Analysis of the intrathecal humoral immune response in Brown Norway (BN) rats, infected with the murine coronavirus JHM

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Summary

Serum and CSF specimens from clinically healthy Brown Norway (BN) rats inoculated intracerebrally with corona virus JHM were analysed with respect to the state of the blood-brain barrier (BBB) and the intrathecal synthesis and isoelectric distribution of immunoglobulins (Ig). Increased CSF/serum ratios for Ig in the context of an intact BBB were never seen in the absence of intrathecal synthesis of virus-specific antibodies. Affinity-mediated immunoblot analysis revealed a broad pattern of virus-specific antibodies with embedded clusters of restricted heterogeneity, but no signs of oligoclonal Ig production carrying non-viral specificity. From these data it was concluded that BN rats do control the intracerebral spread of JHM virus effectively by a strong local virus-specific antibody response, thereby preventing a clinically apparent disease.

Introduction

The murine hepatitis virus MHV 4, strain JHM, is known to cause a subacute demyelinating encephalomyelitis (SDE) in rats after an incubation period of weeks

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or months (Nagashima et al. 1978, 1979; Sorensen et al. 1980; Wege et al. 1982). In one of the most susceptible rat strains, the Lewis rat, SDE is clinically characterized by ataxic gait and hind leg paralysis. Many animals survive this disease but may later develop a relapse of this disorder (Wege et al. 1984a). Histologically perivascular cuffings with well-defined demyelinated plaques are seen predominantly located in the brain-stem and spinal cord with viral antigen present in glia cells in the neighbourhood of plaques. The inflammatory reactions consist of macrophages, lymphocytes and plasma cells which are found in and around demyelinated areas as well as perivascular cuffings (Nagashima et al. 1978, 1979; Sorensen et al. 1980; Wege et al. 1983; Koga et al. 1984). The lesions of perivascular cuffings can be transferred to syngeneic animals by adoptive lymphocyte transfers pointing to a pathogenetic role of a cell-mediated immune response in this disease (Watanabe et al. 1983) whereas the intrathecal viral humoral immune response is weak as almost not noticeable in diseased Lewis rats (Dörries et al. 1986).

In contrast to Lewis rats, the Brown Norway (BN) rat strain behaves differently when infected with wild-type JHM virus. Clinically noticeable diseases are rare despite the occurrence of active demyelination and persistence of JHM virus in brain tissue over weeks (Watanabe 1986). In BN rats demyelination appears as a nodular lesion predominantly located in the periventricular white matter. Perivascular cell infiltrations are infrequent and always directly connected with destructive changes. However, both perivascular cuffings and cellular infiltrations in the nodular lesions in BN rats contain a great number of plasma cells unseen in Lewis rat lesions. Adoptive transfer experiments of lymphocytes from infected BN rats do not lead to any inducible CNS changes in recipient rats.

These different disease patterns in Lewis and BN rats persistently infected with JHM virus suggest that differences in the immune response to the infectious process may be a determinating factor for the development of the CNS disease, particularly since BN rats in contrast to Lewis rats are not susceptible to the induction of autoimmune reactions such as experimental allergic encephalomyelitis (EAE) (Kornblum 1968). Because of the subclinical infection of BN rats and the presence of high numbers of plasma cells in brain lesions of such animals, it was assumed that a pronounced viral humoral immune response may prevent spreading of the virus within the CNS resulting in a persistent infection without severe clinical disease. On the other hand, in Lewis rats a low humoral and a strong T cell response to basic myelin protein (MBP) may be important factors in the generation of the severe disease. Therefore, the present study describes an analysis of the humoral antiviral antibody response in the CNS of JHM-infected BN rats, in an attempt to understand more about the role of JHM virus-specific antibodies in virus infections leading to subacute demyelination.

