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Phenotypic and functional characterization of CD8+ T lymphocytes from the central nervous system of rats with coronavirus JHM induced demyelinating encephalomyelitis

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Intracerebral infection of Lewis (LEW) inbred rats with the neurotropic strain of the murine coronavirus JHM (JHMV) frequently results in a monophasic paralytic disease. In contrast, infection of Brown Norway (BN) inbred rats does not lead to clinical disease. Previous findings indicated that in both rat strains brain-infiltrating leukocytes consisted mainly of CD8+ T lymphocytes. Here, we phenotypically as well as functionally characterised this T cell subset after isolation from the central nervous system (CNS). Using JHMV-infected target cells, MHC class I restricted, cytotoxic T lymphocytes were demonstrated to be present in the leukocyte fraction from the CNS of both, susceptible LEW and disease-resistant BN rats. However, compared to infected, but healthy BN rats, diseased LEW rats generated an enhanced cytotoxic immune response which became most prominent at the maximum of neurological disease. Recently published observations from our laboratory demonstrated a strong virus-specific antibody response in the CNS of BN rats. In LEW rats, however, the response was delayed and of low magnitude. This suggests, that consequences of cytotoxic T lymphocyte action in JHMV-infected CNS tissue largely depend on the efficacy of an accompanying virus-specific humoral immune response.

Keywords: coronavirus; demyelination; cytotoxic T lymphocytes

Introduction

Virus infections of the central nervous system (CNS) may cause a variety of neurological disorders ranging from mild self-limiting meningitis to fatal encephalitis. Occasionally, the disease process is accompanied by intense histopathological changes characterized by loss of myelin sheaths without axonal destruction. The pathomechanisms involved in these demyelinating processes are poorly understood. Thus, animal models of virus-induced demyelination are of particular interest, because they offer the opportunity to investigate comprehensively all aspects of white matter destruction in the course of viral encephalitides (reviewed by Dal Canto and Rabinowitz, 1982). Intracerebral inoculation of rodents with the neurotropic strain of the mouse-hepatitis-virus JHM (JHMV) has been proven to be a valuable experimental system to study the interactions between viral infection, CNS-associated immune response and primary demyelination (Lampert *et al*, 1973; Weiner, 1973; Nagashima *et al*, 1978, 1979; Knobler *et al*, 1982; Sorensen *et al*, 1982; Wege *et al*, 1982; Watanabe *et al*, 1987; Dörries *et al*, 1991).

In rats, JHMV infection leads to a variety of clinical diseases, depending on the rat strain, the age of the animals and the strain of virus (Wege *et al*, 1983; Watanabe *et al*, 1987). Weanling LEW rats may develop a fatal acute encephalitis and a nonlethal subacute demyelinating encephalomyelitis,

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respectively. In contrast, BN rats of the same age remain clinically healthy. Histopathologically, in both rat strains, plaques of primary demyelination can be detected. In BN rats, demyelinated foci are small and predominantly located in the periventricular white matter, whereas in LEW rats extended demyelinated lesions spread throughout the CNS (Watanabe *et al*, 1987). Within demyelinated areas mononuclear cell infiltrates are detectable, consisting of all lymphoid subsets (Dörries *et al*, 1991). The total number of leukocytes entering the affected CNS is up to five times higher in susceptible LEW rats than in disease-resistant BN rats. In LEW rats, the inflammatory process is dominated by CD8+ T cells, whereas BN rats particularly recruit CD4+ T helper cells and only a few CD8+ T lymphocytes. In both rat strains, CD8+ T cells are localized in close contact to virus-infected, MHC class I expressing brain cells (Dörries *et al*, 1991). Given the fact that CD8+ T lymphocytes are able to destroy virally infected glial cells, presence of numerous CD8+ T cells in the CNS of LEW rats might lead to extensive tissue damage, thus contributing to neurological disease.

To address this hypothesis we compared CD8+ T lymphocytes isolated from the CNS of susceptible LEW and disease-resistant BN rats with respect to their ability of lysing JHMV-infected targets *in vitro*. Virus-specific, MHC class I restricted cytotoxicity could be demonstrated within the brain-derived leukocyte-fractions from both rat strains. Compared to BN rats generating a modest CD8+ T cell response, LEW rats exhibited a marked cytolytic activity. Our data support the idea that action of CD8+ cytotoxic T lymphocytes (CTLs) in LEW rats may aggravate neurological signs, whereas in animals of the BN rat strain action of CD8+ effector T cells seems to have no clinical consequences during JHMV-induced demyelinating encephalomyelitis.

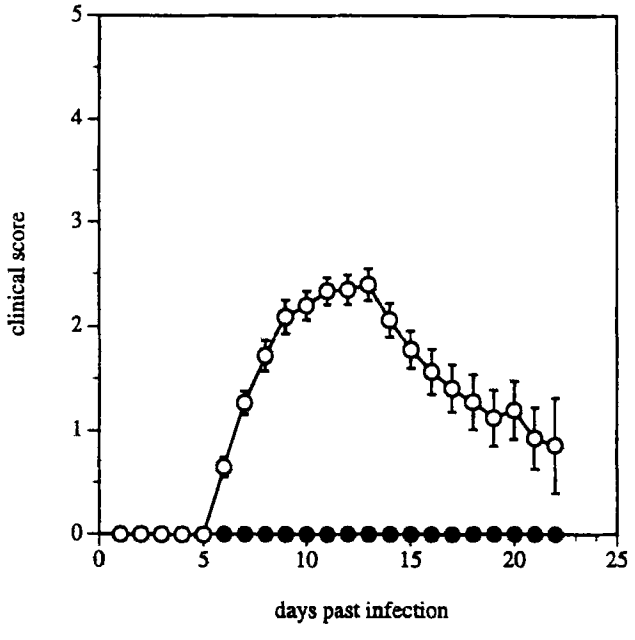

Figure 1 Clinical course of the infection. Neurological signs of intracerebrally inoculated LEW (○) and BN (●) rats were recorded every day past infection and classified according to the score described in 'Materials and Methods'. Data represent the mean values of 93 LEW and 70 BN rats, respectively. Bars indicate the standard error of the mean (± s.e.m.).

