Immunology 1991 **74** 539–545

Population dynamics of lymphocyte subsets in the central nervous system of rats with different susceptibility to coronavirus-induced demyelinating encephalitis

R. DÖRRIES, S. SCHWENDER, H. IMRICH & H. HARMS *Institut für Virologie und Immunobiologie der Universität Würzburg, Würzburg, Germany*

Accepted for publication 1 July 1991

SUMMARY

The inflammatory response in the central nervous system (CNS) of rats with differing susceptibility to demyelinating encephalitis induced by coronavirus MHV4 was characterized. Topographical maps showing the arrangement of infiltrating lymphocyte subsets in virus-infected tissue were developed by digital-image processing of immunohistologically stained CNS sections. The kinetics of the inflammatory process was evaluated by flow-cytometry on lymphocytes isolated from the CNS. Cumulative data obtained with these two techniques demonstrated the following features. In susceptible Lewis (LE) rats, viral antigens were disseminated throughout the CNS, including spinal cord. Onset as well as recovery from neurological disease was associated with a steep rise of infiltrating $CD8^+$ T cells, which localized in close contact to virus-infected cells. Accompanying convalescence was a slight increase in B($OX33^+$) cells in the CNS and the accumulation of immunoglobulin-containing cells in the centre of virus-infected areas. In clinically resistant Brown Norway (BN) rats, virus-infected cells were mainly restricted to small periventricular foci and the extent of lymphocyte infiltration was never as high as that found at any time during the course of infection in LE rats. There were striking differences in the $CD8^+$ T-cell population compared to LE rats. Cells of this phenotype were identified in virus-affected areas of BN rats only early after infection, and their infiltration profile revealed much lower quantities than in the CNS of susceptible LE rats. Although the population dynamics of B($OX33^+$) lymphocytes were comparable in BN and LE rats, as determined by flow-cytometry, less immunoglobulin-containing B cells were detected in virus-infected areas of BN rats.

INTRODUCTION

The intracerebral infection of rats with the murine coronavirus JHM (MHV4) is an excellent animal model to study the pathogenic role of virus-specific immune responses during primary demyelination in the central nervous system (CNS). Different courses of the infection have been reported and these are dependent on the rat strain, the age of the animals and the type of virus used. Lewis (LE) rats tend to develop a subacute demyelinating encephalomyelitis (SDE) accompanied by neurological signs, including hindleg paresis, ataxic gait and tetraplegia. In contrast, Brown Norway (BN) rats very rarely show clinical signs if they are infected later than 21 days post partum, although viral antigens are detectable in CNS tissue. Whereas in clinically healthy BN rats, a limited number of small demyelinated foci are localized around the ventricles, in SDE-diseased LE rats, wide-spread demyelination is seen, extending from the brain to the spinal cord.[1]

CNS-localized immune effector mechanisms must play an important role in the outcome of infection. In contrast to LE rats, strong intrathecal synthesis of virus-specific oligoclonal antibodies is typically observed in BN rats.[2,3] Mononuclear infiltration into the CNS is detectable in both rat strains,[1] and autosensitization of $CD4^+$ T lymphocytes to the oligodendrocyte-specific basic myelin protein (BMP) has been reported in LE rats.[4] Although these data indicate the engagement of the immune system in the CNS, important information about quantity and quality of the lymphoid infiltration process is lacking. This is particularly true for the homing pattern of lymphocyte subsets in brain tissue as well as for their kinetics of infiltration during the course of infection. We therefore characterized the inflammatory lymphocyte response in the CNS of both rat strains during acute encephalitis. Digital image processing of immunohistologically stained brain sections was applied to investigate the spatial arrangement of lymphocyte subsets in

Correspondence: Dr R. Dörries, Institut für Virologie und Immunobiologie der Universität Würzburg, Versbacherstr. 7, 8700 Würzburg, Germany.

virus-infected areas and dynamic changes in the lymphocyte subset populations were recorded by flow cytometry on lymphoid cells isolated from the CNS by density gradient centrifugation.

MATERIALS AND METHODS

Virus

The JHM strain of coronavirus (MHV4) was propagated as described in the accompanying paper (p. 533).[5]

For preparation of virus-specific hyperimmune serum in mice, JHM virus was propagated and purified as described by Wege *et al.*[6]

Animals

Three-week-old specific pathogen-free LE and BN rats were obtained from the Zentralinstitut für Versuchstierzucht (Hannover, Germany). They were inoculated intracerebrally with 60 μl of JHM (HS) containing 800 plaque-forming units (PFU) of virus. Rats were observed daily for appearance of neurological signs and scored according to the following scale: (0) no overt disease; (1) uncommon social behaviour, such as separation from the other rats within the cage; (2) paresis of one or two legs; (3) paresis of more than two legs; (4) paralysis; and (5) moribund. The mean value for clinical signs of all rats was calculated for each day.

