CNS by nylon-wool passage of isolated cells. Fig. 3 shows a representative example of T lymphocyte enrichment at 14 dpi. Cells were extracted from the CNS of a JHMV-infected LEW rat and FACS analysed for rat CD4 and rat α/β TCR. Approximately 20% of the cells expressed α/β TCR (quadrants upper left and upper right). The majority of cells lacked CD4 and α/β TCR and represent mostly microglia and very few B lymphocytes (Sedgwick et al., 1991). Within this fraction, however, a small percentage exhibited a low expression of CD4. As we have shown earlier, these cells are activated microglia cells (Sedgwick et al., 1991). After passage through a nylon wool column, there was a significant increase (to approx. 60%) in the α/β TCR-expressing T lymphocyte fraction (lower panel). Moreover, the CD4^{low}-expressing population was removed from the TCR$^-$ fraction without affecting the CD4/CD8 ratio of the TCR$^+$ fraction, suggesting that activated microglia cells are nylon wool-adherent.
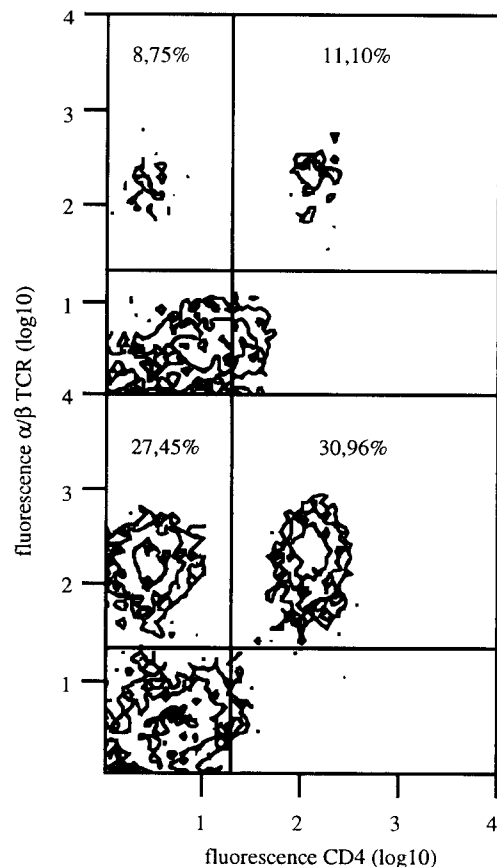

Fig. 3. Enrichment of T lymphocytes from CNS-isolated lymphocytes. Leukocytes isolated from the CNS of a JHM-infected Lewis rat at 14 dpi were double-labelled by mAbs with specificity for CD4 and α/β TCR and subjected to flow cytometry either before (upper panel) or after nylon wool passage (lower panel). Numbers in quadrants refer to the percentage of cells with respect to the total number. Total events were gated for lymphocytes by criteria of forward and side scatter.