Results

Neurological disease

Following intracerebral inoculation neurological signs of individual JHMV-infected LEW (n = 93) and BN (n = 70) rats were scored daily on an arbitrary scale ranging from 0 to 5 during a period of 3 weeks past infection (Figure 1). In line with recently published data (Dörries *et al*, 1991; Schwender *et al*, 1991; Imrich *et al*, 1994) animals of the LEW rat strain developed a monophasic, paralytic disease starting at day 6 past infection. With ongoing infection, clinical signs increased to become maximal at 13 days past infection (dpi). Up to this time, 40% of the rats succumbed to the infection. Thereafter, all of the surviving animals recovered completely. BN rats however, remained clinically healthy.

Cytofluorometric analysis of CNS-extracted leukocytes

In a previous study, we examined the inflammatory response in the CNS of JHMV-infected LEW and BN rats (Dörries *et al*, 1991). During the acute phase of the infection lymphocytic infiltrates were shown to be dominated by CD8+ T lymphocytes homing to virus-infected brain cells. To characterize further this lymphoid subset in the course of the disease, we first quantified the amount of CNS-infiltrating CD8+ T cells employing FACS-analysis. Leukocytes were isolated from the CNS of three JHMV-infected LEW and BN rats, respectively, pooled and subjected to flow cytometry at every second day past infection. Up to 6dpi, no differences to controls could be observed (Figure 2). In parallel with the onset of neurological disease at day 6 past infection (Figure 1), numerous CD8+/α/βTCR+ T lymphocytes were detected in the CNS of LEW rats (Figure 2, upper panel). The number of these cells remained at a high level until 14 dpi before gradually declining. At this time, animals started to recover from the disease (Figure 1). In contrast, few numbers of CD8+ T cells infiltrated the CNS of BN rats throughout the infection (Figure 2, lower panel). Additional cytofluorometric studies revealed, that in both rat strains the majority of infiltrating CD8+ T cells were of a primed phenotype (CD45RC-, CD25-) (Figure 2). However, early past infection (6dpi) a considerable proportion of CD8+ T lymphocytes entering the CNS of LEW rats were of a naive phenotype (CD45RC+,

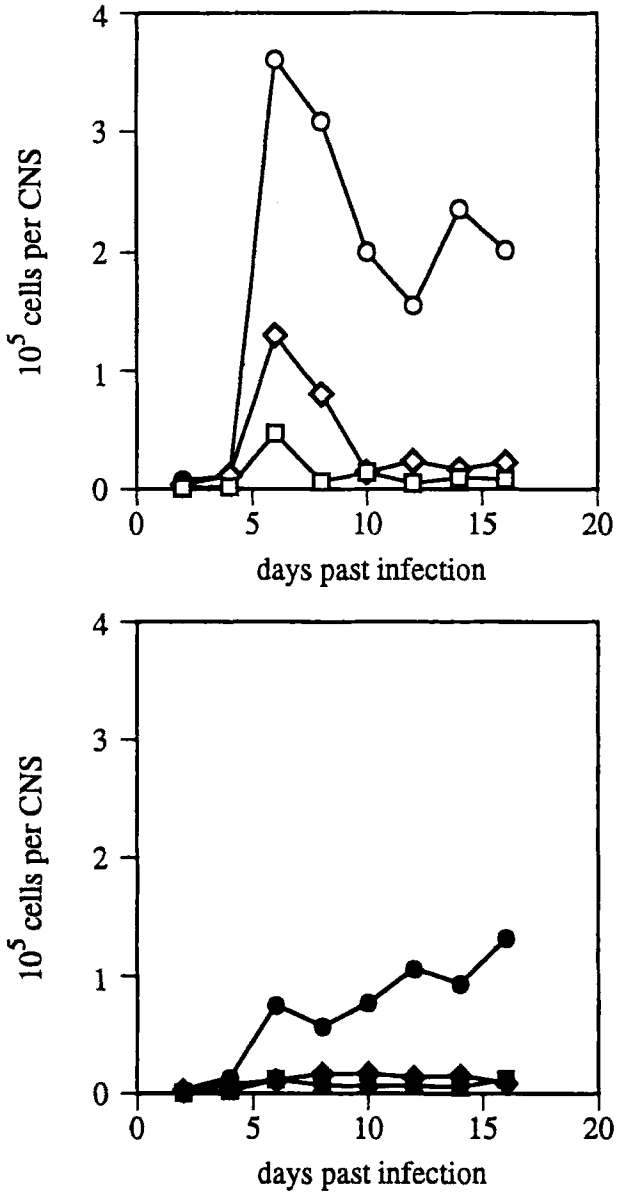

Figure 2 Phenotype of CD8+ T lymphocytes infiltrating the CNS of JHMV-infected LEW (upper panel) and BN rats (lower panel) at different days past infection. Cells of three animals each were pooled, double-immunostained for CD8 and α/βTCR (LEW ○; BN ●), CD8 and CD45RC (LEW ◇; BN ◆), and CD8 and CD25 (LEW □; BN ■), respectively, and analysed by flow cytometry.

CD25+) (Figure 2, upper panel). Influx of these naive cells was probably due to JHMV-induced perturbations of the blood–brain barrier as described previously (Dörries *et al*, 1986). Nevertheless, our data suggested, that during the inflammatory response mainly differentiated, activated CD8+ T cells invaded virus-infected brains. Consequently, we addressed the question of whether JHMV-specific effectors could be found among this lymphoid population.

