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Murine Coronavirus-Induced Encephalomyelitides in Rats: Analysis of Immunoglobulins and Virus-Specific Antibodies in Serum and Cerebrospinal Fluid

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Summary

The humoral intrathecal immune response in coronavirus-induced demyelinating encephalomyelitis in rats associated with an autoimmune reaction to brain antigen, was analysed. The CSF of these animals revealed immune reactions which were directed against coronavirus and other, unknown, antigens. In general, no direct correlation between the disease, the state of the blood-brain barrier (BBB), intrathecal synthesis of Ig and the presence of virus-specific antibodies was detectable, suggesting that the humoral, in contrast to the cellular, immune response does not play a significant pathogenetic role in this CNS disease.

Key words: *Coronavirus JHM – Intrathecal antibody response – Subacute demyelinating encephalitis*

Introduction

Murine coronaviruses are highly neurotropic for mice and rats and cause central nervous system (CNS) disorders ranging from acute to subacute encephalomyelitis

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(Stohlman and Weiner 1981; Knobler et al. 1982a, b; Wege et al. 1982; Lavi et al. 1984; Woyciechowska et al. 1984). Of particular interest, are the subacute infections by the virus strain JHM which can be associated with marked inflammatory demyelinating lesions, especially in rats (Nagashima et al. 1978, 1979; Sorensen et al. 1980). This subacute demyelinating encephalomyelitis (SDE) develops weeks to months after viral inoculation and is characterized neuropathologically by primary demyelination in the brain and the spinal cord and by inflammatory changes consisting mainly of infiltration by macrophages and lymphocytes with perivascular cuffing in the vicinity of plaques. During the clinical disease, the virus can be isolated from the brain and the spinal cord tissue and viral antigens are detectable, predominantly in the glial cells (Nagashima et al. 1978, 1979; Sorensen et al. 1980; Wege et al. 1983; Koga et al. 1984). Rats surviving this disease recover, but may develop a relapse of SDE with fresh demyelinating lesions, weeks to months after the first attack (Wege et al. 1984a). Moreover, during the course of SDE sensitization of lymphocytes to myelin basic protein (MBP) occurs, suggesting that an immunopathological reaction to brain antigens contributes to the development of this demyelinating disease (Watanabe et al. 1983).

The observation of a virus-induced cell-mediated immune reaction (CMI) to a host antigen in the course of a coronavirus infection of the brain has raised a question about the humoral immune reactions in the CNS during SDE. It has been shown, especially in man, that in other acute and chronic viral infections of the brain, antiviral antibodies are synthesized intrathecally by a limited number of cell clones thus leading to a characteristic electrophoretic pattern with restricted heterogeneity of immunoglobulins. These antibodies are predominantly directed against the causative agent but also against other viral antigens which are not involved in the pathogenetic process (Vandvik et al. 1976, 1978a, b; Nordal et al. 1978; Link et al. 1981; Skorr et al. 1981; Vartdal et al. 1982). Therefore, in the current study we collected cerebrospinal fluid (CSF) and blood from SDE animals and analysed it for JHM antibodies and for the presence of restricted heterogeneity in the immunoglobulins. To our surprise, many of SDE animals studied did not reveal an intrathecal JHM immune response despite the presence of viral antigens in the CNS tissue. Other animals showed an oligoclonal pattern of CSF Ig, the specificity of which was not determined. These findings suggest that in SDE induced in rats by coronavirus, humoral antiviral immune response does not contribute directly to the disease. Perhaps the absence of viral antibodies allows the spread of the infection and permits the development of cell-mediated immunity to host antigens. Whether or not the immune response in the CSF of some of these animals is due to autoantibodies, is not yet known.

Materials and Methods

Animals, virus inoculation and sampling of cerebrospinal fluid (CSF) and blood

The inbred strain of Lewis rats was used throughout all experiments. JHM virus TS43 (Wege et al. 1983) was inoculated intracerebrally between 9 and 13 days after

birth in a dose of 10^4–10^5 PFU. After onset of clinical signs of subacute demyelinating encephalomyelitis (SDE) the animals were killed. CSF, approximately 100–200 µl, was collected by puncture of the cisterna magna (Fleming et al. 1983; Reiber and Schunck 1983) and blood by heart puncture. Contamination of CSF by blood was checked by staining nucleated cells with crystal violet (9 µl CSF + 1 µl 2.5% crystal violet). CSF specimens with visible red blood cell (RBC) contamination or RBC counts higher than 1000/µl were excluded from this study.