This result in combination with the high percentage of CNS-extracted T lymphocytes expressing the phenotype $CD4^+$, $CD45RC^-$, $CD25^-$ suggested that the invading cells must have been expanded outside the CNS before they crossed the blood–brain barrier. On the assumption that at least some of $CD4^+$ T lymphocytes that had passed the blood–brain barrier were virus-specific, a proliferative recall response could be expected after a challenge with viral antigens in vitro. Consequently, CNS-isolated T lymphocytes were cultured in the presence of inactivated JHM virus. Stimulation of cell proliferation was monitored by the addition of [^{3}H]thymidine between 48 and 72 h of culture. Surprisingly, neither T cells from BN nor from LEW brain disclosed a proliferative JHMV-specific response (Fig. 4). This failure to respond to a recall antigen could neither be overcome by adding irradiated feeder cells from syngeneic rats (data not shown) nor by
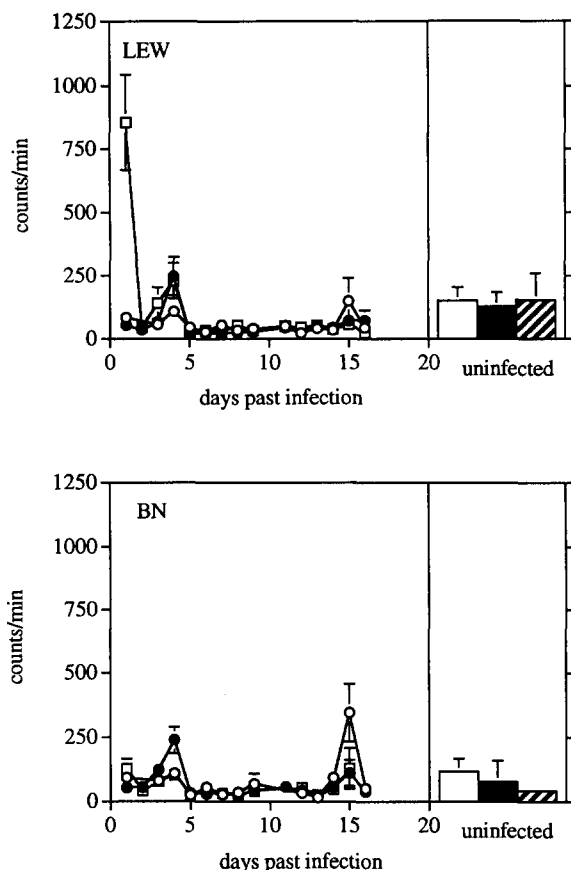
Fig. 4. Proliferation of T cells from the CNS. Leukocytes were isolated from the CNS of JHM virus-infected LEW (upper panel) and BN rats (lower panel) every day post infection and cultured for 72 h at a density of 0.1×10^7/ml either in RPMI medium (o), in the presence of 20 μg/ml of JHM virus (•) or with 2 μg/ml of ConA (□). To monitor proliferation, [^{3}H]thymidine was added to the cultures between 48 and 72 h. Columns in upper and lower right hand panels show [^{3}H]thymidine incorporation in leukocytes from the CNS of uninfected LEW or BN rats (white column, RPMI; black column, 20 μg/ml JHMV; hatched column, 2 μg/ml ConA). Leukocytes were pooled from three animals per strain and cultures were set up at least in duplicates, usually in triplicates or quadruplicates. Vertical bars indicate the SD.

3.4. Proliferation of T lymphocytes from CNS

Enriched T lymphocyte fractions were prepared every dpi and assayed for proliferation $(0.1 \times 10^7$ cells/ml) by incorporation of [^{3}H]thymidine between 48 and 72 h of culture in RPMI medium without addition of growth factors, mitogens or viral antigens. As a reference, lymphocytes from the CNS of uninfected rats were included in this assay. For BN but not for LEW rats a slightly increased rate of proliferation above that of lymphocytes from uninfected animals was only observed 15 dpi (Fig. 4). If the amount of recoverable T lymphocytes permitted, five times more cells were assayed for proliferation at distinct times post infection. However, no increase of [^{3}H]thymidine incorporation was detectable (Fig. 5).

Fig. 5. Proliferative response of T cells from CNS and CLNs in relation to cell density. Leukocytes were isolated from CNS (circles) and CLNs (triangles) of JHM virus-infected LEW (upper panel) and BN rats (lower panel) 9 days post infection and cultured for 72 h in different densities in RPMI medium (open symbols) or in the presence of 20 μg/ml of JHMV (closed symbols). To monitor proliferation [^{3}H]thymidine was added to the cultures between 48 and 72 h. Leukocytes were pooled from three animals per strain and cultures were set up at least in duplicates, usually in triplicates or quadruplicates. Vertical bars indicate the SD.

increasing the cell density per well (Fig. 5). Since the brain-isolated T lymphocytes did also not respond to the T cell mitogen Con A (Fig. 4), it was assumed that the majority of primed infiltrating T lymphocytes was insensitive to further stimulation within the brain parenchyma.

3.5. Proliferation of T lymphocytes from CLNs

Since cervical lymph nodes are known to drain the head area (Harling-Berg et al., 1989; Doherty et al., 1990; Cserr and Knopf, 1992), it seemed possible that prior to the infiltration in the CNS, sensitisation and expansion of virus-specific T lymphocytes had occurred in these lymphoid organs. Therefore, we assayed CLN lymphocytes taken from the same animals as for isolation of lymphocytes from the CNS. In contrast to brain-extracted lymphocytes, a clear dose–response curve was detected when the proliferation signal was examined in relation to the seeded cell density, giving the best signal at 1.0×10^7 cells/ml (Fig. 5). This result reflects the fact that in lymphoid tissue the majority of cells consists of naive recirculating cells, whereas in tissue primed and activated lymphoid cells are preferentially accumulated. Thus, it needs much more lymphocytes from secondary lymphoid tissue to obtain an amount of primed and activated cells comparable to tissue-infiltrating leukocytes.

