# Relapsing Subacute Demyelinating Encephalomyelitis in Rats during the Course of Coronavirus JHM Infection

Helmut Wege, Rihito Watanabe and Volker ter Meulen

*Institute of Virology and Immunobiology, University of Würzburg, D-8700 Würzburg (F.R.G.)*

## Summary

Temperature-sensitive mutants of the murine coronavirus JHM induced a sub-acute demyelinating encephalomyelitis (SDE) in young rats. Neurological symptoms were associated with marked lesions of primary demyelination in the white matter of the central nervous system (CNS), and developing after an incubation time of several weeks to months. Many rats survived this infection and recovered completely from this CNS disease. Among 43 survivors of SDE, 9 rats developed a relapse 27–153 days after onset of the first attack. Neuropathological examination of these animals revealed areas of fresh demyelination together with old remyelinated lesions. Viral antigens were detectable in the neighbourhood of fresh lesions and in some cases infectious virus was re-isolated from rats revealing low antibody titers to JHM virus. These results demonstrate that mutants of JHM virus can induce a relapsing demyelinating disease process, associated with a persistent infection, which possesses some similarities to chronic experimental allergic encephalomyelitis.

## Introduction

Relapsing demyelination is an important feature of certain chronic central nervous system (CNS) diseases in both animal and man for which etiology and/or

pathogenesis are unknown. The most prominent example is multiple sclerosis (MS) which is thought to be associated with an immune pathological process possibly acting in concert with a virus infection (Waksman 1981). It has been shown that an autoimmune reaction against CNS tissue can lead to chronic relapsing demyelination as seen in chronic experimental allergic encephalomyelitis (EAE) whereas the role of a virus infection in the induction of such a condition has not so far been directly demonstrated (McFarlin et al. 1974; Raine et al. 1978; Lassmann and Wisniewski 1979; Traugott et al. 1982). It is therefore desirable to develop animal models of relapsing demyelination associated with a virus infection in order to analyse the mechanisms by which virus-induced neuropathological changes may occur.

Viruses can induce demyelination which is interpreted as either the consequence of cell death in the course of viral replication, or the result of dysfunction in persistently infected oligodendroglia cells. Immunopathological reactions could also play a role in virus-induced demyelination as studies with Theiler's virus infection in mice suggest. Relapsing demyelination associated with a clinical disease as seen in chronic EAE has been postulated in viral infections of the CNS, especially in man, but not studied experimentally. In the following we describe a model in which certain mutants of murine coronavirus JHM cause clinical relapsis of a subacute demyelinating encephalomyelitis (SDE) in rats. After virus infection in rats inflammatory disseminating CNS lesions of marked demyelination develop accompanied by clinical signs of a subacute disease after varying incubation times. A considerable percentage of diseased animals survive and recover from this infection and some come down with a second attack of SDE after a period of weeks to months. Such animals show both, fresh demyelinating lesions with infiltrations, and remyelinated areas in the brain and spinal cord. Infectious JHM virus can be reisolated from brain tissue and virus antigens demonstrated in brain cells. These findings demonstrate that a clinically recognizable relapsing CNS disease can develop in association with exacerbations of demyelination in the course of a persistent viral infection of brain tissue.

## Materials and Methods

### Animals

Outbred specific pathogen-free rats (CHBB/Thom) were purchased from Thomae (Biberach, F.R.G.). Lewis rats were obtained from the Zentralinstitut für Versuchstiere (Hannover, F.R.G.). 10–15 day old rats received $4 \times 10^3$ PFU of JHM virus in 30 $\mu$l tissue culture medium into the left brain hemisphere using a dispenser syringe.

### Virus

The temperature-sensitive (ts) mutants ts6, ts42 and ts43, which were selected from the murine coronavirus JHM after growth in presence of fluorouracil have been previously described (Wege et al. 1983). The viruses were propagated on monolayers of Sac(-) cells at 34°C with Eagle's minimal essential medium (MEM) and 5% fetal calf serum.