Characterization of antiviral cytotoxicity in the CNS of JHMV-infected rats

Effector function of CD8+ T lymphocytes is typically assayed by their ability to kill appropriate target cells in an antigen-specific, MHC class I restricted manner *in vitro*. Although primary rat glial cells can principally serve as JHMV-infectable targets, a number of drawbacks are associated with the use of these cells in a cytotoxicity assay. Glial cell lines are difficult to establish, uncomfortable to handle and tend to release spontaneously cell labels resulting in background problems during cytotoxicity tests. Peripheral rat cells, however, are not permissive for JHM-virus (unpublished observation). To overcome problems, we established LEW as well as BN MHC-compatible permanent cell lines by cell-to-cell fusion of primary rat spleenocytes with JHMV-susceptible mouse myeloma SP2/0-Ag14. Of two hybridomas, designated LEW1B4 and BN3B2, single cell clones were expanded and subsequently phenotyped by flow-cytometry. As summarized in Table 2, both cell lines are transformed rat leukocytes (CD45+) expressing rat (RT1.A+) as well as mouse (H-2D^{d+}) MHC class I antigens. In contrast, MHC class II antigens were not detected (RT1.B$^-$, H-2IE^{d-}).

Virus-specific cytotoxic activity of lymphocytes isolated from the CNS of both rat strains could never be detected directly *ex vivo* at any given time point past infection. Neither purification of CD8+ T cells before the assay nor *in vitro* stimulation by T cell growth factors (TCGF), ie CASUP, or by syngeneic feeder cells disclosed JHMV-specific cytotoxic T lymphocytes among CNS-derived leukocytes (data not shown). However, after short-term *in vitro* stimulation of brain-isolated leukocytes of both, LEW and BN rats with irradiated xenogeneic feeder cells in the presence of mitogens (ConA) and TCGF, antiviral cytoxicity could be demonstrated (Figure 3). During the first 2 weeks of infection, CTL activity in diseased LEW rats was found to be just above background levels. Maximal virus-specific cytotoxicity was detected at 14 dpi. Thereafter, CTL activity dropped gradually to an undetectable level at 21 dpi (Figure 3, upper panel). Profile of virus-specific CTL response in JHMV-infected BN rats resembled those found in LEW rats. Up to 14 dpi and later than 17 dpi, antiviral cytolytic activity did not appear within the brain-derived leukocyte population from BN rats. Analogous to LEW rats, maximal cytotoxicity was detected at 14 dpi (Figure 3, lower panel). However, level of virus-specific cytotoxicity among the leukocyte-fraction from the CNS of BN rats was four times lower in comparison with LEW rats at similar effectors:targets ratios (Figure 3). These results were verified by repetition of the whole CTL kinetics using identical experimental settings. Additionally, killing capacity was analyzed

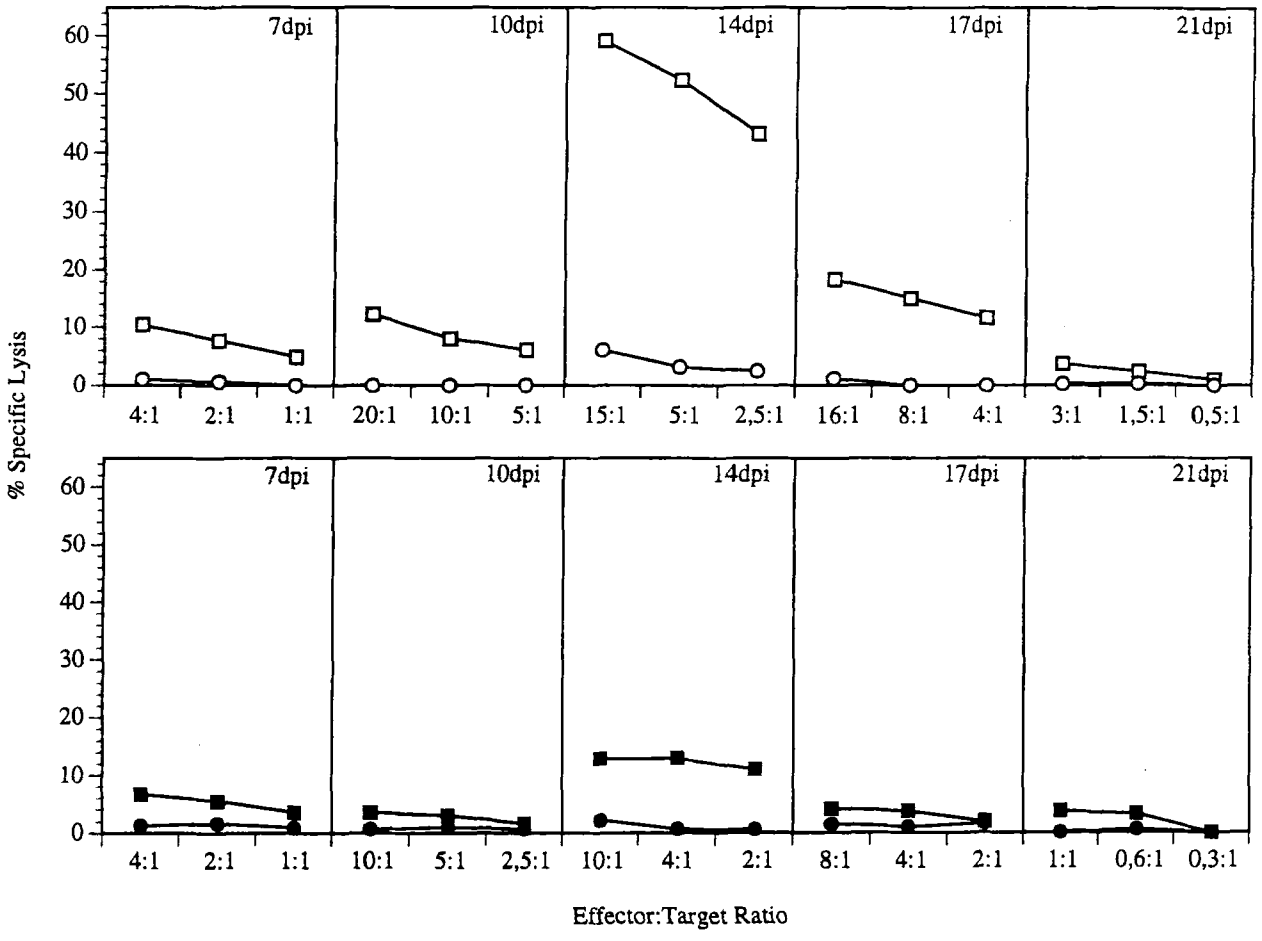

Figure 3 Virus-specific cytotoxicity in the CNS of JHMV-infected LEW (upper panel) and BN rats (lower panel) in the course of the disease. After short-term *in vitro* stimulation cytolytic activity of brain-derived leukocytes was assayed against histocompatible JHMV-infected (LEW1B4 □, BN3B2 ■) and uninfected targets (LEW1B4 ○; BN3B2 ●), respectively. At the time points indicated, lymphocytes from the CNS of at least three animals were pooled before testing their cytotoxic capacity.

multiple times at distinct time points past infection. No divergent results were obtained (data not shown). Thus, in BN rats $CD8^+$ T cell-mediated cytolytic activity played a minor role during the antiviral immune attack compared to LEW rats.