Compared to uninfected rats, assays of CLN lymphocytes at a density of 1.0×10^7/ml without further stimulation revealed proliferation at day 4 in BN and at 12 dpi in LEW rats ex vivo (Fig. 6). Most remarkable, LEW rats revealed a later but stronger proliferation compared to BN rats. The highest [^{3}H]thymidine incorporation rates in LEW rats were detected at a time when the neurological disease was already fully developed (Fig. 6, upper panel; and Fig. 1). In contrast, the maximal response of BN rats was seen shortly before the first clinical signs of the infection were detectable in LEW rats (Fig. 6, lower panel; and Fig. 1). These findings suggest that BN rats probably reacted earlier to the viral challenge compared to LEW rats. To verify this assumption, CLN cells were stimulated by the same viral antigen batch used for T lymphocytes extracted from the CNS. The results of this assay in Fig. 6 support our hypothesis. CLN cells from BN rats could be stimulated to strong virus-specific proliferation already at day 5 post infection. At this time the response of CLN lymphocytes from LEW rats was significantly lower. It took 6–7 additional days until CLN cells responded in these rats as detectable in BN rats. Nevertheless, in contrast to CLN cells from BN rats, enhancement of proliferation above the level observed in ex vivo assayed CLN cells was only marginal after restimulation with JHMV. Moreover, lymphocytes from BN rats revealed a second peak of JHMV-
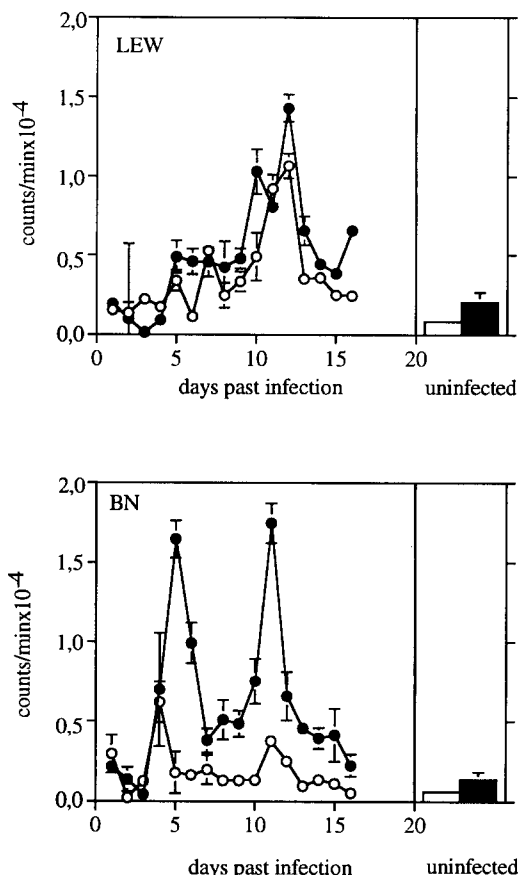

Fig. 6. Proliferation of T cells from CLNs. Leukocytes were isolated from CLNs of JHM virus-infected LEW (upper panel) and BN rats (lower panel) every day post infection and cultured for 72 h in a density of 1×10^7/ml either in RPMI medium ($\bigcirc$) or in the presence of 20 μg/ml of JHM virus ($\bullet$). To monitor proliferation [^{3}H]thymidine was added to the cultures between 48 and 72 h. Columns in upper and lower right hand panels show [^{3}H]thymidine incorporation in leukocytes from CLNs of uninfected LEW or BN rats (white column, RPMI; black column, 20 μg/ml JHMV). Leukocytes were pooled from three animals per strain and cultures were set up at least in duplicates, usually in triplicates or quadruplicates. Vertical bars indicate SD.

specific proliferation concomitantly with the observed increase of [^{3}H]thymidine incorporation in LEW rats. This indicates that in JHMV-infected LEW rats the proliferative response of CLN cells is vigorous, but slower and less specific compared to BN rats.

4. Discussion

Upregulation of MHC class II antigens on ramified microglia cells in the inflamed virus-infected CNS and a phenotype of resting microglia reminiscent of dendritic cells is indicative of a potential immunoregulatory role of this CNS-resident cell (Lowe et al., 1989; Weinstein et al., 1990; Griffin et al., 1992). This is strongly supported by studies that demonstrate the