*Virus isolation attempts*

Samples of brain and spinal cord were homogenized immediately after dissection and adsorbed for 1 h at 34°C on monolayers of Sac(-) cells in 24 well cluster plates (Wege et al. 1983). The number of wells in which syncytia developed was scored after 3 days incubation at 34°C. The supernatants from positive wells were harvested, pooled and titrated at 34°C as well as 39.5°C to measure the temperature sensitivity. Cells from reisolation attempts which yielded only very few or small syncytia were trypsinised, mixed with normal Sac(-) cells and passaged further.

*Antibody determination*

To measure neutralizing antibodies, the sera were inactivated for 30 min at 56°C, mixed in serial dilutions with 100 pfu of wild type JHM virus (final volume 200 $\mu$l) and incubated for 1 h at 4°C. Neutralization was assayed using Sac(-) cells in 24-well microplates. The cultures were incubated for 20 h at 37°C without agar overlay. Microplaques were counted after staining with May–Grünwald and Giemsa, the serum dilution resulting in 50% plaque reduction ($ND_{50}/0.1$ ml) was thus calculated.

Total JHM-virus antibodies were titrated using a solid phase enzyme immunoassay (ELISA), performed according to standard procedures (Voller et al. 1976). Antigen was prepared from infected or control Sac(-) cells, which were disrupted by dounce homogenisation in RSB (Tris–HCl 20 mM, pH 7.4; sodium chloride 100 mM, magnesium chloride 5 mM) and 0.2% Nonidet P40. The homogenate was centrifuged 5 min at $10000 \times g$ and the antigens were pelleted by ultracentrifugation at $100000 \times g$ for 90 min. Antigens and indicator immunoglobulins (horseradish peroxidase-labelled anti-rat globulin, Dako) were calibrated by block titrations for reproducible sensitivity. The positive anti-JHM virus serum was prepared by immunisation of rats with density gradient purified virus (Wege et al. 1979) and had a neutralisation titre 1 : 1250. The antigen block titrations were calibrated with a serum dilution of 1 : 400 to give a netto absorbance value (we mean of virus antigen-coated wells minus mean of control antigen-coated wells) of 1.0. Flat bottom microplates (Dynatech) were coated with antigen and control antigen in 0.05 M sodium carbonate buffer, pH 9.6 overnight at 4°C (100 $\mu$l/well), and unbound proteins removed by washing with Tris buffer (0.05 M, pH 7.4) containing 0.15 M sodium chloride and 0.1% Tween 20. Serum dilutions were made in the same buffer containing 5% newborn calf serum and 0.002% phenol red. After incubation for 1 h at 37°C the plates were washed again, then incubated with peroxidase-labelled rabbit anti-rat globulin and after a final wash orthophenylene diamine (0.5 mg/ml) in citric acid (35 mM, dinatrium hydrogen-phosphate 66.6 mM, hydrogene peroxide 0.01%, pH 5) was added as substrate. The reaction was stopped after 1 h at room temperature by addition of 50 $\mu$l sulfuric acid (3 M) and the colour measured with a multichannel spectrophotometer (Titertek, Flow Labs.) at 492 nm. The endpoint of the titration (ELISA titre) was defined as that serum dilution which yielded a netto absorbance value more than 3-fold higher than the negative control sera at a dilution of 1 : 10. The positive and negative serum pool which was used for the calibration of the assay by block titrations was included in each experiment.

*Histology*

Tissue was fixed in buffered 10% formalin and embedded in paraffin for histological examination. Sections were stained with hematoxylin–eosin and luxol fast blue. For epon embedding, animals were perfused with 2% glutaraldehyde and 2% paraformaldehyde, 0.5% sucrose in 0.1 M phosphate buffer solution. The tissue was postfixed by osmium, dehydrated, embedded in epon and 1 μm sections were stained with toluidine blue for light microscopy. Ultrathin sections were stained with uranylacetate and lead citrate for electron microscopy.