Intraperitoneal immunization of rats

Rats were immunized intraperitoneally with 400 µg of alaun-precipitated protein (measles virus, keyhole limpet hemocyanine or JHM virus, 0.5 ml/animal). A booster injection was given 4 weeks later with soluble non-precipitated proteins (400 µg/0.5 ml/animal). Animals were sacrificed 14 days later, serum and CSF samples were taken and specific antibody titers to the corresponding antigen measured by microenzyme immunoassay. Calculation of end-point titers was carried out as described below.

Virus strain, propagation and purification

The temperature-sensitive mutant of the coronavirus TS43 was propagated in murine Sac^- cells as described earlier (Wege et al. 1983). For inoculation of animals cell-free tissue culture supernatant from infected Sac cells was used. Viral antigen for enzyme immunoassays or immunoblot technique was prepared from cytolytic extracts of infected Sac cells (Wege et al. 1984a). Control antigen was purified simultaneously in the same manner as the viral antigens from the uninfected Sac cells.

Determination of albumin and immunoglobulin in the CSF and serum specimens

Albumin concentration in CSF and serum was determined by rocket electrophoresis as described by Laurell (1966). For Ig determination in the CSF and serum samples a quantitative solid-phase sandwich enzyme immunoassay was established. Round-bottom micro-ELISA plates (Nunc) were coated with the IgG fraction of rabbit anti-rat Ig (10 µg IgG/ml, 100 µl/well) by overnight incubation at 4°C in 0.05 M Na_2CO_3 buffer, pH 9.6. For control purposes, wells were coated with the IgG fraction of rabbit preimmune serum under the same conditions. After washing the wells with Tris-buffered sodium chloride (TBSC) (50 mM Tris, 350 mM NaCl, 0.1% Tween-20, pH 7.4), 100 µl samples of diluted CSF or serum in TBSC + 5% rabbit serum were incubated for 1 h at 37°C in anti-rat Ig-coated wells and preimmune IgG-coated wells. After a further washing cycle, rabbit anti-rat Ig conjugated with horseradish peroxidase (diluted 1 : 4000) was added to all the wells for another hour at 37°C. After washing off unbound enzyme-labeled detector immunoglobulin, the wells were developed with *o*-phenylenediamine × 2 HCl (OPD). A reference curve with a rat Ig standard was established which showed proportionality between the optical density at 492 nm and the Ig concentration between 40 ng and 1 ng by log-log plotting. Ig concentrations were calculated with reference to the standard curve. CSF/serum ratios were calculated for albumin and Ig according to

Reiber (1980): $A = (albumin)_{CSF} \times 10^3/(albumin)_{serum}$, and $I = (Ig)_{CSF} \times 10^3/(Ig)_{serum}$. Albumin (A) and immunoglobulin (I) ratios were plotted within an X/Y coordinate system.

Enzyme immunoassay (EIA) for JHM-specific antibodies

EIA to detect JHM-specific antibodies was basically carried out as previously described (Wege et al. 1984b). To determine antibody titers in CSF and serum specimens 25 μl samples were used in duplicates. End-point titers were defined by the dilution of CSF or serum exhibiting an optical density $ABS_{492\ nm} \geqslant 0.2$ calculated by: $ABS_{492}(JHM) - ABS_{492}(Sac)$. Specific antibody indices were calculated according to Arnadottir et al. (1982): $Sab_{index} = (titer)_{CSF} \times (albumin)_{serum}/(titer)_{serum} \times (albumin)_{CSF}$.

Immunoblot technique for isoelectric distribution of Ig and JHM-specific antibodies

Electrophoretic distribution of Ig and JHM-specific antibodies was analysed by immunoblot technique as previously described (Dörries and ter Meulen 1984). Thus, serum specimens were adjusted to contain the same amount of Ig as the CSF by dilution with distilled water. If Ig concentration in the CSF was higher than 5 μg/ml, the CSF as well as the serum was diluted to contain 5 μg Ig/ml. A sample of 20 μl was isoelectrically focused in an 0.5 mm thick agarose gel (pH gradient 3–10.5) and the total Ig fraction was transferred by affinity from the gel to a nitrocellulose (NC) filter coated either with the IgG fraction of rabbit anti-rat Ig (10 μg Ig/cm^2 of filter area) or with JHM antigen (EIA grade, 25 μg protein/cm^2 filter area). All transferred immunoglobulins, including the JHM-specific Ig were visualized by incubation of the filter with peroxidase-labeled rabbit anti-rat Ig and the subsequent development of the filter in a precipitating peroxidase product (4-chloro-1-naphthol).