capacity of microglia cells to induce a proliferative response of antigen-specific CD4$^+$ T lymphocytes in vitro (Frei et al., 1987; Matsumoto et al., 1992; Williams et al., 1992). However, the data presented here do not corroborate a stimulating influence of activated microglia on proliferation of brain-infiltrating T lymphocytes in vivo. We have previously documented upregulation of MHC class II antigens on brain-resident microglia cells of rats infected intracerebrally with the murine coronavirus strain JHM (Sedgwick and Dörries, 1991; Sedgwick et al., 1991) as well as presence of JHM virus antigen in the CNS for at least 3 weeks post infection (Dörries et al., 1991). Additionally, as shown in this paper, CD4$^+$ T cells infiltrating into the CNS of these animals are of primed or memory phenotype by virtue of a low expression of CD45RC. Nevertheless, we were unable to demonstrate a proliferative response of CNS-isolated T lymphocytes ex vivo. Concurring with this result is the fact that after a week post infection less than 5% of CNS-recoverable CD4$^+$ T cells expressed the IL-2R. Both low expression of CD45RC and IL-2R are not confined to CD4$^+$ T lymphocytes from virus-infected rat brain. As shown by McCombe et al. (1992), the majority of T lymphocytes that can be detected in spinal cord of rats suffering from experimental allergic encephalomyelitis (EAE) is of the same phenotype. This suggests that the CD4$^+$ T lymphocyte population infiltrating the brain in the course of acute inflammatory CNS disorders is generally dominated by primed and already differentiated cells.

On the other hand, infiltrating CD4$^+$ T lymphocytes usually home to virus-infected areas of the brain (Dörries et al., 1991), making it very likely that they are indeed virus-specific. In this context, our failure to restimulate these cells in vitro with viral antigens was surprising. For an explanation of this phenomenon at least two aspects have to be discussed: (i) Viability of cells could be impaired by the extraction procedure. Beside the fact that we recover roughly 80% of viable cells from a Percoll gradient when we mix viable spleen cells into the CNS extraction procedure (unpublished observation), cell counts for proliferation assays are expressed as viable cells by Trypan blue exclusion immediately before stimulation experiments. Nevertheless, in view of recent data from the EAE rat model (Schmied et al., 1993) we cannot rule out that a certain percentage of cells is apoptotic. Indeed, we have observed in preliminary experiments by FACS analysis, that up to 40% of CNS extracted cells can show DNA fragmentation without being necessarily recognised as dying cell by Trypan blue exclusion (unpublished observation). In any case, 60% of living cells should be enough cells to give a proliferative response. (ii) As hypothesised by Cross et al. (1991), only a minority of CNS-infiltrating T cells may be antigen-specific in au-

toimmune EAE. Although we cannot exclude the possibility that this could also be the case in virus-induced encephalomyelitis, absence of a dose–response relationship between amount of CNS-extracted T cells and virus-specific proliferation argues against it. Since attempts to induce proliferation by ConA were also unsuccessful, we believe indeed that the majority of CD4$^+$ T lymphocytes entering the virus-infected brain is unresponsive to further proliferation signals that probably could be given by MHC class II expressing brain-resident cells.

Apparently conflicting data have been published recently by Williamson (1992), who reported a proliferative response of leukocytes from the CNS of JHMV-infected mice after in vitro stimulation with viral antigen or ConA. However, careful evaluation of these investigations reveals that CNS-extracted leukocytes were assayed only on a single day post infection (day 5) and that compared to unstimulated cells the inducible proliferative response was marginal (2-fold enhancement). In this context, it is most remarkable that we also observed a slight (2-fold) enhancement of virus-inducible proliferation in CNS-extracted leukocytes of LEW and BN rats 4 dpi (Fig. 4). However, this very low and statistically doubtful induction of proliferation is the only exemption from the rule of proliferative unresponsiveness within 14 dpi. Probably, a transient increased permissiveness of the blood–brain barrier as observed by us in rats early post intracerebral JHMV infection (Dörries et al., 1986) opens a small window allowing a few activated but not fully differentiated T cells to gain access to the brain parenchyma, where they can be detected for a very limited time as a small T cell population reactive to proliferation-inducing signals.

The assumption that the majority of CNS-extractable T cells is refractory to further antigen or mitogen-inducible proliferation is supported by findings in other animal models. Nelson et al. (1990) reported low proliferative activity of CD4$^+$ T lymphocytes isolated from lungs, and Ohmori et al. (1992) used the bromodeoxyuridine labelling technique to demonstrate in situ that the majority of T lymphocytes in the CNS of LEW rats suffering from EAE do not proliferate. Moreover, proliferating T lymphocytes that were transferred from EAE animals to healthy syngeneic hosts stopped proliferation after entry into the CNS. Recent in vitro data from Matsumoto et al. (1992) suggest that astrocytes probably exhibit inhibitory functions on T lymphocyte proliferation, comparable to alveolar macrophages that probably suppress a proliferative response of non-recirculating T lymphocytes in the lung (Holt, 1986).