MHC restriction of CNS-derived cytotoxic lymphocytes

Because of the lack of MHC class II antigens at the surface of the target cell lines, contribution of MHC class II restricted antiviral cytolytic activity could be excluded. However, natural killer (NK) cell-mediated lysis of target cells could not be ruled out. To distinguish between NK and T cell-mediated cytotoxicity, *in vitro* stimulated leukocytes from the CNS of JHMV-infected rats were coincubated with infected and uninfected, syngeneic as well as infected, allogeneic targets. In another set of experiments, MHC class I restricted killing of histocompatible, JHMV-infected hybridoma was blocked by addition of rat MHC class I specific antibodies. Representative for three independent assays, Figure 4 illustrates that CNS-derived leukocytes from LEW rats failed to lyse virus-infected targets of the BN rat haplotype, whereas JHMV-infected, syngeneic targets were destroyed. Presence of RT1.A specific immunoglobulins completely inhibited antiviral cytotoxicity. Variability in magnitude of cytolytic activity was low between individual experiments. Specific lysis of JHMV-infected, syngeneic targets varied no more than 5% at each effector:target ratio tested and amount of detectable radioactivity released in control and blocking assays was less than 3% of the maximum release. Altogether, these results indicated that cytolytic activity within the brain-extracted leukocyte fraction was not due to NK cells but to MHC class I restricted $CD8^+$ T lymphocytes (Figure 4).

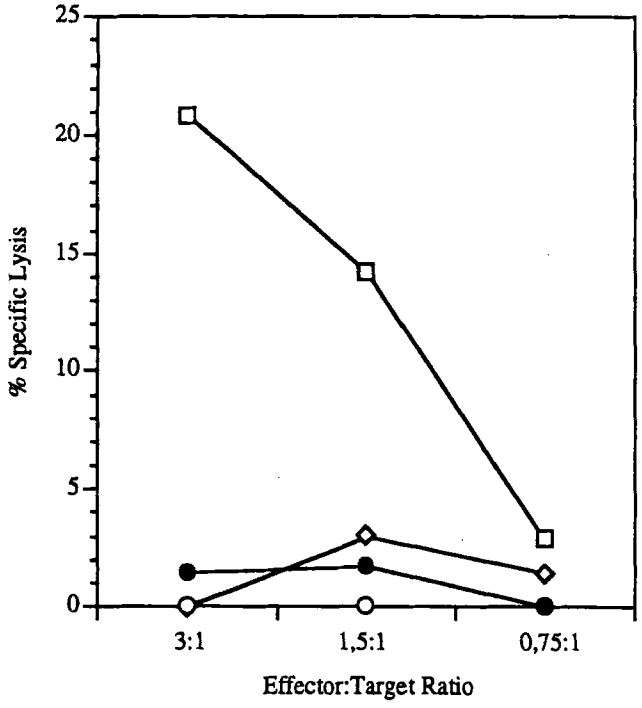

Figure 4 MHC class I restriction of JHMV-specific CTLs from the CNS of LEW rats at 14 dpi. *In vitro* restimulated lymphocytes were coincubated with JHMV-infected syngeneic (LEW1B4 □) or allogeneic (BN3B2 ●) and syngeneic uninfected (LEW1B4 ○) targets, respectively. JHMV-specific cytotoxicity was abrogated by addition of mab specific for rat MHC class I antigens (OX18, 1mg ml^{-1}, 30 μg $test^{-1}$) to JHMV-infected, syngeneic targets (LEW1B4◇).

Discussion

Since the first report of active CTLs in the CSF of mice infected with lymphocytic choriomeningitis virus (LCMV) (Zinkernagel and Doherty, 1973), CNS-localized CTL activity has been identified in several animal models of virally induced CNS disorders (Liu *et al*, 1989; Planz *et al*, 1993; Hudson and Streilein, 1994). Although there is circumstantial evidence that cytotoxic T cells might play a pivotal role in the course of virus-induced demyelinating encephalomyelitides (Rodriguez and Sriram, 1988; Fleming *et al*, 1990; Williamson and Stohlman, 1990; Yamaguchi *et al*, 1991), functional data of brain-infiltrating $CD8^+$ T lymphocytes in demyelination are rare (Lindsley *et al*, 1991). More recently, virus-specific CTLs from the CNS of JHMV-infected mice have been described (Stohlman *et al*, 1993), but a correlation between antiviral cytotoxicity and clinical symptomatology following JHMV infection has not been established. To assess more precisely the pathogenic role of CTLs during JHMV-induced demyelination, we analyzed the biological properties of CNS-extracted $CD8^+$ T lymphocytes from both, susceptible and disease-resistant rat strains at various times past infection.