Results

Diseased animals

A group of 12 Lewis rats which developed clinically the characteristic signs of SDE after inoculation with JHM virus TS43 was selected for this study (Table 1). Clinical diagnosis was verified by histological examination of the brain. All animals revealed the typical neuropathological changes consisting of inflammatory demyelinating lesions in the spinal cord and the brain (data not shown). As can be seen in Table 1, onset of the symptoms varied between 21 and 128 days after virus inoculation. Animals were sacrificed within 1–6 days after the clinical disease was noticed and the CSF and serum specimens were collected and stored at $-20°C$. These materials were used to analyse the state of the blood-brain barrier (BBB), especially the intrathecally synthesized immunoglobulins and the clonal distribution of the total and the JHM-specific immunoglobulins.

TABLE 1

ANIMALS SELECTED FOR THE STUDY

Animal No.	Virus inoculation (age in days)	Onset of disease (days P.I.)	Sampling of blood and CSF (days P.I.)
G826	9	128	128
G946	9	50	50
G4011	12	45	51
G4054	11	74	75
G4091	12	20	22
G4232	10	29	34
G4251	11	21	24
G4252	11	21	24
G4261	13	27	27
G4262	13	28	29
G4263	13	32	36
G4264	13	35	36

P.I. = post-inoculation.

State of the blood-brain barrier

To evaluate the state of BBB, CSF/serum ratios for albumin and immunoglobulin were calculated and plotted according to Reiber (1980).

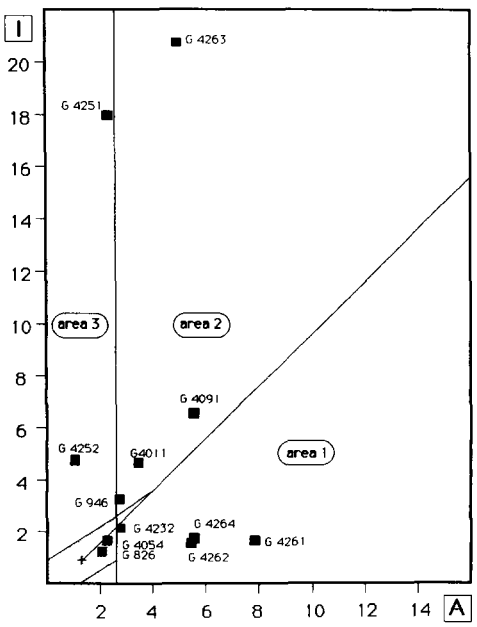

Fig. 1. Graphical evaluation of the state of BBB in SDE diseased Lewis rats. Albumin (A) and immunoglobulin (I) CSF/serum ratios were plotted according to Reiber (1980).

To establish a normal range of both ratios, the CSF and serum samples of 10 non-diseased animals (aged 50–60 days) were analysed. Mean values obtained were 1.3 ± 0.65 for CSF/serum ratios of albumin (A) and 0.87 ± 0.43 for immunoglobulin (I). By adding 2 standard deviations to the mean values, we established the limits of normal range, represented by the shaded box in Fig. 1. Outside this normal range animals investigated fall into 3 different areas of the plot. Animals in area 1 revealed an increase in albumin of the CSF as a result of a severe BBB dysfunction without intrathecal Ig synthesis; those in area 2 are characterized by a BBB dysfunction with additional local Ig production within the CNS; those in area 3 display an intrathecal Ig synthesis without BBB disturbances.

Most animals (8 rats in areas 1 and 2) do exhibit dysfunction of BBB. Half of the animals (6 rats in areas 2 and 3) synthesized Ig intrathecally, regardless of the state of the BBB: whether intact (2 rats in area 3) or disturbed (4 rats in area 2). Two animals (G4054, G826) within the normal range, although clearly diagnosed as having SDE, showed no pathological findings in the A and I ratios. These were the animals with the longest incubation periods, 74 (G4054) and 128 days (G826).