Monoclonal antibodies and polyclonal IgG fractions

Monoclonal antibodies (mAb) and polyclonal IgG fractions, which were used as primary antibodies to stain lymphocytes or viral antigens, are summarized in Table 1.

Table 1. Monoclonal antibodies and polyclonal IgG fractions used for staining of lymphocytes and viral antigens

Antibody	Specificity
W3/25[7]	Rat CD4 molecule
OX8[8]	Rat CD8 molecule
OX12[9]	Rat immunoglobulin κ-chain
OX21[10]	Human C3b receptor
OX33[11]	Rat LCA on B lymphocytes
ED1[12]	Rat macrophages
HIS14[13]	Rat LCA on B lymphocytes
R73[14]	Rat α/β T-cell antigen receptor
Mouse anti-MHV4 (IgG)	MHV4 proteins

Preparation of the IgG fraction from mouse anti-MHV4 serum

BALB/c mice bred in this Institute were immunized by intraperitoneal (i.p.) injection of 100 μg of UV-inactivated virus (2500 mW/cm^2 for 5 min) precipitated in alum[15] and supplemented with inactivated *Pertussis bordetella* (10^{11} particles/ml). Intraperitoneal booster injections were done 3 weeks and 4 weeks after primary immunization with 50 μg of inactivated virus in phosphate-buffered saline (PBS). Blood was collected from the animals 4 days after the last booster injection. After clotting overnight at 4°, serum was separated by centrifugation (1500 *g*, 10 min, 4°) and kept at −20°. The IgG fraction was prepared by three repeated precipitations with ammonium sulphate, followed by ion-exchange chromatography on DEAE–Sephacel.[15]

Localization of lymphocytes and virus-infected cells in situ

For immunohistological studies the brain was removed from killed animals after perfusion with 200 ml of 20% sucrose in PBS. The CNS material was cut into six pieces in a caudorostral direction (approximately 5 mm thick) and snap-frozen in melting 2-methyl butane. Frozen sections were kept in liquid nitrogen for several hours and finally stored in air tight vials at −80°.

Serial sections (6 μm) were prepared from frozen CNS using a Leitz cryostat 1720 (Leitz, München, Germany) equipped with a C-shaped knife at −20°. Sections were air-dried on glass slides previously cleaned once with 1 M HCl (Merck, Darmstadt, Germany), twice with H_2O and once with ethanol (Roth, Karlsruhe, Germany) and coated with gelatine-chromalum (0·5%, 0·2%, respectively). After fixation in dehydrated acetone (acetone saturated with $CuSO_4$) at −20° for 10 min, these slides were stored in a dry atmosphere until use.

Viral antigens and lymphocyte subsets were detected in serial CNS tissue sections by an indirect staining procedure using primary mouse antibodies and alkaline phosphatase-labelled rat anti-mouse IgG as a secondary antibody. Each section of a series was incubated with a different primary antibody in the sequence anti-MHV4, anti-CD4 (W3/25), anti-CD8 (OX8), anti-macrophage (ED1), anti-B cell (His 14) and anti-κ chain (OX12).

Primary antibodies were diluted in TBS (0·05 M Tris, 0·15 M NaCl, pH 8·0) supplemented with 20% normal rat serum (NRS). In the case of OX12, 20% of bovine serum albumin (BSA) was substituted for NRS. After incubation overnight at 4° in a humid atmosphere, the slides were washed three times in TBS (10 min each cycle at room temperature). Subsequently, as a secondary antibody the IgG fraction of rat anti-mouse IgG serum labelled with alkaline phosphatase (AP) (Dianova, Hamburg, Germany) diluted in supplemented TBS was applied to the sections. After 1 hr at room temperature the slides were washed as described and incubated with the substrate Neufuchsin prepared according to a technical note released by the Dako company (Hamburg, Germany). The substrate was applied to the sections for 30 min at room temperature on a rocker platform. To stop substrate conversion, the slides were washed in distilled H_2O and counterstained in Ehrlich's haematoxilin for 3 min according to Romeis.[16] For microscopic examination the slides were mounted in buffered glycerol (pH 8·0, adjusted with saturated Tris solution).

Computer-aided cytophotometry and image processing

Serial CNS sections stained by the immune alkaline phosphatase technique were examined under an Axiomat microscope (Zeiss, Oberkochen, Germany). Pictures of these sections were digitized by a video camera model DXG M2 (Sony, Köln, Germany) mounted on the microscope and processed on a VAX computer (Digital Equipment Company, München, Germany) using an algorithm for colour analysis from H. Harms (Würzburg, Germany).