In susceptible LEW rats, absence of an early virus-specific antibody response (Dörries *et al*, 1986; Schwender *et al*, 1991) presumably allowed widespread infection of the CNS. Despite a delayed and marginal intracerebral humoral immune response the majority of infected LEW rats recovered from neurological disease indicating that cellular mediated immune reactions were recruited to clear virus from infected tissue. This might explain that in animals of the LEW rat strain the disease process was associated with the infiltration of numerous, fully differentiated $CD8^+$ T cells into the CNS. Because of their unique homing pattern to virus-affected areas of the brain (Dörries *et al*, 1991) it has been assumed that this lymphocyte population is involved in histopathological alterations by killing virally infected glial cells. Indeed, our results demonstrate that CNS-extracted $CD8^+$ T cells exhibited typical CTL activity with respect to antigen specificity and MHC class I restriction *in vitro*. Highest virus-specific cytotoxicity coincided with maximal neurological signs suggesting that CTLs potentially mediated immunopathology and thereby enhanced clinical disease. Additionally, intense CTL-induced white matter destruction might have been accompanied by the release of high doses of myelin basic protein (MBP) resulting in sensitization of autoreactive T cells (Watanabe *et al*, 1983). Unlike LEW, disease-resistant BN rats generate a strong intracerebral antibody response (Dörries *et al*, 1987; Schwender *et al*, 1991). As shown previously, neutralization titres in BN rats on average are at least 30 times higher in magnitude compared to LEW rats. This difference in the CNS-localized humoral immune response is most prominent during the early phase of the infection (7–10 dpi) (Schwender *et al*, 1991). This could limit extracellular viral spread to a few and small periventricular sites as described by Watanabe and coworkers (1987). As a corollary, numbers of virally affected brain cells remained low so that few $CD8^+$ T effector cells were sufficient to eliminate JHM-virus from the CNS. Thus, in contrast to LEW, action of $CD8^+$ T cells in BN rats most likely had no clinical consequences.

In analogy to findings of Stohlman *et al* (1993) who demonstrated virus-specific CTLs from the CNS of JHMV-infected mice exclusively after *in vitro* expansion of brain-derived leukocytes, we also failed to disclose CTLs in freshly isolated brain lymphocytes. Strong proliferation inducing stimuli were needed to expand virus-specific CTLs *in vitro*. This was surprising, because the majority of infiltrating $CD8^+$ T lymphocytes were of primed and activated phenotype. At least three possibilities should be considered to explain this phenomenon: (1) A trivial, but likely explanation is that the proportion of virus-specific cells at the CNS-derived $CD8^+$ T cell population is too low to be detected *ex*

vivo. Thus, *in vitro* expansion is required to generate a measurable CTL signal. (2) In view of previously published data from the experimental allergic encephalomyelitis (EAE) rat model indicating that apoptosis of brain-infiltrating T lymphocytes is an important mechanism in controlling CNS-inflammation (Schmied *et al*, 1993), unresponsiveness of CNS-extractable CD8+ T cells might be due to programmed cell death. (3) Effector function of CD8+ T cells *in vivo* is not lysis of targets by direct cell-to-cell contact in a MHC-restricted manner but virostatic action by means of soluble factors. This hypothesis is substantiated by the recent observation that adoptive transfer of virus-specific CTLs can result in virus-clearance from infected neurons without tissue destruction (Tishon *et al*, 1993) suggesting a cytokine-mediated mechanism. Furthermore, Schijns *et al* (1991) provided evidence that the interleukins γ-IFN and TNF α, which both can be synthesized by CD8+ T lymphocytes, individually or in combination are capable of inhibiting growth of pseudorabies virus in brain cell cultures. In mice, infection with a neurotropic variant of mouse-hepatitis-virus causes CNS-localized transcriptional upregulation of different lymphokine-specific gene sequences including TNF α and γ-IFN (Pearce *et al*, 1994). In accordance with these data preliminary results from our laboratory obtained by RT-PCR on brain-isolated leukocytes demonstrate a marked γ-IFN mRNA-expression following intracerebral JHMV-challenge (Dörries *et al*, 1993). With regard to its antiviral capacity, intrathecal production of γ-IFN by CD8+ T lymphocytes might be important for virus clearance from infected CNS as proposed recently (Ramsay *et al*, 1993). All these studies would fit with our observation that brain-derived CD8+ T lymphocytes did not exhibit CTL activity *ex vivo* favoring the idea that MHC-restricted cytotoxicity is not the major role of this lymphoid subset within CNS tissue.

In view of the striking differences in virus-specific humoral as well as CD8+ T cell-mediated immune reactions between the two rat strains the role of CD4+ T cells during JHMV-infection has to be discussed. This lymphoid subpopulation is mainly responsible for regulating the different arms of the immune system. Previously published data from our laboratory (Imrich *et al*, 1994) elucidated that vigorous intrathecal synthesis of virus-specific antibodies is preceded by a strong proliferative response of JHMV-specific CD4+ T lymphocytes in the draining cervical lymph nodes (CLN). In LEW rats, however, a significantly reduced viral antigen-driven expansion of this T cell subset could be detected in CLNs (Imrich *et al*, 1994). Thus, distinct antibody and CD8+ T cell responses in LEW and BN rats following JHMV challenge may be the consequence of different priming and differentiation events within the CD4+ T lymphocyte compartment.

Aside from their regulatory role in the immune system response CD4+ T lymphocytes can exert pathogenic effector functions in the course of JHMV-induced demyelinating CNS disorder. This idea is supported by preliminary results from our laboratory demonstrating that reconstitution of γ-irradiated LEW rats by a pure fraction of naive, syngeneic CD4+ T lymphocytes shortly before JHMV-infection resulted not only in an earlier onset of disease but in an enhanced neurological symptomatology compared to unmanipulated, virus-infected control animals (Schwender *et al*, 1993). Watanabe *et al* (1983) described in JHMV-infected LEW rats the generation of myelin-specific, MHC class II restricted spleenocytes which after adoptive transfer into histocompatible, normal recipients induced white matter damage comparable to EAE. BN rats, however, never showed any autosensitization after JHMV challenge (Watanabe *et al*, 1983). Additionally, as shown by Imrich *et al* (1994), sensitization of CD4+ T cells in CLNs of LEW but not of BN rats is characterized by a broad polyclonal *ex vivo* proliferation. From these data one could speculate that virus-specific as well as myelin-specific CD4+ T effector cells are expanded in the course of JHMV-induced encephalomyelitis in LEW rats.