Material and methods

Animals

A total of 33 Brown Norway (BN) rats (Zentralinstitut für Versuchstierzucht, Hannover, F.R.G.; Repgo Institutes, Rijswijk, Netherlands) were analysed in this

study. One group of ten animals, aged between 7 and 10 weeks, served as control for determination of the normal CSF/serum ratios of albumin (A) and immunoglobulin (Ig). A second group of ten animals, aged between 4 and 5 weeks was hyperimmunized either with JHM virus or keyhole limpet hemocyanin (KLH) (Calbiochem, Frankfurt/M., F.R.G.) intraperitoneally according to the following immunization scheme: A primary injection of 0.5 ml was given containing 400 μg of protein precipitated in aluminium hydroxyde and 10^{10} inactivated *Pertussis bordetella* particles (Calbiochem, Frankfurt/M., F.R.G.). Between 1 and 3 weeks later the animals were challenged intraperitoneally with soluble protein (0.5 ml, 400 μg). Between 1 and 2 weeks later serum and CSF samples were taken to determine CSF/serum ratios of albumin and antibody titres specific for the antigen used for hyperimmunization.

A third group of 13 animals was inoculated at the age of 4–6 weeks intracerebrally by injection of 40 μl of cell-free infectious tissue culture supernatant, adjusted to contain 10^5 plaque-forming units (PFU). At different times after infection animals were killed and CSF and serum were sampled to determine CSF/serum ratios of albumin, immunoglobulin and JHM virus-specific antibodies. Those samples which revealed virus-specific antibodies were analysed for the isoelectric distribution of total as well as virus-specific immunoglobulins. Brains were removed for histological examinations. CSF specimens with red blood cell contamination higher than 1000 red blood cells/μl were not included in this study.

Virus

JHM wild-type virus was propagated in murine sac cells and processed for enzyme immunoassay (EIA) or immunoblot techniques as described previously (Wege et al. 1983, 1984b). Virus-free control antigen was produced in the same way as EIA antigen.

Determination of albumin and immunoglobulin concentrations

Albumin concentration in CSF and serum specimens was determined by rocket electrophoresis (Laurell 1966) and immunoglobulin concentrations by an EIA as described in detail in a previous paper (Dörries et al. 1986).

Calculation of immunoglobulin and albumin CSF/serum ratios and specific antibody indices

CSF/serum ratios for immunoglobulins (I) and albumin (A) were calculated by the equations (Reiber 1980):

$$I = \frac{\text{immunoglobulin}_{CSF} \ (\mu g/ml)}{\text{immunoglobulin}_{serum} \ (\mu g/ml)} \times 10^3$$

and

$$A = \frac{\text{albumin}_{CSF} \ (\mu g/ml)}{\text{albumin}_{serum} \ (\mu g/ml)} \times 10^3$$

Specific antibody indices (SAB) for JHM virus or KLH were calculated according to Arnadottir et al. (1982):

$$SAB = \frac{\text{antibody titre}_{CSF} \times \text{albumin}_{serum} \; (\mu g/ml)}{\text{antibody titre}_{serum} \times \text{albumin}_{CSF} \; (\mu g/ml)}$$

Enzyme immunoassay to detect JHM-specific antibodies

Standard techniques for EIA were used to determine JHM-specific antibody titres in CSF and serum (Wege et al. 1984b). The reciprocal of the last dilution of the specimen showing a negative control antigen-corrected optical density higher than 0.2 $A_{496 \; nm}$ was considered to be the titre.