Quantification and phenotypic characterization of isolated CNS lymphocytes

Lymphocytes were isolated from the CNS as described in the accompanying paper (p. 533).[5] Briefly, spinal cord and brain were removed from killed animals after extensive perfusion with PBS. Brain and spinal cord were mechanically disrupted and

subjected to enzymatic digestion. Lymphocytes were isolated from this homogenized brain material by density gradient centrifugation.

Lymphocytes isolated from the CNS were quantified and phenotypically identified by flow-cytometry. For single-colour fluorescence, 10^5 lymphocytes were suspended in 100 μl of a primary antibody diluted in Hanks' buffer supplemented with 3% FCS, 10% NRS, 10% normal goat serum and 0·02% NaN_3 (dilution buffer). After incubation for 20 min at 4° the cells were diluted to 4 ml in Hanks' buffer (3% FCS, 0·02% NaN_3), centrifuged (6 min, 170 ***g***) and resuspended in 100 μl of phycoerythrin (PE)-coupled goat anti-mouse IgG antibody (Dianova, Hamburg, Germany) in dilution buffer. After another 20 min at 4° the cells were washed, resuspended in 500 μl of Hanks' buffer (3% FCS, 0·02% NaN_3) and analysed. In the case of two-colour fluorescence these indirectly stained lymphocytes were processed further by incubation in 100 μl of a primary FITC-labelled antibody in Hanks' buffer (3% FCS, 0·02% NaN_3, 20% normal mouse serum) followed by washing, resuspension in 500 μl of Hanks' buffer (3% FCS, 0·02% NaN_3) and flow-cytometric analysis.

B-lymphocytes were identified by single-colour analysis using the mAb OX 33 (Serotec, Wiesbaden, Germany). T-lymphocyte subpopulations were stained by two-colour immunofluorescence. In the case of CD4$^+$ T-lymphocytes the mAb R73 specific for the α/β chain of the rat T-cell antigen receptor (TcR) was used in combination with the FITC-labelled W3/25 mAb specific for the rat CD4. Cytotoxic (CD8$^+$) T lymphocytes were detected by the same procedure but using the FITC-labelled mAb OX 8 against rat CD8. As a control, the mouse mAb OX 21, specific for the human complement receptor C3b, was used as primary antibody.

Flow-cytometry was carried out on a FACScan (Becton-Dickinson, Heidelberg, Germany). Collected data were processed by a Hewlett Packard 6000 computer and the Consort 30 software package. The number of B(OX33$^+$), T(CD4$^+$, α/β TcR$^+$) and T(CD8$^+$, α/β TcR$^+$) cells per CNS was determined by multiplying the percentage of positive cells by the total number of lymphocytes recovered from the CNS of individual animals.

RESULTS

Course of the infection

MHV4-infected animals were observed over 27 days post-infection (p.i.) and neurological signs were scored daily. Out of 54 LE rats, 49 animals (91%) developed signs of disease. Roughly 41% (22 rats) became moribund within the first 16 days p.i., 50% (27 rats) recovered from disease and approximately 9% (5 rats) remained asymptomatic up to 27 days p.i. Overt neurological signs appeared 6 days p.i. and increased until 12 days p.i. Thereafter animals almost completely recovered up to 27 days p.i. (Fig. 1). None of the BN rats (41 in total) revealed any clinical signs of infection during the time of observation.

Lymphocyte distribution in virus-infected areas

To identify infiltrating lymphocyte subsets *in situ* we applied immunohistochemistry to localize lymphoid cells in relation to virus-infected target cells. By computer-aided image processing,

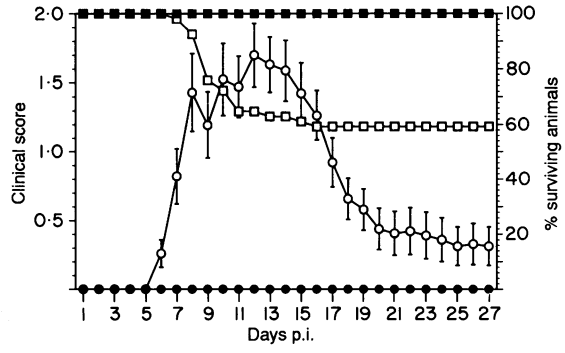

Figure 1. Course of the infection. Neurological signs of individual animals were scored daily in MHV4-infected LE ($n=54$) and BN rats ($n=41$). The average symptomatology of each rat strain is given by the arithmetic mean value. (○), LE rats; (●), BN rats; days p.i., days post-infection. Vertical bars indicate the standard error. Due to the fatal outcome of the infection in some of the LE rats, the number of animals scored per day decreases with increasing time of infection. The percentage of surviving animals is indicated by solid and open squares; (□) LE rats; (■) BN rats.

topographical maps of virus-affected areas were created at 7, 14 and 21 days p.i.