In line with these observations, our data add more support to the idea that inside a target organ of lymphoid infiltration priming and expansion of CD4$^+$ T lymphocytes is rare. Under these circumstances the

absence of a lymphoid drainage system in the CNS raises the question for the peripheral sites that will allow priming and expansion of CD4$^+$ T lymphocytes that subsequently will enter the CNS. Data published by Harling-Berg et al. (1989), Doherty et al. (1990) and Cserr and Knopf (1992) show that CLNs are the most important secondary lymphoid organ collecting antigens applied to the brain. Most likely, these antigens will leak with cerebrospinal fluid through the cribriform plate where olfactorial nerves cross the blood–brain barrier and leave the base of the skull to penetrate the mucosa of the nose. From there lymphatic drainage will take antigens to CLNs where priming and differentiation of antigen-specific lymphocytes occur. Our data from CLNs of JHMV-infected rats are consistent with this hypothesis. T lymphocytes extracted from CLNs of animals that were taken to isolate lymphocytes from the CNS do proliferate ex vivo and can be stimulated to JHMV-specific proliferation in vitro. Since the dynamics of this CLN response reveal distinct maxima that precede onset as well as recovery from neurological disease, it seems very likely that the clinical course of the infection is closely related to the sensitisation event. These relationships are further substantiated by differences between LEW and BN rats with respect to the JHMV-specific in vitro response of CLN lymphocytes. Whereas in BN rats a strong proliferative response can be provoked by viral antigens early post infection, in LEW rats at the same time only minor virus-specific proliferation was inducible in vitro. It is of note, however, that minor in vitro induction of virus-specific proliferation in CLN cells from LEW rats is not due to a general incapability of these cells to proliferate. It seems rather the strong ex vivo proliferation of CLN cells from JHMV-infected LEW rats that prevents further enhancement of proliferation by exposure to viral antigens. Thus, it is likely that in contrast to BN rats the T cell compartment of LEW rats responds less specific with a long-lasting broad polyclonal proliferation to the viral challenge.

As we have reported previously (Dörries et al., 1991) in infected but healthy BN rats, viral spread in the CNS is limited to few and small periventricular sites. In contrast, large virus-infected areas spread through the CNS of LEW rats, including brain stem, cerebellum and spinal cord. Most likely, restricted viral spread in the BN rat is associated with the humoral immune response because virus-specific antibody-secreting plasma cells rapidly increase in the brain parenchyma post infection, whereas LEW rats show a considerable delay in the appearance of such cells in the CNS (Schwender et al., 1991). Additionally, more of these plasma cells can be recovered from the infected BN brain and their capacity to secrete virus-specific antibodies as determined on a single-cell basis is much higher compared to LEW rats. These differences result

in a rapid accumulation of virus-specific neutralising antibodies in the cerebrospinal fluid of BN rats (Schwender et al., 1991). Differentiation of B lymphocytes into antibody-secreting plasma cells is highly dependent on the help from interleukins provided by T lymphocytes expressing the CD4, α/β T cell antigen receptor (α/β TCR) phenotype. Hence, the observed differences between BN and LEW rats with respect to kinetics, magnitude and quality of virus-specific antibody responses in the CNS could possibly be explained by the more rapid and specific sensitisation of the CD4$^+$, α/β TCR$^+$ T lymphocyte fraction in CLNs of BN rats. However, since there is no simple quantitative relationship between local virus-specific antibody response and CNS-invading CD4$^+$, α/β TCR$^+$ T lymphocytes, this hypothesis remains to be clarified on a qualitative basis.

In conclusion, CLNs are an important site of T lymphocyte priming and expansion during virus-induced encephalitis. Speed and intensity of T cell sensitisation in these organs are most likely of major importance for the clinical outcome of the infection. Primed T lymphocytes enter the CNS and acquire a state of unresponsiveness to antigen- or mitogen-induced proliferation by a yet unknown regulatory event. Of course, this silent state with respect to proliferation does not necessarily exclude secretion of cytokines by these cells. Preliminary, so far unpublished investigations of cytokine-specific mRNA expression performed in our laboratory by RT PCR on CNS extracted leukocytes revealed a strong interferon-γ response in the CNS of both LEW and BN rats. Thus, future examinations of functional properties of CNS-infiltrating CD4$^+$ T cells especially with respect to interleukin secretion and to consequences of their possible interaction with upregulated MHC class II molecules on CNS-resident cells will further elucidate their important role as effector cells in virus-induced demyelinating encephalomyelitis.

Acknowledgements

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