Immunoblot to detect the isoelectric distribution of Ig clones

A modification of the affinity-mediated immunoblot published earlier was used (Dörries and ter Meulen 1984; Dörries et al. 1986). Generally 20 μl aliquots of serum and CSF samples, diluted to contain 100 ng of total Ig were isoelectrically focused in 0.5 mm-thick agarose gels. CSF specimens containing less than 100 ng Ig/20 μl were focused as taken from the animal. After focusing total or virus-specific Ig was transferred by affinity to a nitrocellulose filter (BA85, Schleicher & Schüll, Dassel, F.R.G.), coated either with the IgG fraction of a rabbit anti-rat Ig serum (100 μg/ml/10 cm^2 of filter area, Dako Patts, Hamburg, F.R.G.) or with JHM virus (EIA grade, 500 μg/ml/10 cm^2 of filter area). Filter-bound Ig was identified with the IgG fraction of rabbit anti-rat Ig serum labeled with peroxidase (1 : 100, 1 ml/10 cm^2 of filter area, Dako Patts, Hamburg, F.R.G.). To allow only high affinity reactions to take place in this detection procedure, stringency was kept high by dilution of all components of the detection system in 1% Tween 20, 5% bovine serum albumin (BSA) and 0.4 M sodium chloride in 150 mM Tris-HCl, pH 7.5. Detection of the transferred clones was achieved by incubating the filter in 4-chloro-naphthol substrate, which was converted by the peroxidase into an insoluble blue-violet precipitate on the filter.

Results

Neuropathological findings in animals inoculated with JHM virus

Animals were killed between 26 days and 9 months after virus inoculation to collect CSF, serum and brain specimens. None of the 13 animals showed clinical signs of disease but all revealed brain lesions of either mild demyelinating encephalomyelitis (MDE) or scars of remyelination (SCR) without inflammatory changes (Table 1). Histologically, MDE consisted of nodular demyelinating lesions with prominent infiltration of plasma cells whereas small lymphocytes were only occasionally detectable. The demyelination was restricted to distinct perivascular areas of the brain revealing always signs of remyelination. No extension of the demyelinated area into the spinal cord could be noticed. Those rats with scars of

TABLE 1

HISTOLOGICAL AND CLINICAL FINDINGS IN BN RATS INFECTED INTRACEREBRALLY
WITH JHM VIRUS

Rat	Inoculation of virus (d.p.p.)	Collection of specimens (d.p.i.)	Clinical disease	Histological changes	Detection of viral antigens in brain tissue
1	32	270	NAD	SCR	−
2	32	270	NAD	SCR	−
3	32	48	NAD	SCR	−
4	32	28	NAD	MDE	+
5	32	28	NAD	MDE	+
6	32	28	NAD	MDE	+
7	32	31	NAD	MDE	+
8	32	31	NAD	MDE	+
9	39	53	NAD	SCR	−
10	39	53	NAD	SCR	−
11	25	53	NAD	SCR	−
12	25	54	NAD	ND	ND
13	25	54	NAD	MDE	+

NAD = no apparent disease; SCR = scars of remyelination; ND = not done; MDE = mild demyelinating encephalomyelitis; d.p.p. = days post-partum; d.p.i. = days post-infection.

remyelination did not show signs of inflammatory changes or active demyelination. Viral antigens were detectable in the neighbourhood of lesions in glia cells of animals with MDE but not in those with SCR.

Virus-specific antibodies in serum and CSF

Sera and CSF samples were titred for JHM antibodies by a microenzyme immunoassay and the results are shown in Table 2. All animals revealed virus-specific antibody titres in the serum between 1 : 100 and 1 : 12 800. It is most remarkable that two animals showing a titre of 1 : 3200 (rat No. 1 and rat No. 2) were analysed 9 months after inoculation with the virus. However, not all animals showing JHM antibodies in the serum were positive for JHM Ig in the corresponding CSF sample. Only eight animals gave a positive reading (titre > 1 : 10) on EIA. In the remaining five animals virus-specific titres seemed to have dropped beyond the detection limit of the assay.

The presence of virus-specific antibodies in the CSF could be explained solely by diffusion from vascular space into the CNS or by some antibodies synthesized within the CSF. These alternative explanations were analysed by calculating the specific antibody indices (SAB) as outlined by Arnadottir et al. (1982). The normal reference range was established by analysing healthy animals hyperimmunized with JHM virus or a strong immunogen, keyhole limpet hemocyanin (KLH) intraperitoneally. The mean value for SAB was found to be 0.96 for KLH and 0.41 for JHM virus. SAB indices higher than those reference values would indicate intrathecal synthesis of antibodies specific for the antigen in question. As can be seen in Table 3, all animals positive for JHM-specific antibodies in the CSF revealed