During initial screening for virus-affected areas in the CNS, a fundamental difference was noticed between BN and LE rats at 7 days p.i. Viral antigens in BN rats were mostly limited to foci around the ventricles extending from the ependymal cell layer into the white matter. In contrast, LE rats revealed a disseminated distribution of viral antigens throughout the CNS, including the spinal cord. The typical arrangement of lymphocytes and viral antigens which was repeatedly detected in LE and BN rats is shown in Fig. 2a–f. CD8$^+$ lymphocytes were the major lymphocyte subtype in apposition to virus-infected cells in tissue lesions of both rat strains at this stage of the infection (Fig. 2 a,b) and were surrounded by a few CD4$^+$ cells. B(κ-chain$^+$) lymphocytes were very rarely seen and their localization revealed no constant pattern.

This picture completely changed by 14 days p.i., especially in LE rats which usually started to recover from neurological disease at this time (Fig. 2c). B(κ-chain$^+$) lymphocytes appeared in the centre of infected areas and the number of surrounding CD4$^+$ cells increased. It is noteworthy that, *in situ*, B lymphocytes were only stained by the immunoglobulin κ-chain-specific mAb OX12, but not by either the mAb HIS 14 nor by the B-cell specific mAb OX33, both of which detect the B-cell specific leucocyte common antigen (LCA). Occurrence of CD8$^+$ lymphocytes was reduced in the limited areas of viral antigen deposits. In BN rats, virus-infected cells were even less frequently detected than in LE rats. The lesions were now dominated by CD4$^+$ cells in close contact with virus-infected cells and a few B lymphocytes were present in the surrounding parenchyma (Fig. 2d).

Viral antigen was almost undetectable in LE and BN rats 21 days p.i. Only small scattered areas of virus-infected cells were present in LE rats, accompanied by a few B lymphocytes, very small numbers of CD8$^+$ cells and some CD4$^+$ cells (Fig. 2e). At this time animals had completely recovered from clinically visible disease. In BN rats the very few virus-infected areas which could still be detected were clearly dominated by the CD4$^+$ cell population (Fig. 2f). Interestingly, they were seen in