JHM virus-specific antibodies in serum and CSF samples

The observation that intrathecal Ig synthesis takes place in rats with SDE has led to the question of the antigen specificity and clonal distribution of these immunoglobulins. Because viral antigen is detectable in the brain of the animals at the state of the disease, the CSF of the animals was analysed for JHM-specific Ig by viral immunoblot technique (Dörries and ter Meulen 1984). Before the assay, we adjusted the serum samples to the same Ig concentration as that of the CSF sample of the same animal. Due to a shortage of CSF, this analysis could only be completed for 8/12 animals. Only 3 rats (G826, G4232, G4263) exhibited virus-specific Ig in the CSF but not in the corresponding serum specimen. Typical results of this technique are given in Fig. 2 which demonstrates the isoelectric pattern of total and JHM-specific Ig in CSF and serum samples of animal No. G4263. In the CSF an oligoclonal distribution of total Ig towards the cathodic part of the gradient can be seen beside a polyclonal pattern at the neutral pH. However, only part of the total CSF Ig shows a virus-specific activity. Because virus-specific Ig bands could not be detected analysing the same quantity of serum-derived Ig from the same animal, we concluded that virus-specific Ig in CSF must have been synthesized within the CNS compartment.

To verify this observation JHM-specific antibody indices (Arnadottir et al. 1982) of those animals that showed JHM-specific antibodies in CSF by immunoblot analysis (G826, G4232, G4263) were compared with specific antibody indices of disease-free control animals hyperimmunized with different viral and non-viral antigens. As can be seen in Table 2, specific antibody indices were significantly higher in diseased animals. Although one of these animals (G4263) did reveal significant BBB dysfunction which may contribute to the increased specific antibody index, we do not believe that the BBB disturbance alone explains this finding. The increase of the albumin CSF/serum ratio of the diseased animal as compared to the control JHM hyperimmunized animal (N22) is only 5-fold whereas the

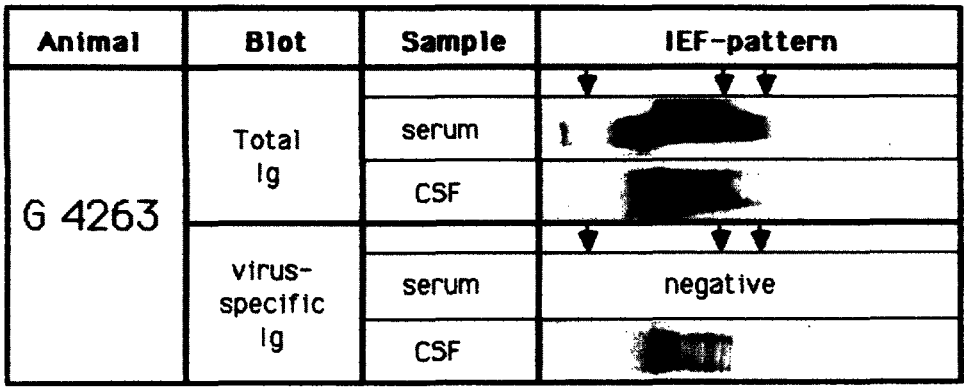

Fig. 2. Immunoblot of total and virus-specific Ig. CSF and serum (20 μl each) were isoelectrically focused and blotted either with an anti-rat Ig or a JHM virus-coated nitrocellulose filter. Transferred Ig was detected by peroxidase-labeled rabbit anti-rat Ig (peroxidase-specific substrate: 4-chloro-naphthol). Black arrows indicate the pH gradient, from left to right: pH 5.2, 8.2, 8.7.

increase in CSF/serum ratio of JHM antibody titer is 250-fold. Taken together the results of immunoblot and antibody index calculation we assume that presence of JHM-specific antibodies in the CSF can be explained by intrathecal synthesis of these antibodies.