TABLE 2

VIRUS-SPECIFIC ANTIBODY TITRES IN PAIRED SERUM AND CSF SPECIMENS FROM BN RATS INFECTED INTRACEREBRALLY WITH JHM VIRUS

Rat	Reciprocal of JHM virus-specific antibody titres	
	Serum	CSF
1	3 200	40
2	3 200	< 10
3	3 200	< 10
4	12 800	1 280
5	3 200	< 10
6	12 800	40
7	800	40
8	6 400	160
9	1 600	< 10
10	400	40
11	100	< 10
12	400	10
13	6 400	640

TABLE 3

SPECIFIC ANTIBODY INDICES IN BN RATS, INFECTED INTRACEREBRALLY WITH JHM VIRUS OR HYPERIMMUNIZED INTRAPERITONEALLY

Rat	Antigen	Route	CSF/serum ratio of		SAB index	Mean SAB index
			Antibody titre ($\times 10^3$)	Albumin conc. ($\times 10^3$)		
1	JHM	i.c.	12.50	1.27	9.84	
4	JHM	i.c.	100.00	2.00	50.00	
6	JHM	i.c.	3.20	1.78	1.80	
7	JHM	i.c.	50.00	1.43	34.97	37.72
8	JHM	i.c.	25.00	1.55	16.13	
10	JHM	i.c.	100.00	0.83	120.48	
12	JHM	i.c.	25.00	2.33	10.73	
13	JHM	i.c.	100.00	1.73	57.80	
N3	KLH	i.p.	1.56	1.20	1.30	
N7	KLH	i.p.	0.40	1.00	0.40	
N8	KLH	i.p.	1.56	0.71	2.20	0.96
N10	KLH	i.p.	0.78	1.47	0.53	
N14	KLH	i.p.	0.78	2.10	0.37	
N15	JHM	i.p.	0.40	1.00	0.40	
N45	JHM	i.p.	0.39	1.54	0.25	
N46	JHM	i.p.	1.56	6.00	0.26	0.41
N48	JHM	i.p.	1.56	2.50	0.62	
N52	JHM	i.p.	1.56	3.15	0.50	

i.c. = intracerebral; i.p. = intraperitoneal.

increased SAB ratios (mean value: 37.72). Therefore it has to be concluded that beside those antibodies which entered the CNS by diffusion, additional antibodies had been synthesized within the CNS compartment.

State of the blood-brain barrier (BBB)

To evaluate if the intrathecal synthesis of JHM virus-specific antibodies is always reflected in an increased CSF/serum ratio of total immunoglobulins, the state of the blood-brain barrier was analysed according to Reiber (1980), showing the protein profile for CSF immunoglobulin and albumin.

CSF/serum ratios of albumin (A) and immunoglobulin (I) were plotted into an x/y-coordinate system (Fig. 1), whereby each individual animal is characterized by the position of the corresponding dot in a certain area of this plot. A reference area of normal animals was established by calculating CSF/serum ratios of A and I in ten non-inoculated BN rats at the age of 7–10 weeks. The mean values determined were 2.00 ± 0.86 for A and 0.65 ± 0.28 for I. These values plus two times the standard deviation were taken as the normal range represented in Fig. 1 by the shaded box. Animals in area I are characterized by a disproportional increase in the CSF/serum ratio for albumin, whereas animals in area II represent a proportional increase in the BBB permeability for small proteins like albumin as well as for immunoglobulins. Area III is difficult to interpret since animals with an increased BBB permeability may either have synthesized Ig intrathecally or reflect a disproportional increase in the BBB permeability for large proteins. Unrestricted diffusion
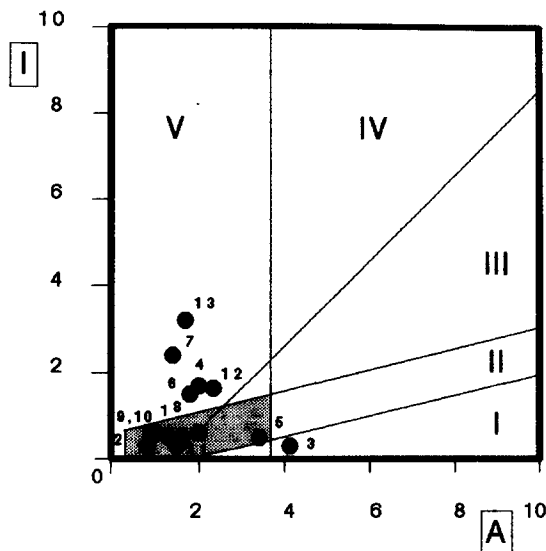