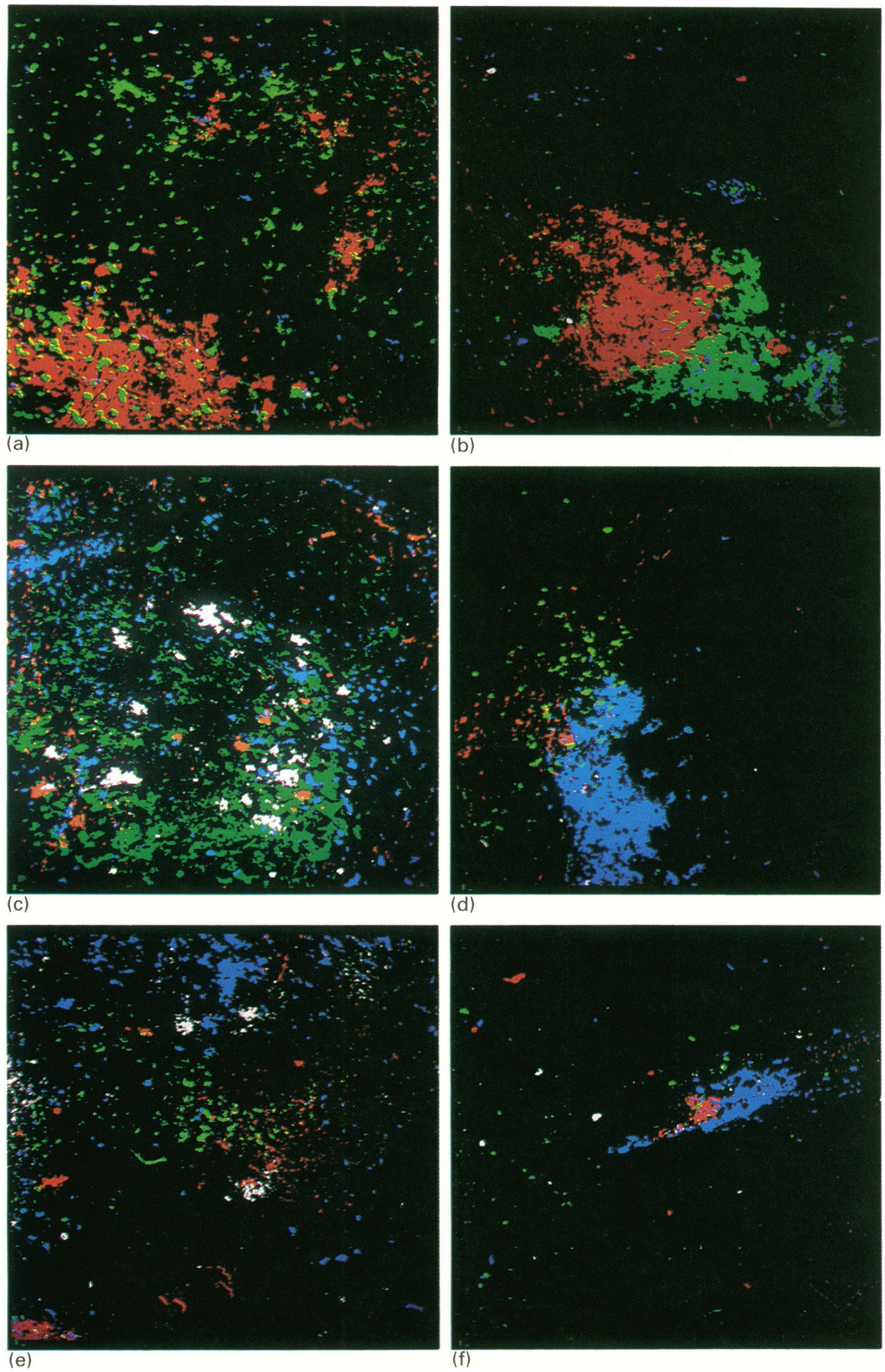

Figure 2. Digitized pictures of lymphocytes in virus-infected CNS tissue. Digitized pictures (512 × 512 pixels displayed per picture, 0·45 pixel/μm, thus a CNS area of approximately 1 mm square is shown) of individual serial sections stained by the immune AP technique were processed using a colour analysis program from H. Harms (Würzburg, Germany). Staining of the CNS tissue itself was changed in all pictures of a series to a black background colour. Staining of viral antigens is shown in red, the colour green was assigned to $CD8^+$ T cells, blue to $CD4^+$ T cells and white to B(κ-chain$^+$) cells. Individual pictures of a series of sections were assembled into a single picture. Frontal sections from the mesencephalon of a LE rat at 7, 14 and 21 days p.i. respectively (a, c, e). Frontal sections from the diencephalon of a BN rat at 7, 14 and 21 days p.i., respectively (b, d, f).

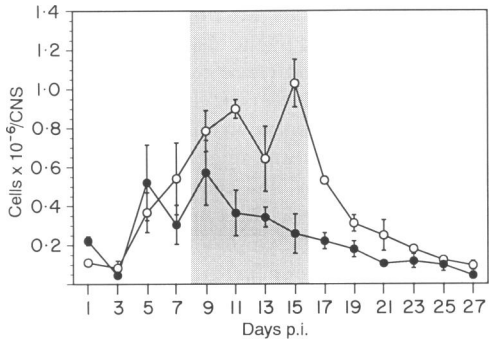

Figure 3. Number of CD4$^+$ T lymphocytes from the CNS at different times post-infection. Each point represents the average number of cells isolated from the CNS of three animals. (○) LE rats; (●) BN rats; days p.i., days post-infection; the shaded area indicates the time period when LE rats were diseased with an average score ≥ 1·0. Vertical bars indicate the standard error.

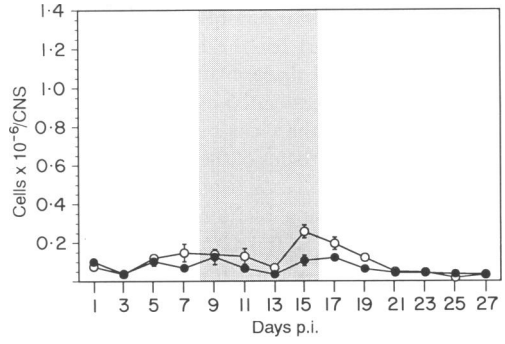

Figure 5. Number of B(OX33$^+$) lymphocytes from the CNS at different times post-infection. Each point represents the average number of cells isolated from the CNS of three animals. (○) LE rats; (●) BN rats; days p.i., days post-infection; the shaded area indicates the time period when LE rats were diseased with an average score ≥ 1·0. Vertical bars indicate the standard error.

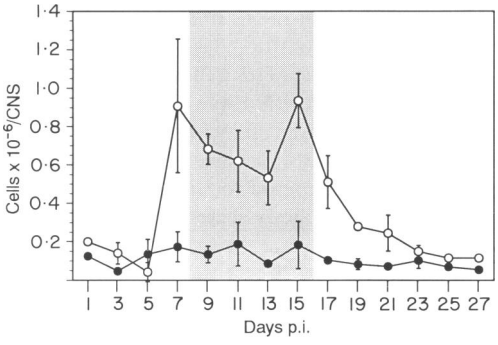

Figure 4. Number of CD8$^+$ T-lymphocytes from the CNS at different times post-infection. Each point represents the average number of cells isolated from the CNS of three animals. (○) LE rats; (●) BN rats; days p.i., days post-infection; the shaded area indicates the time period when LE rats were diseased with an average score ≥ 1·0. Vertical bars indicate the standard error.

close contact with virus-infected cells, comparable to the intimate proximity of CD8$^+$ cells to virus-infected cells at 7 days p.i.