*Histochemistry*

Appropriate tissue blocks were fixed in buffered 2% paraformaldehyde for 4 h to overnight, washed by graded sucrose, finally by 10% glycerol and 20% sucrose in 0.1 M phosphate buffer at the temperature of 4°C, embedded in OCT, frozen by liquid nitrogen and kept at −70°C until 10-μm sections were prepared by cryostat. The PAP method (Sternberger et al. 1979) was applied by using rabbit anti-JHM serum (having a neutralization titre of 1:1860) at a dilution of 1:1000. The rabbit had been immunised with density gradient purified JHM virus (Wege et al. 1979) and the serum was absorbed with acetone-extracted rat brain powder. For control staining, normal preimmune rabbit serum and an anti-measles virus rabbit serum was used. As a second antibody anti-rabbit IgG (Dako Z147) and PAP complex (Dako Z113) were included. Counter staining was achieved with methyl green.

## Results

*Subacute demyelinating encephalomyelitis (SDE)*

Intracerebral inoculation of 10–15 days-old Thomae or Lewis rats with temperature-sensitive (ts) mutants was followed by the development of SDE after an incubation period of several weeks (Wege et al. 1983). This disease started quite often with a mild to moderate hindleg weakness with ataxic gait which causes the rat considerable difficulties in righting itself up when flipped onto its back. At variable times up to several days these signs were followed by severe hindleg weakness leading to hindleg paralysis. At such a stage no voluntary movement of the hindlimbs could be observed although some reflex stiffening occurred. Approximately 60–70% of the diseased animals died within a few days after onset of disease, whereas the surviving animals recovered completely, or with a stabilized disability.

The most prominent histological finding consisted of demyelinating plaques located in the white matter (Fig. 1) primarily of the optic nerve, midbrain, pons, cerebellum and spinal cord. Within the demyelinated plaque, axons and neurons were well preserved. In addition, cell infiltrations were observed consisting of lymphocytes, plasma cells and macrophages. Infectious virus could be isolated by conventional methods, and viral antigens were easily detectable in glia cells in the neighbourhood of demyelinating plaques (Nagashima et al. 1978, 1979; Sörensen et al. 1980, 1982; Wege et al. 1983).
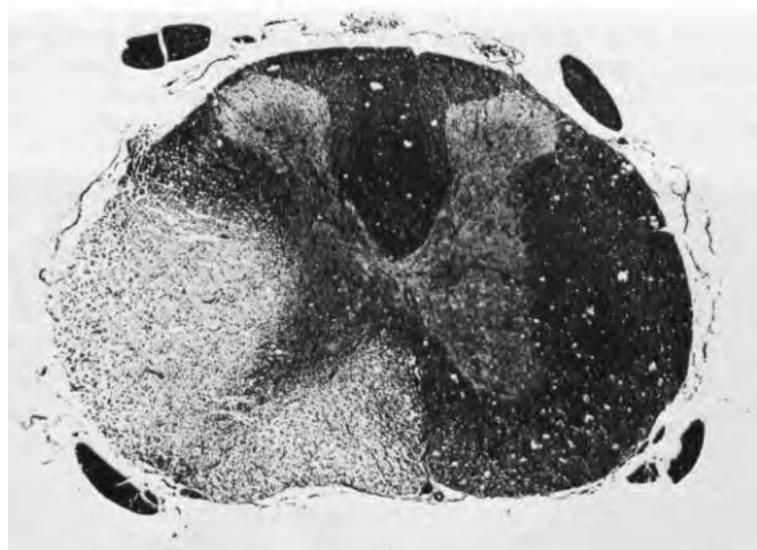
Fig. 1. Large demyelinating plaque located in the white matter of the spinal cord in a Lewis rat with SDE. Hematoxylin–eosin and luxol fast blue staining, ×28.

## Relapsing SDE

Of particular interest is the observation that some of the animals which survived and recovered from SDE revealed later a second attack of this disease. Of 276 rats infected with different ts-mutants of JHM virus 57% developed clinical signs of SDE within an incubation period of 14–158 days. 36% of the diseased animals survived this infection.