Fig. 1. CSF protein profile of 13 BN rats infected intracerebrally with coronavirus JHM. A and I ratios are plotted according to Reiber (1980). A = CSF/serum ratios for albumin$\times 10^3$, I = CSF/serum ratio for immunoglobulin$\times 10^3$.

of immunoglobulins and albumin into the cerebral space is theoretically possible if the CSF/serum ratios of albumin and Ig are equal, a situation indicated by a line with a slope of 45°, separating areas III and IV of the plot. Therefore animals falling in area IV must have synthesized Ig intrathecally in addition to their increased BBB permeability, whereas animals in area V represent a state of intact BBB in conjunction with intrathecal synthesis of Ig.

As can be seen in Fig. 1, out of 13 animals only one exhibited a BBB dysfunction with respect to small proteins like albumin in the context of a normal immunoglobulin ratio. All others had an intact BBB, but five animals out of these could be detected in that area of the plot indicating intrathecal Ig synthesis (area V, Fig. 1). Correlation of these data with the presence of intrathecal synthesis of JHM virus-specific antibodies revealed that intrathecal synthesis of total Ig concentration in the CSF was only noticed in animals showing intrathecal synthesis of JHM-specific antibodies as well (rats No. 4, 6, 7, 12, 13). However, intrathecal JHM-specific antibody synthesis was not always reflected by an increased CSF/serum ratio of total immunoglobulin (rats No. 1, 8, 10). These data show that absence of increased total Ig ratios and clinically inapparent state do not exclude intrathecal synthesis of agent-specific antibodies in virus-induced chronic demyelination.

Clonal distribution of virus-specific antibodies

In analogy to the oligoclonal pattern of virus-specific immunoglobulins found in cases of chronic persistent virus infections in the CNS of man (Johnson 1982), the presence of locally synthesized high titre virus-specific antibodies in the CSF of our rats led to a series of experiments characterizing the clonal distribution of these immunoglobulins. The electrophoretic distribution of CSF-derived JHM-specific antibodies according to their isoelectric point was analysed by affinity-mediated immunoblots. Representative results of this technique from animals revealing MDE or SCR are shown in Figs. 2 and 3.

As can be seen, a pronounced virus-specific Ig pattern is detectable in the CSF of the selected animals (rat No. 8 and rat No. 10). The fact that clusters of distinct

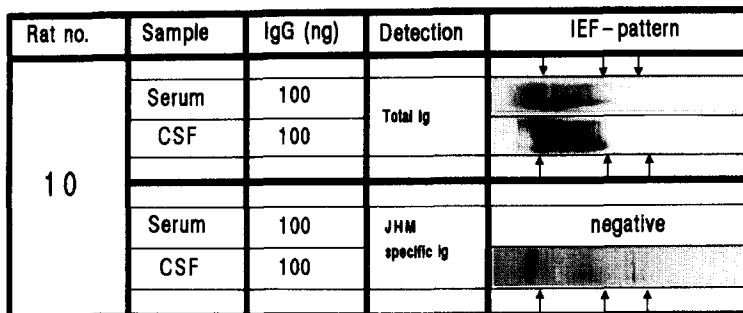| Rat no.	Sample	IgG (ng)	Detection	IEF – pattern
10	Serum	100	Total Ig	
	CSF	100		
	Serum	100	JHM specific Ig	negative
	CSF	100		

Fig. 2. Isoelectrical pattern of total and JHM virus-specific immunoglobulin in CSF and serum specimens from a rat with scars of remyelination (SCR). Established pH gradients are indicated by the black arrows, from left to right: pH 6.6, 8.2, 8.7. Ig (ng) = amount of total Ig focused per track.