Although staining of macrophages (mAb ED1) was included in these experiments, the results were not merged into the final topographical map, because the high number of these cells at any time during infection obscured the picture of the lymphocyte subset arrangements. It is possible that a few of the CD4$^+$ staining cells may have been due to macrophages as these cells are CD4$^+$ in the rat[17] but our histological data indicated that ED1 and CD4 staining was mostly on mutually exclusive populations (not shown).

Kinetics of the inflammatory response

The results obtained from *in situ* localization of lymphocytes in virus-infected areas of the CNS suggested distinct changes in the population dynamics of individual lymphocyte subsets in the course of the infection. To monitor these changes, lymphocytes were isolated every second day post-infection from the CNS of three LE and three BN rats at each time-point and analysed for the presence of B(OX33$^+$) lymphocytes, CD4$^+$ T(W3/25$^+$, R73$^+$) lymphocytes and CD8$^+$ T(OX8$^+$, R73$^+$) lymphocytes by flow cytometry.

CD4$^+$ T lymphocytes were the dominant cell type infiltrating the brain of BN rats (Fig. 3). Their numbers increased up to 5 days p.i., remained at that level for another 4 days and subsequently dropped to very low numbers by 27 days p.i. In contrast, CD8$^+$ T lymphocytes were rarely isolated from BN CNS (Fig. 4). It is nevertheless clear that CD8$^+$ T cells did respond to the infection, as shown in Fig. 2b, but compared to LE rats foci were less in number. Therefore, on a whole CNS basis (Fig. 4), only relatively few cells were detected by flow cytometry. B(OX33$^+$) lymphocytes formed a slight peak roughly 2 weeks p.i. and dropped thereafter to low numbers (Fig. 5).

In LE rats the infiltration profiles of CD4$^+$ T and CD8$^+$ T lymphocyte subsets were quite different to those observed in BN rats (Figs 3 and 4). Generally the numbers of T lymphocytes were higher compared to the BN rat. Initially the increase of CD4$^+$ T cells followed that of BN rats, but after 7 days p.i. their numbers continued to rise up to 11 days p.i. (Fig. 3). This first peak was followed by a slight drop at Day 13 and a second even higher peak at 15 days p.i. This was exactly the date when animals started to recover from neurological disease. The biphasic nature of the T-lymphocyte infiltration process was even more pronounced within the CD8$^+$ T-cell subset (Fig. 4). After a sharp rise early at 7 days p.i., numbers dropped until 13 days p.i. followed by a second increase at 15 days p.i. Whereas the first peak corresponded to the time-point when animals became diseased, the second peak appeared coincidentally with the second peak of CD4$^+$ T cells. The B(OX33$^+$) lymphocyte infiltration paralleled that of the BN rats, except that the number was slightly higher in LE rats (Fig. 5).

DISCUSSION

It has generally been believed that the lytic infection of oligodendrocytes is the main pathogenic mechanism causing primary demyelination and overt signs of neurological disease during MHV4-induced encephalomyelitis in mice.[18] It is only recently, however, that the pathogenic role of local immune effector mechanisms has been considered of importance in

determining the course and outcome of infection.[19,20] Our data provide additional support for the idea that susceptibility or resistance to neurological disease during demyelinating encephalitides might not be associated exclusively with the neuropathogenic properties of the virus but may, rather, be heavily influenced by the characteristics of the inflammatory response in the CNS.

Digital image processing of immunohistologically stained frozen sections, as shown in this paper for the first time in the CNS of MHV4-infected rodents, revealed the dominance and close association of $CD8^+$ T lymphocytes to virus-infected cells in the CNS of LE rats 7 days p.i. This indicated a very rapid recruitment of cytotoxic effector cells into virus-affected areas of the brain. The sharp and rapid increase of this lymphocyte subset, positively identified as T cells rather than natural killer cells by virtue of expression of the α/β TcR[21] by flow cytometry, strongly supported this assumption. The biphasic nature of the $CD8^+$ T-lymphocyte infiltrate in the CNS was closely related to the onset of neurological signs and also recovery from disease. This may indicate that early in infection, when large areas of the tissue are virus-infected, effector function of these cells could contribute to neurological symptomatology, as is the case, for example, in lymphocytic choriomeningitis virus (LCMV) infection in mice.[22]