TABLE 1

HISTORY OF CORONAVIRUS JHM-INDUCED RELAPSING SDE

Rats were inoculated at an age of 10–15 days with $4 \times 10^3$ pfu of virus (i.c.).

| Case No. | Mutant inoculated | Rat strain | Onset of symptoms (days p.i.) | Duration of symptoms (days) | Relapse of SDE (days p.i.) | Clinical condition at autopsy |
|---|---|---|---|---|---|---|
| 1 | ts6 | Thomae | 14 | 9 | 70 | Severe tetraplegia |
| 2 | ts6 | Thomae | 14 | 15 | 47 | Paresis of hindlegs, circling movements |
| 3 | ts6 | Lewis | 14 | 8 | 41 | Severe paralysis of hindlegs |
| 4 | ts42 | Thomae | 19 | 18 | 56 | Paralysis of hindlegs |
| 5 | ts43 | Thomae | 21 | 23 | 71 | Severe paresis of hindlegs |
| 6 | ts43 | Lewis | 22 | 15 | 50 | Circling movements, severe paresis |
| 7 | ts43 | Lewis | 33 | 22 | 95 | Circling movements, severe tetraparesis |
| 8 | ts43 | Lewis | 33 | 12 | 138 | Severe paralysis of hindlegs |
| 9 | ts43 | Thomae | 20 | 25 | 173 | Paresis of hindlegs |

Among the 56 survivors of SDE, 13 rats continuously exhibited clinical signs of hindleg paresis or paralysis. The remaining 43 animals were apparently healthy, but 9 of these animals developed a second attack of SDE 2–18 weeks after recovery from the first attack. As documented in Table 1 these animals had developed their first attack of SDE after an incubation period of 14–33 days. Clinical symptoms of this
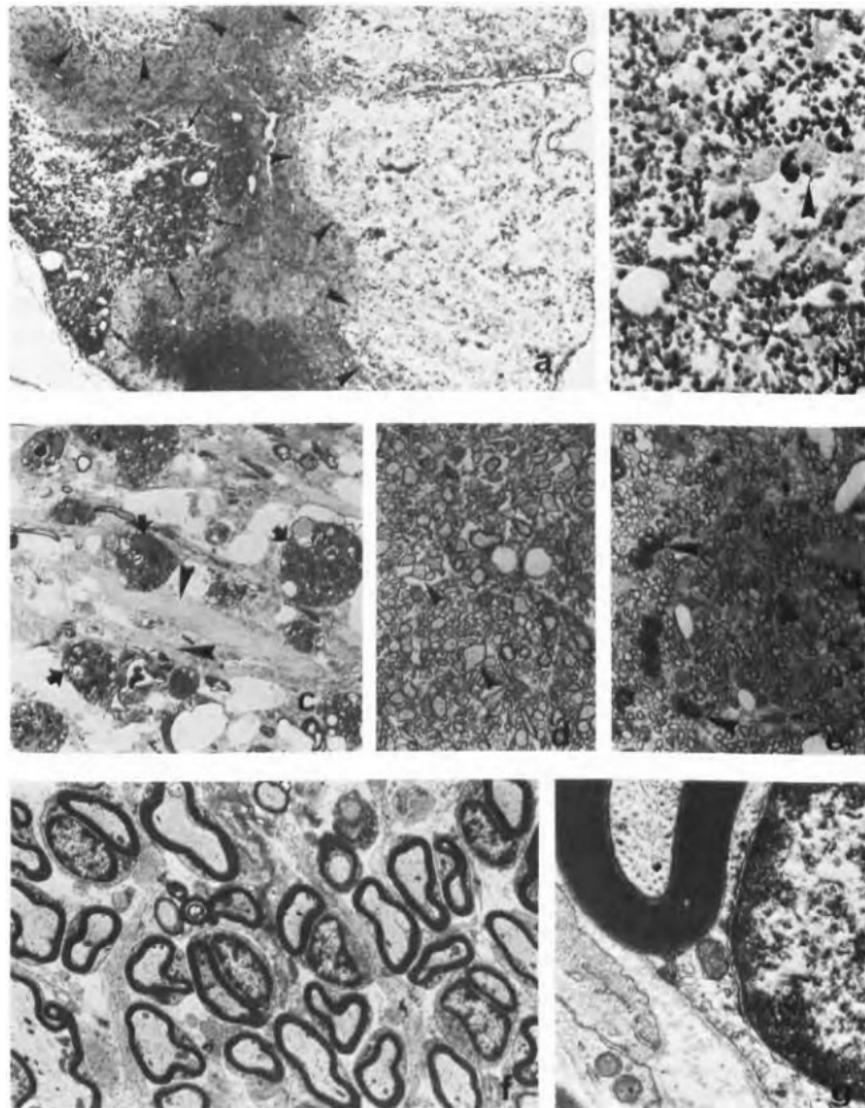
first attack lasted from 8 to 25 days until they recovered. In all cases recovery was complete and no disability was noted. These animals came down with a second attack 41–173 days p.i., however, with an average incubation time between onset of the first attack of disease and its exacerbation, of 61 days. The clinical picture of the second attack was similar to the first, except the course seemed to be more severe. Since these animals were needed for experimental analysis it is not known whether they would have recovered again.