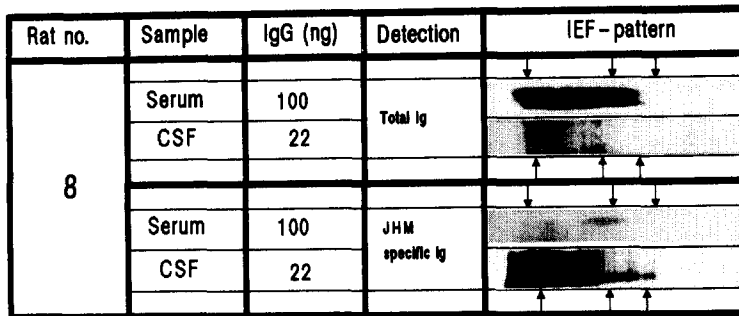| Rat no.	Sample	IgG (ng)	Detection	IEF-pattern
8	Serum	100	Total Ig	
	CSF	22		
	Serum	100	JHM specific Ig	
	CSF	22		

Fig. 3. Isoelectrical pattern of total and JHM virus-specific immunoglobulin in CSF and serum specimens from a rat with mild demyelinating encephalitis (MDE). Established pH gradients are indicated by the black arrows, from left to right: pH 6.6, 8.2, 8.7. Ig (ng) = amount of total Ig focused per track.

virus-specific antibody bands can be seen in the CSF with negative or only slightly positive stainings in a comparable or even higher amount of serum-derived Ig again strengthened the assumption that some antibody clones have been synthesized locally in the CSF.

Analysing the distribution of total rather than the virus-specific Ig in the CSF, no oligoclonal distribution of Ig was seen in the absence of virus-specific antibody bands. This observation made it very unlikely that an autosensitization to brain antigen had taken place in our animals.

Taken together these results demonstrate that an intrathecal antibody response to JHM virus takes place, detectable even months after virus inoculation. Clusters of restricted heterogeneity of these antibodies might reflect a persistent infection of these rats, characterized by a continuous expression of relevant viral antigens.

Discussion

In this study the humoral immune response of BN rats, infected intracerebrally with the murine corona virus JHM, was analysed for the presence of virus-specific immunoglobulins in the CSF with respect to the site and intensity of synthesis and clonal heterogeneity. From the group of infected animals, sacrificed within 28–270 days after viral inoculation, 50% still revealed an inflammatory CNS disease process with presence of viral antigen, whereas in the rest of the animals neither viral antigen nor inflammatory changes or active demyelination could be detected. Yet, all animals exhibited a strong virus-specific immune response in the serum and eight out of 13 animals in the CSF. However, no correlation between the presence of viral antibodies in CSF and inflammatory brain lesions was noticed since intrathecal virus-specific immunoglobulins were found in both groups.

Calculation of specific antibody indices in those BN rats showing JHM-specific antibodies in the CSF, indicated that a high proportion of these antibodies must have been synthesized locally in the CNS. Local production of JHM-specific

antibodies was found in one rat up to 9 months post-infection. The same data were obtained when the isoelectric heterogeneity of CSF-derived virus-specific antibodies was determined by affinity immunoblotting. Restricted clusters of antibodies in a broad pattern of virus-specific antibodies were seen in most CSF specimens. However, this pattern looks quite different from that usually described as oligoclonal in human CSF specimens from patients with persistent virus infection of the brain. Whereas in man oligoclonal Ig is focused predominantly at the very cathodic side of the pH gradient, in our rats at least three groups of oligoclonal bands are embedded in a broad polyclonal pattern of JHM-specific Ig in the CSF.