With proceeding infection in LE rats, B(κ-chain$^+$) lymphocytes settled in areas of virus infection. Since they neither stained with the pan-B-specific mAb HIS 14 nor with the mAb OX33, it is assumed that they represented differentiated B cells. This circumstance might explain why equivalent numbers of these cells could not be detected in the CNS of LE rats by flow cytometry using the OX33 antibody. Surprisingly, B(κ-chain$^+$) lymphocytes were detected more frequently in virus-affected tissue of LE rats compared to BN rats (Fig. 2c, d). This was unexpected because we have reported previously that BN rats produce a high-titred virus-specific antibody response in the CSF and such a humoral response is barely detectable in LE rats suffering from severe neurological disease.[2,3] It is likely, therefore, that differentiation of B lymphocytes into antibody-secreting cells with virus-specificity is less effective in LE rats. This assumption is supported by data in the accompanying study, where it is demonstrated that virus-neutralizing antibody titres rise much faster and reach higher levels in the CSF of BN rats compared to LE rats. Furthermore, the number of antibody-secreting plasma cells with specificity for MHV4 is roughly threefold higher in the CNS of BN rats. It is only after about 3 weeks p.i. that virus-specific antibody-secreting plasma cells can be isolated from the CNS of LE rats in a quantity comparable to that of BN rats present by 11 days p.i.[5] Thus, the vast majority of B(κ-chain$^+$) cells in the tissue of LE rats most likely represent cells which have not received signals driving them into antibody-secreting plasma cells. Nevertheless, due to the action of the few virus-specific antibody secretors present at 3 weeks p.i. neutralizing antibody titres rise in the CSF of LE rats,[5] which helps to limit spread of the infection to unaffected areas of the CNS. This presumably allows a continuous eradication of virus-infected cells by cytotoxic T cells which, finally, may produce a complete recovery from neurological disease. This view is in agreement with others who have reported that neurological signs of infection as well as clearance of virus from the brain is highly dependent on cytotoxic T lymphocytes in MHV4-induced encephalomyelitis in mice.[19,20]

Additional support for this hypothesis is produced by the data emerging from the BN rat population. Early action of $CD8^+$ T cells, as suggested by the histopathology of an infected area 7 days p.i. could also cause rapid elimination of virus-infected cells. However, due to the limitation of virus-infected cells to a few small periventricular foci, this did not result in overt neurological disease and might explain why on a whole CNS basis only relatively few cells of this phenotype were detected by flow cytometry at this time post-infection. With ongoing infection $CD8^+$ T lymphocytes were almost totally replaced by $CD4^+$ T-lymphocytes homing to virus-infected areas and we do not have a suitable explanation for these unusual kinetics. Nevertheless, their appearance is probably highly significant. In mice, two subtypes of T-helper lymphocytes (Th1, Th2) have been identified.[23] Th1 cells predominantly act as effector cells in delayed-type hypersensitivity (DTH) and in the differentiation of $CD8^+$ T lymphocytes into cytotoxic T cells (CTL) by secretion of interleukin (IL)-2 and interferon-gamma. In contrast, Th2 cells are thought to be the main providers of help for B lymphocytes by secretion of cytokines such as IL-4 and IL-5. BN rats produce much higher levels of antibody than most other rat strains upon antigenic challenge. This is particularly true for the IgE isotype,[24] which is controlled by $CD4^+$T lymphocytes (Th2 cells) secreting IL-4.[25] Additionally, we know that (i) BN rats vigorously synthesize virus-specific antibodies of an oligoclonal nature in CSF;[3] (ii) antibodies secreted by BN plasma cells in the CNS probably have higher affinity compared to LE rats;[5] (iii) in BN rats the level of virus-specific antibody-secreting cells is threefold higher than in the tissue of LE rats at the time of maximal disease,[5] and (iv) neutralizing antibody titres in the CSF of BN rats appear 4–5 days earlier than in LE rats and reach much higher titres.[5] From these data one may speculate that with proceeding infection in BN rats a Th2-like lymphocyte population may localize in virus-infected areas of the CNS, providing help for differentiation of virus-specific B lymphocytes into antibody-secreting plasma cells and thus effectively limit the spread of virus.

In summary, we favour the hypothesis that elimination of coronavirus from an infected rat brain is associated with $CD4^+$ and $CD8^+$ T lymphocyte action, probably as it has been described for the clearance of MHV4 from the brain of infected mice.[20] However, substantial demyelination and neurological disease might result from this action if the virus is allowed to disseminate in the CNS early in infection, due to a delayed differentiation and maturation of B lymphocytes into virus-neutralizing antibody-secreting cells. Currently this hypothesis is under investigation in our laboratory by functional studies on T lymphocytes isolated at different times post-infection from the CNS of both LE and BN rats.

ACKNOWLEDGMENTS

Marion Zips, Barbara Bayer and Andrea Deutscher are gratefully acknowledged for their expert technical assistance. We thank Drs ter Meulen and Sedgwick for critical reading of the manuscript. The work was supported by the Bundesminister für Forschung und Technologie (grant no. KI 01 8839-2).