Whereas exact effector functions of both, CD8+ and CD4+ T lymphocytes *in vivo* will require further studies, on the basis of our present data and previously published findings we propose the following sequence of events emerging after intracerebral JHMV-infection of rats. Resistant BN rats respond immediately to viral CNS-infection by a rapid and efficient intrathecal synthesis of virus-neutralizing antibodies thereby limiting infection to small areas of the brain. Under the umbrella of this vigorous humoral immune response, moderate CD8+ T cell activity is sufficient to eliminate virus-infected cells subclinically. In contrast, a delayed onset of antiviral immune reactions in LEW rats allows virus-spreading throughout the CNS. Subsequently, numerous infiltrating CD8+ T lymphocytes may contribute to extensive tissue damage and thereby to paralytic disease. However, if in parallel to CD8+ T effector cells a moderate virus-specific antibody response is recruited, this seems to be sufficient for recovery from the disease.

Materials and methods

Virus

Coronavirus JHM used for intracerebral infection of animals was propagated and purified as previously described (Wege *et al*, 1978). Target cells used in cytotoxic assays were infected with a tissue culture adapted JHMV-variant obtained after several passages through the myeloma cell line (SP2/0-Ag14 (Shulman *et al*, 1978).

Animals

Specific pathogen free rats of the inbred strains

LEW ($RT1^l$) and BN ($RT1^n$) were purchased at 3 weeks of age from the 'Zentralinstitut für Versuchstierzucht' (Hannover, Germany) and the 'Charles River GmbH' (Sulzfeld, Germany), respectively. After intracerebral inoculation with approximately 10^3 plaque forming units (PFU) of JHMV in a volume of 50µl each animal was examined daily for neurological signs and scored according to the following scheme (Dörries *et al*, 1991): (0) no overt signs of disease, (1) unusual seclusion from other rats, (2) ataxia, (3) paralysis of one or two legs, (4) paralysis of three legs or tetraplegia, (5) moribund.

Cell lines

All cell lines were grown in complete growth medium (CGM), ie RPMI 1640 (Serva, Heidelberg, Germany) supplemented with 10% FCS (Serva, Heidelberg, Germany), 2mM L-Glutamin, 2mM sodium pyruvate, non-essential amino acids (Biochrom, Berlin, Germany), 50µM 2-mercaptoethanol (Merck, Darmstadt, Germany) and 100 units ml^{-1} penicillin and 100µg of streptomycin per ml at 37°C in a humidified atmosphere containing 5% CO_2.

The lymphoid cell lines LEW1B4 ($RT1.A^l$) and BN3B2 ($RT1.A^n$) were used as target cells in cytotoxic assays. They were obtained by electrofusion (Schmitt and Zimmermann, 1989; for review see Zimmermann, 1986) or primary rat spleenocytes with the mouse myeloma SP2/0-Ag14. Briefly, lymphocytes and myeloma cells were mixed at a proportion of 2:1 and washed twice in fusion medium (1 mg ml^{-1} bovine serum albumin (BSA); 75mM D (-)-sorbitol; 0,5mM magnesium acetate; 0,1mM calcium acetate). Cells were resuspended in fusion medium at a concentration of $3 \cdot 10^6$ cells ml^{-1} and 200µl of this cell suspension was transferred into a fusion chamber. After aligning the cells in an alternating electrical field of 1,5MHz at 300V/cm for 30s cell-to-cell fusion was achieved by two square pulses of 1,25kV/cm and 15µs duration each at a 1s interval. Subsequently, the alternating field was applied for another 30 s. All steps were carried out at room temperature. To allow membrane resealing fusion chambers were kept undisturbed for 30 min before opening and rinsing with 1ml CGM. 300µl of this cell suspension was added to one well of a 24 well cloning plate (Greiner, Nürtingen, Germany) containing 1ml CGM. Following a 24 h incubation period at 37°C and 5% CO_2 growth medium was replaced by CGM supplemented with 0,1mM/l hypoxanthine, 0,4µM/l aminopterin and 16µM/l thymidine (HAT, Boehringer Mannheim, Mannheim, Germany). Growing hybridomas were cloned and subcloned and assayed for rat MHC class I expression by FACS analysis.

A mixture of several herpes papio transformed monkey B-cell lines was used as xenogeneic stimulators in lymphocyte cultures.

Isolation of CNS-infiltrating leukocytes

At different times past infection animals were sacrificed and extensively perfused with PBS. Brain and spinal cord were removed and lymphocytes were isolated according to Schwender *et al* (1991) with minor modifications. After mechanical dissociation and enzymatic digestion CNS material was resuspended in 35ml isotonic Percoll® (Pharmacia, Freiburg, Germany) at a density of 1,060 g ml^{-1}. The suspension was underlayered with 5ml Percoll® at 1,098 g ml^{-1} and subsequently overlayered by 5ml Hank's buffer. After 20 min of centrifugation (1250g)

Table 1 Monoclonal antibodies and polyclonal IgG fractions used for immunostaining of lymphocytes and fusion hybrids

Antibody	*Specificity*
OX1	Rat CD45
OX33	Rat CD45RA or A/B (B-cell specific)
Goat anti rat IgG+IgM	Rat IgG+IgM
R73	Rat α/β T-cell antigen receptor
1F4	Rat CD3 complex
W3/25	Rat CD4 molecule
OX8	Rat CD8 molecule
3.2.3	Triggering structure on rat NK-cells
OX22	Rat CD45RC
OX39	Rat IL-2 receptor (CD25)
OX21	Human C3b inactivator (control mouse mab)
I169.1	Rat $RT1.A^l$
OX27	Rat $RT1.A^a$
OX6	Rat RT1.B[I-A]
HB102	Mouse $H\text{-}2D^d$
13/4	Mouse $H\text{-}2IE^{k,d}$
Rabbit anti rat whole serum	Rat immunoglobulins
Goat anti mouse IgG	Mouse IgG

Table 2 Phenotype of hybridoma cell lines used as targets in cytotoxic assays

	Hybridoma cell line	
Antigen	*LEW1B4*	*BN3B2*
Rat CD45	+	+
Rat CD3 complex	–	–
Rat α/βTCR	–	–
Rat CD4 molecule	–	–
Rat CD8 molecule	–	–
Rat B cell LCA	–	–
Rat surface Ig	–	–
Antibody secretion	–	–
Rat NK cell	–	–
$Rat\ RT1.A^a$	–	+
$Rat\ RT1.A^l$	+	–
$Rat\ RT1.B^a$	–	–
$Rat\ RT1.B^l$	–	–
$Mouse\ H\text{-}2IE^d$	–	–
$Mouse\ H\text{-}2D^d$	+	+

lymphocytes were collected from the top of the 1,098 g ml^{-1} Percoll® cushion, washed once in Hank's buffer and counted.