Non-virus-specific immunoglobulins in the CSF samples

In addition to the animals that had elevated Ig concentrations and intrathecal Ig production specific for JHM, we observed animals with increased or normal Ig levels in CSF, but with no virus-specific antibodies either in serum or in the CSF. Examination of the clonal distribution of total Ig in the serum and CSF of these animals revealed oligoclonal Igs in CSF, as shown in Fig. 3. These bands could not

TABLE 2

SPECIFIC ANTIBODY INDICES IN RATS WITH SDE AND HYPERIMMUNIZED CONTROL RATS

Animal	Disease	Antigen	Route	CSF/serum ratios of		SAb index
				Antibody titer	Albumin titer	
G826	SDE	JHM	i.c.	50.0	2.06	24.33
G4232	SDE	JHM	i.c.	12.5	2.76	4.53
G4263	SDE	JHM	i.c.	100.0	5.00	20.00
N20	–	Measles	i.p.	0.39	1.64	0.24
N21	–	KLH	i.p.	0.39	1.75	0.22
N22	–	JHM	i.p.	0.39	1.00	0.39

SAb = specific antibody; i.c. = intracerebral; i.p. = intraperitoneal; – = no disease; KLH = keyhole limpet hemocyanine.

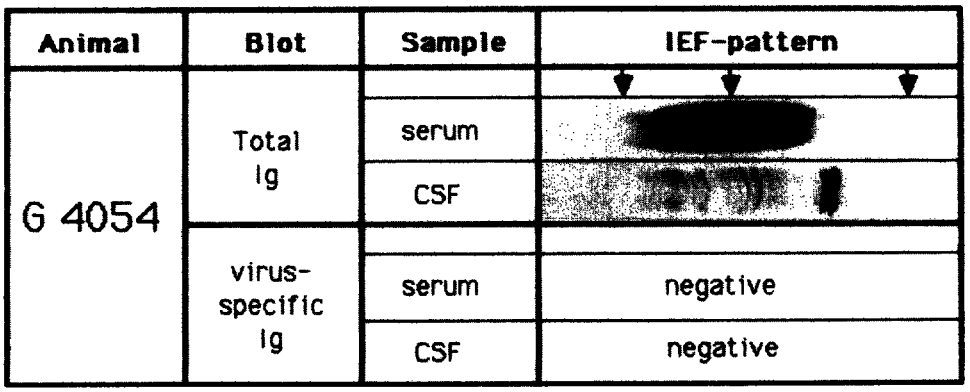

Fig. 3. Immunoblot of total and virus-specific Ig. CSF and serum (20 μl each) were isoelectrically focused and blotted either with an anti-rat Ig or a JHM virus-coated nitrocellulose filter. Transferred Ig was detected by peroxidase-labeled rabbit anti-rat Ig (peroxidase-specific substrate: 4-chloro-naphthol). Black arrows indicate the pH gradient, from left to right: pH 5.2, 6.6, 8.2.

be detected in the matched serum specimens. Thus far the nature of the antigen that causes a local antibody response within the CNS is unknown. Screening assays using different types of neural and glial cell antigens from embryonic Lewis rats aimed at the identification of the postulated non-viral antigen, are in progress.

Discussion

In this study we attempted to analyse the serum and CSF immunoglobulins in a group of rats with a subacute demyelinating encephalomyelitis. The disease was a result of an infection with a temperature-sensitive mutant of JHM virus (TS43), selected for its capacity to induce this type of disorder (Wege et al. 1983). One of the major problems in this analysis was the very limited amount (100–200 μl) of blood-free cerebrospinal fluid that could be obtained from the diseased animals, forcing us to use highly sensitive detection techniques such as EIA and virus-specific immunoblot technique (Dörries and ter Meulen 1984). Between 10 and 100 ng of Ig per assay was sufficient to determine such important factors as the state of the BBB, intrathecal synthesis of Ig and the clonal distribution of virus-specific, as well as total Ig, in unconcentrated CSF specimens from individual animals.

We analysed the state of the BBB by the plot described by Reiber (1980). Out of 12 animals, 6 showed increased CSF/serum ratios of immunoglobulin either with or without a disturbed BBB; 4 revealed BBB dysfunction without an increased CSF/serum Ig ratio, and the remaining 2 animals showed no pathological changes in the BBB or the CSF/serum Ig ratio, despite the CNS disease. It is interesting that clinical disease in these two animals had an unusually long incubation period, suggesting that changes in the BBB may be detectable only within a certain time after the inoculation of the virus. The proportion of animals with increased

CSF/serum Ig ratios approximately one month after inoculation of the virus is high. This suggested to us that intrathecal Ig synthesis may be an important marker of the disease. If this assumption is correct, it would be expected that the number of animals with an increased CSF/serum ratio of Ig will be even higher when CSF specimens are taken at a later time in the disease.