The histopathological investigation of relapsing SDE in these rats revealed the following changes as documented in Fig. 2. All rats studied showed fresh demyelinating lesions with infiltrations of mononuclear cells primarily located in spinal cord and brain stem. In the same animals, old lesions were also found in pons, thalamus, cerebellum or spinal cord. These old lesions in the white matter revealed extended remyelination of the CNS and/or PNS type. In some cases proliferation of astroglia was observed. A very pronounced fresh, demyelinated lesion in the spinal cord at the same level as a remyelinated area is shown in Fig. 2a. This rat developed a relapse of SDE 138 days p.i. (No. 8 in Table 1). A higher magnification of a thin section from the demyelinated plaque clearly reveals naked axons in the presence of macrophages (Fig. 2c). An extended area of remyelination by Schwann cells (PNS type) was noted at the same level of this spinal cord. Higher magnification and electron microscopy clearly demonstrated the association of Schwann cells with axons and the basal membrane (Figs. 2b,e,f,g). Even in the remyelinated areas infiltrating macrophages were detectable. Furthermore, remyelinated areas of the CNS type were found at different levels of this spinal cord (Fig. 1d). In addition, remyelinated areas of the PNS type and macrophage infiltrations with myelin debris were encountered in the pons.

Attempts to re-isolate JHM virus from these animals yielded different results (Table 2). Despite the fact that all animals tested revealed viral antigens in glia cells

Fig. 2. Relapse of SDE 138 days p.i. following inoculation of ts43. The Lewis rat was infected at an age of 10 days.

   a: Large demyelinated plaque in the anterior and lateral area of white matter (arrow heads). The anterior white matter is unusually swollen by edema which leads to deformation of the grey matter. However, in this area neurons are well preserved. In the posterior column (surrounded by arrows) PNS type remyelination was observed. Hematoxylin-eosin and luxol fast blue staining of paraffin-embedded section. The rat was perfused with glutaraldehyde-paraformaldehyde solution. ×64.

   b: Same level as a, higher magnification from remyelinated area. Numerous nuclei of Schwann cells are detectable indicating a PNS type remyelination. Note infiltration by macrophages (arrow heads) even in this area. ×420.

   c: Same level of spinal cord with demyelinating plaque as a, 1-μm section after embedding in epon. Naked axons (arrow heads) and infiltration by macrophages (arrows) with myelin debris indicate fresh demyelination. Stained with toluidine blue. ×625.

   d: Thoracic spinal cord, upper level. Remyelination of the CNS type is indicated by thinly myelinated axons (arrow heads). 1-μm section after embedding in epon, stained with toluidine blue. ×550.

   e: 1-μm section after embedding in epon from same level with remyelination of PNS type as a and b. Nuclei of Schwann cells associated with axons. Macrophage infiltration (arrow heads). ×324.

   f and g: Electron microscopy of remyelinated area (same level as a and b). Schwann cells in association with axons are surrounded by a basal membrane. ×4400 and ×26000.