Preparation of cytotoxic T effector cells
Brain-isolated lymphocytes were mixed with irradiated (30 Gy) xenogeneic stimulator cells to give a final ratio of 2:1. Cells ($5 \cdot 10^6$) were placed into one well of a 12 well flat bottom plate (Costar, Cambridge, USA) in a volume of 4ml CGM supplemented with 2 µg ml^{-1} concanavalin A (ConA) and 50% tissue culture supernatant obtained from ConA activated spleenocytes (CASUP). Responder cells were harvested by BSA density gradient centrifugation 4 days later and cultured overnight in the presence of 50% CASUP at 37°C and 5% CO_2.

Monoclonal and polyclonal antibodies
Table 1 summarizes the panel of mouse monoclonal antibodies (mabs) and polyclonal IgG fractions used for phenotyping of CNS-derived leukocytes and mouse-rat hybridoma cell lines. The phenotypic characteristics of the cell lines LEW1B4 and BN3B2 are presented in Table 2.

Flow cytometry
Fusion hybrids as well as lymphocytes were stained by single-color and two-color immunofluorescence, respectively. Cells ($2 \cdot 10^5$) were incubated with primary mab for 20 min at 4°C. Subsequently, they were washed and fluorescence intensity was measured using a FACScan® (Becton Dickinson, Heidelberg, Germany). In the case of dual-labeling an unconjugated primary mouse mab was detected by secondary PE-labeled goat anti mouse antibodies. After a washing step free binding sites of secondary antibodies were saturated by 20% normal mouse serum before adding the second antibody, conjugated with FITC. Collected data were processed by a Hewlett Packard 6000 computer and the Lysis II software package. $CD8^+/\alpha/\beta TCR^+$ cells/CNS of individual animals were quantified by multiplying the percentage of positive cells by the total number of CNS-extracted leukocytes.

Cytotoxicity assay
The hybridoma cell lines LEW1B4 and BN3B2 were infected with a tissue culture adapted variant of JHMV at a multiplicity of infection (moi) of 10–15 for 5 h at 37°C. Cells were washed once with CGM and infected as well as uninfected cells were labeled with 3,7MBq ^{51}Cr (DuPont, Dreieich, Germany)/$1 \cdot 10^6$ cells for 1h at 37°C. Subsequently, targets were washed twice with CGM, resuspended with effectors at various ratios in a final volume of 200µl CGM and seeded in a 96 well V-bottom plate (Greiner, Nürtingen, Germany). After a 5h incubation period at 37°C, 5% CO_2, 100µl of cell free supernatant was removed and radioactivity released was determined in a γ-counter (LKB-Wallac, Turku, Finland). Percent specific lysis was calculated according to the formula: [(cpm in experiment - cpm spontaneous release)/(cpm in 2% Triton X 100 - cpm spontaneous release)]·100. Spontaneous release from all target cells was always less than 25% of the maximum release.

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References

Dal Canto MC, Rabinowitz SG (1982). Experimental models of virus-induced demyelination of the central nervous system. *Ann Neurol* **11**: 109–127.

Dörries R, Watanabe R, Wege H, ter Meulen V (1986). Murine coronavirus induced encephalomyelitides in rats: analysis of immunoglobulins and virus-specific antibodies in serum and cerebrospinal fluid. *J Neuroimmunol* **12**: 131–142.

Dörries R, Watanabe R, Wege H, ter Meulen V (1987). Analysis of the intrathecal humoral immune response in Brown Norway (BN) rats, infected with the murine coronavirus JHM. *J Neuroimmunol* **14**: 305–316.

Dörries R, Schwender S, Imrich H, Harms H (1991). Population dynamics of lymphocyte subsets in the central nervous system of rats with different susceptibility to coronavirus-induced demyelinating encephalitis. *Immunology* **74**: 539–545.

Dörries R, Schwender S, Imrich H, Hein A, Czub S, Bartz R (1993). Characterization of the inflammatory T-lymphocyte response in coronavirus-induced demyelinating encephalomyelitides of rats (abstr). *Abstr IXth Intern Cong Virol 1993* W55-4, p 88.

Fleming JO, Wang FI, Trousdale MD, Hinton DR, Stohlman SA (1990). Immunopathogenesis of demyelination induced by MHV-4. In Cavanagh D, Brown TDK (eds.). Coronaviruses and their diseases. *Adv Exp Med Biol* **276**: 565–572, Plenum Press, New York.

Hudson SJ, Streilein JW (1994). Functional cytotoxic T cells are associated with focal lesions in the brains of SJL mice with experimental herpes simplex encephalitis. *J Immunol* **152**: 5540–5547.

Imrich H, Schwender S, Hein A, Dörries R (1994). Cervical lymphoid tissue but not the central nervous system supports proliferation of virus-specific T lymphocytes during coronavirus-induced encephalitis in rats. *J Neuroimmunol* **53**: 73–81.

Knobler RL, Tunison LA, Lampert PW, Oldstone MBA (1982). Selected mutants of mouse hepatitis virus type 4 (JHM strain) induce different CNS diseases. *Am J Pathol* **109**: 157–168.

Lampert PW, Sims JK, Kniazeff AJ (1973). Mechanism of

demyelination in JHM virus encephalomyelitis. *Acta Neuropath (Berl)* **24**: 76–85.

Lindsley MD, Thiemann R, Rodriguez M (1991). Cytotoxic T cells isolated from the central nervous system of mice infected with Theiler's virus. *J Virol* **65**: 6612–6620.