Similar persistence of intrathecal, virus-specific antibody synthesis as seen in our rats was reported after recovery from acute herpes virus encephalitis, mumps virus and varicella zoster meningoencephalitis (Vandvik et al. 1982, 1985; Vartdal et al. 1982) in man, but the mechanism leading to that prolonged antibody synthesis is still unclear. It has been proposed that presence of intrathecal antibody production over long time periods reflects the persistence of virus infection, but our studies suggest that presence of viral antigens in brain tissue is not mandatory as exemplified in animals No. 1 or 10. However, one has to keep in mind that by immunofluorescence one cannot exclude unequivocally the presence of viral antigen in CNS cells, and more sensitive techniques have to be applied for detection of viral antigens in brain tissue.

Graphic analysis of the protein profile showed that despite the intracerebral infection approximately half of all animals exhibited no pathological changes with respect to intrathecal Ig synthesis or increased BBB permeability. Disproportional increase in albumin permeability was noticed only in one animal, whereas five animals synthesized Ig intrathecally without any disturbance of BBB permeability. Interestingly, all of those five animals have been identified to synthesize JHM virus-specific antibodies intrathecally. However, intrathecal production of virus-specific antibodies did not necessarily result in an increased CSF/serum ratio for total immunoglobulin. Rats No. 1, 8 and 10 raised high titres of JHM virus-specific antibodies intrathecally without any noticeable changes in the CSF protein profile. This observation is not unique to our animal model, since patients suffering from mumps meningitis may reveal the same phenomenon (Link et al. 1981; Ukkonen et al. 1981). Obviously the amount of virus-specific antibody synthesized within the CSF is not always sufficient to be noticed as increased CSF/serum ratio for total Ig. These data clearly stress that the absence of clinical disease and a normal state of the BBB in animal models of virus-induced demyelinating diseases do not exclude histopathological changes and strong agent-specific antibody synthesis within the CNS compartment.

By comparing these data with those obtained in our previous study in Lewis rats with SDE in the course of JHM virus infection (Dörries et al. 1986) distinct differences between the two rat strains can be noticed. Lewis rats with SDE show an intrathecal synthesis of immunoglobulins with almost the same frequency as clinically inapparent BN rats, but in the majority of diseased Lewis rats no antiviral antibodies were found despite presence of viral antigens in brain tissue indicating an antibody production against unknown antigens. Since Lewis rats are susceptible to

EAE (Kornblum 1968) and SDE Lewis rats develop a cell-mediated immune reaction against basic myelin protein after JHM virus infection (Watanabe et al. 1983) one could assume that the unidentified intrathecal antibodies may be directed against autoantigens, a phenomenon which is not seen in JHM-infected BN rats. In addition, a correlation between a significant disturbance of the BBB permeability in SDE Lewis rats in relation to active demyelination can be found, but not in infected BN rats. It is conceivable that in the infected BN rats the very efficient intrathecal virus-specific antibody response restricts the intracerebral spread of the virus and overcomes the infectious process as seen in those BN rats without inflammatory lesions. Although it is presently not known how in coronavirus-induced SDE of Lewis rats the virus infection elicits a T cell-mediated autoimmune response, a strong antiviral immune reaction detectable in the infected organ could have some regulatory functions. Massa and coworkers (1986) recently showed that coronavirus particles are capable of inducing Ia antigens on astrocytes in culture, which might allow such cells to present antigens for T helper lymphocytes. These reactions can be prevented by monoclonal antibodies directed to the peplomer protein E2 of JHM virus. Since hyper-presentation of Ia molecules is probably connected with autoimmune diseases (Janeway et al. 1984) viral antibodies in the extracellular space of brain cells may be beneficial not only in neutralizing infectious virus, but also by blocking a virus-induced Ia expression on astrocytes. On the basis of these observations, studies are in progress in coronavirus-infected Lewis and BN rats to evaluate the pathogenetic role of viral antibodies with respect to the different JHM virus structural proteins. Certainly, information obtained in this animal model will be of help in the study of human demyelinating disease processes associated with viral infections.

Acknowledgements

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