TABLE 2

VIROLOGICAL AND IMMUNOLOGICAL FINDINGS

| Case | Virus antigen detected in: | | Virus isolation | | Antiviral antibodies | |
|------|---------|-----------------|-------------------|---------------------|---------------------|----------------|
| | Neurons | Non-neural cells | Infectious virus | Cell-associated virus | Neutralizing titer | ELISA titer |
| 1 | − | + | − | + | 1:120 | 1:640 |
| 2 | − | + | − | + | 1:8 | 1:160 |
| 3 | − | + | − | − | 1:980 | 1:2560 |
| 4 [a] | n.d. | n.d. | n.d. | n.d. | 1:13 | n.d. |
| 5 | n.d. | n.d. | − | + | 1:1000 | 1:640 |
| 6 | − | + | + | − | <1:8 | 1:40 |
| 7 | − | + | + | − | <1:8 | 1:40 |
| 8 [a] | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 9 | − | + | n.d. | n.d. | 1:200 | 1:640 |

[a] These rats were perfused with glutaraldehyde–paraformaldehyde for electron microscopy.

n.d. = not done.

in the neighbourhood of demyelinating plaques (Fig. 3), only from 2 animals (animal Nos. 6 and 7) was infectious virus obtained after homogenization of spinal cord and brain specimens. Only non-infectious virus was recovered from the other animals.
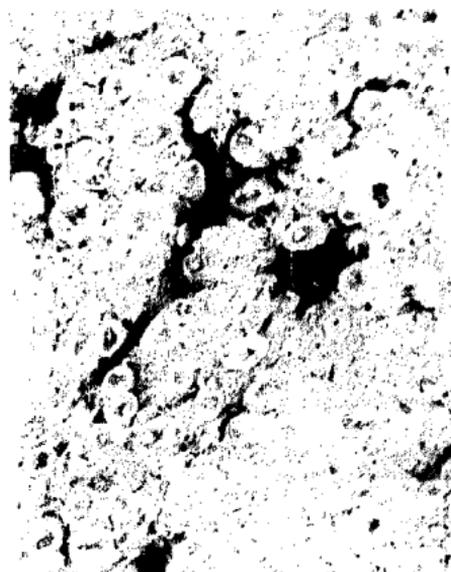


Fig. 3. Virus antigen in the white matter of the spinal cord in the neighbourhood of a fresh demyelinating plaque. Antigen-positive cells are closely related to the myelin sheath around the axons. Frozen section, ×725.

Sac(-) cells which were used for those isolation attempts, developed cytopathic effects consisting of giant cell formation, a typical CPE of coronavirus infection. in which viral antigens were present. No infectious virus was detectable in these cultures, however, either in the supernatant or in a cell-associated form. A non-yielding persistent infection was apparently established in these tissue culture cells. With regard to these findings, the immune response of these animals against JHM virus is of interest. All animals tested except 2 revealed high antibody titers against JHM virus detectable by ELISA and neutralizing assay. Only serum samples from rat Nos. 6 and 7 showed a low reaction in the ELISA and no detectable neutralizing antibodies. Yet, infectious virus was isolated only from these 2 animals which lacked neutralizing antibodies. This suggests that the failure to recover infectious virus from the other rats might be related to the presence of neutralizing antibodies which inactivate free infectious virus.

## Discussion

Intracerebral inoculation of 10–15-days-old Thomae or Lewis rats with certain neurotropic ts-mutants of murine coronavirus JHM leads to the development of a subacute demyelinating encephalomyelitis which may or may not be fatal. Animals surviving this infection can recover completely and reveal no clinical signs of disability, but under conditions which are presently not understood a second attack of this CNS disease can develop which is very similar to the first one.