Liu Y, Blanden RV, Müllbacher A (1989). Identification of cytolytic lymphocytes in West Nile virus-infected murine central nervous system. *J Gen Virol* **70**: 565–573.

Nagashima K, Wege H, Meyermann R, ter Meulen V (1978). Corona virus induced subacute demyelinating encephalomyelitis in rats: A morphological analysis. *Acta Neuropathol (Berl)* **44**: 63–70.

Nagashima K, Wege H, Meyermann R, ter Meulen V (1979). Demyelinating encephalomyelitis induced by a long-term corona virus infection in rats. *Acta Neuropathol (Berl)* **45**: 205–213.

Pearce BD, Hobbs MV, McGraw TS, Buchmeier MJ (1994). Cytokine induction during T-cell-mediated clearance of mouse hepatitis virus from neurons in vivo. *J Virol* **68**: 5483–5495.

Planz O, Bilzer T, Sobbe M, Stitz L (1993). Lysis of major histocompatibility complex class I-bearing cells in Borna disease virus-induced degenerative encephalopathy. *J Exp Med* **178**: 163–174.

Ramsay A, Ruby J, Ramshaw I (1993). A case for cytokines as effector molecules in the resolution of virus infection. *Immunol Today* **14**: 155– 157.

Rodriguez M, Sriram S (1988). Successful therapy of Theiler's virus-induced demyelination (DA strain) with monoclonal anti-Lyt-2 antibody. *J Immunol* **140**: 2950–2955.

Schijns VECJ, Van der Neut R, Haagmans BL, Bar DR, Schellekens H, Horzinek MC (1991). Tumour necrosis factor-α, interferon-γ and interferon-β exert antiviral activity in nervous tissue cells. *J Gen Virol* **72**: 809–815.

Schmied M, Breitschopf H, Gold R, Zischler H, Rothe G, Wekerle H, Lassmann H (1993). Apoptosis of T lymphocytes in experimental autoimmune encephalomyelitis. Evidence for programmed cell death as a mechanism to control inflammation in the brain. *Am J Pathol* **143**: 446–452.

Schmitt JJ, Zimmermann U (1989). Enhanced hybridoma production by electrofusion in strongly hypo-osmolar solutions. *Biochim Biophys Acta* **983**: 42–50.

Schwender S, Imrich H, Dörries R (1991). The pathogenic role of virus-specific antibody-secreting cells in the central nervous system of rats with different susceptibility to coronavirus-induced demyelinating encephalitis. *Immunology* **74**: 533–538.

Schwender S, Hein A, Imrich H, Dörries R (1993). On the role of different lymphocyte subpopulations in the course of coronavirus MHV IV (JHM)-induced encephalitis in Lewis rats. In: Laude H, Vautherot JF (eds). Coronaviruses: molecular biology and virus-host interactions. *Adv Exp Med Biol* **342**: 425–430, Plenum Press, New York.

Shulman M, Wilde CD, Köhler G (1978). A better cell line for making hybridomas secreting specific antibodies. *Nature* **276**: 269–270.

Sorensen O, Dugre R, Percy D, Dales S (1982). *In vivo* and *in vitro* models of demyelinating disease: endogenous factors influencing demyelinating disease caused by mouse hepatitis virus in rats and mice. *Infect Immun* **37**: 1248–1260.

Stohlman SA, Kyuwa S, Polo JM, Brady D, Lai MMC, Bergmann CC (1993). Characterization of mouse hepatitis virus-specific cytotoxic T cells derived from the central nervous system of mice infected with the JHM strain. *J Virol* **67**: 7050–7059.

Tishon A, Eddleston M, de la Torre JC, Oldstone MBA (1993). Cytotoxic T lymphocytes cleanse viral gene products from individually infected neurons and lymphocytes in mice persistently infected with lymphocytic choriomeningitis virus. *Virology* **197**: 463–467.

Watanabe R, Wege H, ter Meulen V (1983). Adoptive transfer of EAR-like lesions by BMP stimulated lymphocytes from rats with coronavirus-induced demyelinating encephalomyelitis. *Nature* **305**: 150–153.

Watanabe R, Wege H, ter Meulen V (1987). Comparative analysis of coronavirus JHM-induced demyelinating encephalomyelitis in Lewis and Brown Norway rats. *Lab Invest* **57**: 375–384.

Wege H, Müller A, ter Meulen V (1978). Genomic RNA of the murine coronavirus JHM. *J Gen Virol* **41**: 217–227.

Wege H, Siddel S, ter Meulen V (1982). The biology and pathogenesis of coronaviruses. In Cooper M (ed). *Curr Topics Microbiol Immunol* **99**: 165–200, Springer-Verlag, Berlin.

Wege H, Watanabe R, Koga M, ter Meulen V (1983). Coronavirus JHM-induced demyelinating encephalomyelitis in rats: influence of immunity on the course of disease. In: Behan PO, ter Meulen V, Clifford Rose F (eds). Immunology of Nervous System Infections. *Prog Brain Res* **59**: 221–231, Elsevier Science Publishers BV, Amsterdam.

Weiner LP (1973). Pathogenesis of demyelination induced by a mouse hepatitis virus (JHM virus). *Arch Neurol* **28**: 298–303.

Williamson JSP, Stohlman SA (1990). Effective clearance of mouse hepatitis virus from the central nervous system requires both $CD4^+$ and $CD8^+$ T cells. *J Virol* **64**: 4589–4592.

Yamaguchi K, Goto N, Kyuwa S, Hayami M, Toyoda Y (1991). Protection of mice from a lethal coronavirus infection in the central nervous system by adoptive transfer of virus-specific T cell clones. *J Neuroimmunol* **32**: 1–9.

Zimmermann U (1986). Electrical breakdown, electropermeabilization and electrofusion. *Rev Physiol Biochem Pharmacol* **105**: 175–256.

Zinkernagel RM, Doherty PC (1973). Cytotoxic thymus-derived lymphocytes in cerebrospinal fluid of mice with lymphocytic choriomeningitis. *J Exp Med* **138**: 1266–1269.