Local synthesis of Ig in the CNS of diseased animals led to the question of the antigen specificity and the clonal distribution of this CSF Ig. In experimental allergic encephalomyelitis (EAE) or in human neurological disorders, such as viral meningitis, encephalitis and multiple sclerosis (MS), antibodies of restricted heterogeneity can be observed in the CSF (Lowenthal et al. 1984). Whereas in virus-induced disorders most of the oligoclonal Ig reacts with the causative agent (Vandvik et al. 1976, 1978a, b; Nordal et al. 1978; Link et al. 1981; Skorr et al. 1981; Vartdal et al. 1982), in EAE the antigen specificity of major oligoclonal Ig bands may be to *Mycobacterium tuberculosis* antigens given as adjuvants during sensitization of the animal (Glynn et al. 1982). In MS, no major antigen recognized by oligoclonal Ig of CSF, has so far been identified although this immune response is very pronounced and detectable in almost every case (Lowenthal et al. 1984).

In our rats, clonal distribution of virus-specific Ig in serum and CSF was analysed by isoelectric focusing and subsequent virus-specific immunoblot (Dörries and ter Meulen 1984). Several important observations were made. First, virus-specific Ig was synthesized in the CSF, a conclusion derived from the observation that — by equal Ig concentration in the serum and CSF specimens — only CSF Ig revealed antiviral activity in immunoblot analysis. This interpretation was further supported by the calculation of JHM-specific antibody indices in the CSF specimens of animals with SDE and without CNS disease but hyperimmunized with different antigens intraperitoneally. Antibody indices of diseased animals were significantly higher than those of non-diseased animals, a fact that supported the view that JHM-specific antibodies in the CSF are a result of intrathecal synthesis. Similar observations have been made by Sorensen et al., who reported intrathecal JHM-specific Ig synthesis in Wistar Lewis rats, calculated from increased CSF/serum ratios of JHM-specific antibodies (Sorensen et al. 1984).

Secondly, only a few animals revealed JHM antibodies in the CSF despite the fact that JHM antigen was present in the brain tissue of each of the animals included in this study. This is in contrast to other subacute viral infections of the central nervous system, such as subacute sclerosing panencephalitis (SSPE) or progressive rubella panencephalitis, in which measles or rubella virus antibodies are found in the CSF of each patient (Johnson 1982). Thirdly, electrophoretic distribution of virus-specific Ig exhibited an oligoclonal pattern of a broader pH range than that observed in viral CNS infections in men. This phenomenon cannot be interpreted at present since no comparable data from other animal models are available. Perhaps antibodies synthesized intrathecally as a response to viral antigen in rats do not focus as cathodic, unlike in men. Fourthly, there was no correlation between increased total Ig in the CSF and the presence of virus-specific antibodies. This observation supports the interpretation that viral antigens are not the only ones that can evoke a local humoral immune response; non-viral antigens can also cause

intrathecal Ig synthesis. Moreover, the presence of virus-specific antibodies in the CSF with a normal Ig concentration shows clearly that intrathecal virus-specific Ig is not necessarily reflected in an increased total Ig concentration. A similar observation has been made in human mumps meningitis, in which mumps-specific IgG titers in the CSF are not always accompanied by an increased IgG concentration in CSF (Link et al. 1981; Ukkonen et al. 1981). It seems that, either the contribution of virus-specific antibodies to the total Ig concentration in the CSF is low, or virus-specific immunoglobulin replaces non-viral immunoglobulins in the CSF.

On the basis of these data we assume that antiviral antibodies, although synthesized intrathecally, do not contribute significantly to the increase in Ig. It is most likely that these specific antibodies reflect, in some way, the accessibility of viral antigens within the CNS to the immune system and that they disappear if viral antigen is no longer expressed on the cell surfaces or is released.

By applying the same technique to the evaluation of the clonal distribution of total Ig rather than that of virus-specific Ig in CSF and serum specimens, oligoclonal Ig was detected in some animals. Since these Ig bands do not have antiviral activity and are not present in the serum, it seems likely that during the course of SDE with a long incubation period, B cell clones are switched on to synthesize Ig of an unknown specificity. As had been observed with the antiviral antibodies, however, the presence of oligoclonal Ig was not correlated with an increased Ig concentration in CSF. The question of a possible autoreactivity by these clones cannot be answered at present. Studies are now in progress in our laboratory, screening brain antigens derived from Lewis rat glial cells.

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