REFERENCES

1. 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.

2. 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.
3. 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.
4. WATANABE R., WEGE H. & TER MEULEN V. (1983) Adoptive transfer of EAE-like lesions from rats with coronavirus-induced demyelinating encephalomyelitis. *Nature,* **305,** 150.
5. 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.
6. WEGE H., MÜLLER A. & TER MEULEN V. (1978). Genomic RNA of murine coronavirus JHM. *J. gen. Virol.* **41,** 217.
7. WILLIAMS A.F., GALFRE G. & MILSTEIN C. (1977). Analysis of cell surfaces by xenogenic myeloma-hybrid antibodies: differentiation antigens of rat lymphocytes. *Cell,* **12,** 663.
8. BRIDEAU R.J., CARTER P.B., MCMASTER W.R., MASON D.W. & WILLIAMS A.F. (1980) Two subsets of rat T lymphocytes defined with monoclonal antibodies. *Eur. J. Immunol.* **10,** 609.
9. HUNT S.V. & FOWLER M. (1981) A repopulation assay for B and T lymphocytes stem cells employing radiation chimeras. *Cell Tissue Kinetics,* **14,** 445.
10. HSIUNG L.-M., BARCLAY A.N., BRANDON M.R., SIM E. & PORTER R.R. (1982) Purification of human C3b inactivator by monoclonal antibody affinity chromatography. *Biochem. J.* **203,** 293.
11. WOOLLETT G.R., BARCLAY A.N., PUKLAVEC M. & WILLIAMS A.F. (1985) Molecular and antigenic heterogenity of the rat leucocyte-common antigen from thymocytes and T and B lymphocytes. *Eur. J. Immunol.* **15,** 168.
12. DIJKSTRA C.D., DÖPP E.A., JOLING P. & KRAAL G. (1985) The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2 and ED3. *Immunology,* **54,** 589.
13. KROESE F.G.M., WUBBENA A.S., OPSTELTEN D., DEENEN G.J., SCHWANDER E.H., DE LEIJ L., VOS H., POPPEMA S., VOLLBERDA J. & NIEUWENHUIS P. (1987) B lymphocyte differentiation in the rat: Production and characterization of monoclonal antibodies to B lineage-associated antigens. *Eur. J. Immunol.* **17,** 921.
14. HÜNIG T., WALLNY H-J., HARTLEY J.K., LAWETZKY A.G. & TIEFENTHALER A. (1989) A monoclonal antibody to a constant determinant of the rat T cell antigen receptor that induces T cell activation. *J. exp. Med.* **169,** 73.
15. HARLOW E. & LANE D. (1988) *Antibodies: a Laboratory Manual,* pp. 298 and 302. Cold Spring Harbour Laboratory, New York.
16. ROMEIS B. (1968) *Mikroskopische Technik,* p. 165. R. Oldenbourg Verlag, München, Wien.
17. JEFFERIES W.A., GREEN J.R. & WILLIAMS A.F. (1985) Authentic T helper CD4 (W3/25) antigen on rat peritoneal macrophages. *J. exp. Med.* **162,** 117.
18. WEINER L.P. (1973) Pathogenesis of demyelination induced by a mouse hepatitis virus (JHM virus). *Arch. Neurol.* **28,** 298.
19. WANG F., STOHLMAN S.A. & FLEMING J.O. (1990) Demyelination induced by murine hepatitis virus JHM strain (MHV-4) is immunologically mediated. *J. Neuroimmunol.* **30,** 31.
20. WILLIAMSON J.S.P. & STOHLMAN S.A. (1990) Effective clearance of mouse hepatitis virus from the central nervous system requires both $CD4^+$ and $CD8^+$ T-cells. *J. Virol.* **64,** 4589.
21. LAWETZKY A., TIEFENTHALER G., KUBO R. & HÜNIG T. (1990) Identification and characterization of rat T cell subpopulations expressing T cell receptors α/β and γ/δ. *Eur. J. Immunol.* **20,** 343.
22. DOHERTY P.C., ALLAN J.E., LYNCH F. & CEREDIG R. (1990) Dissection of an inflammatory process induced by $CD8^+$ T cells. *Immunol. Today,* **11,** 55.
23. MOSMAN T.R. & COFFMAN R.L. (1989) TH1 and TH2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Ann. Rev. Immunol.* **7,** 145.
24. SEDGWICK J.D. & HOLT P.G. (1984) Suppression of IgE responses in inbred rats by repeated respiratory tract exposure to antigen: responder phenotype influences isotype specificity of induced tolerance. *Eur. J. Immunol.* **14,** 893.
25. PAUL W.E. & OHARA J. (1987) B-cell stimulatory factor-1/interleukin 4. *Ann. Rev. Immunol.* **5,** 429.