These observations demonstrate that JHM-persistent infection in rat CNS tissue can result in demyelinating diseases, with remission and relapse of clinical symptoms, in connection with exacerbations of neuropathological changes. Evidence for chronic, recurrent demyelination by coronavirus JHM has been obtained in infections of mice but no significant clinical changes were observed in this animal (Herndon et al. 1975; Haspel et al. 1978; Knobler et al. 1981, 1982; Stohlman and Weiner 1981). A biphasic disease course with clinical symptoms and recurrent demyelination has been described in Theiler's virus infection of mice (Lipton and Dal Canto 1976; Dal Canto and Lipton 1979; Dal Canto 1982). In this infection, young adult mice develop a flaccid paralysis from which the majority of animals recover. Surviving mice subsequently come down after weeks with a spastic, paralytic gait disorder. In the early disease the grey matter is predominantly involved, resembling human poliomyelitis, whereas in the later disease primary demyelination is found related to perivascular mononuclear cell infiltrations. In this virus infection the early and late disease are neuropathologically quite different, whereas in JHM virus-induced recurrent demyelination the first and second disease are clinically and neuropathologically very similar. Some evidence has been provided that Theiler virus induces an immune-pathological reaction which could contribute to demyelination, since immunosuppression decreases or prevents demyelination, but the exact pathogenetic mechanism is unknown.

The induction of a progressive demyelinating, or relapsing demyelinating, disease

process, in JHM virus-infected rats has some parallels to chronic EAE, an animal model based on sensitation against CNS tissue extracts or myelin basic protein (McFarlin et al. 1974; Raine et al. 1978; Lassmann and Wisniewski 1979; Traugott et al. 1982). The mechanisms by which a JHM infection leads to SDE with a remitting-relapsing course is unknown at present. Immunological studies carried out in our laboratory at the first attack of SDE demonstrate that lymphocytes are sensitized against myelin basic protein in the course of JHM infection. Adoptive transfer of these lymphocytes, restimulated against myelin basic protein in vitro, was accompanied by EAE-like lesions in CNS tissue as well as clinical symptoms (Watanabe et al. 1983). These findings suggest that JHM virus infection in brain tissue may have a similar effect to Freund's complete adjuvant plus myelin basic protein, or CNS-extracts, in EAE. During replication of JHM virus, an autoimmune reaction is triggered which may contribute not only to the development of SDE, but also to the observed relapses. In other experimental infections by neurotropic viruses, an indication of the development of a humoral autoimmune reaction to CNS tissue has been observed. During replication of a neurotropic vaccinia virus in mice, and in chronic canine distemper virus infections of dogs, antibodies against myelin basic protein and oligodendrocytes were detected (Steck et al. 1981; Vandevelde et al. 1982). These data suggest that different virus infections of the CNS can be associated with an autoimmune response against brain tissue.

On the other hand, it is conceivable that the virus infection of CNS cells also contributes to the relapsing disease course. It has been shown in visna infection in sheep that the antiviral immune response is not able to control the CNS infection. In the presence of antibodies to visna virus, mutants continuously develop which cannot be neutralized by the antibodies directed against the parental virus (Narayan et al. 1978). The emergence of each new mutant permits the infection of new areas of the brain leading to inflammatory reactions and CNS damage. In coronavirus infection of rats, JHM virus can be re-isolated after several months of persistence. So far, the antigenicity and biological properties of such re-isolated virus have not been compared to those of the virus used for inoculation. In relation to other persistent infections of the CNS such as subacute sclerosing panencephalitis, it is remarkable that infectious JHM virus can be isolated: this suggests that antigenic changes may occur during persistency.

The occurrence of chronic viral infection, the relapsing clinical course with the presence of old and fresh demyelinating plaques and the evidence of a cell-mediated immune reaction to myelin basic protein during the first attack of SDE (Watanabe et al. 1983), suggest that this disease process is a result of a very complex virus-host interaction, in which host factors play an important role. It is conceivable that the autoimmune reaction against myelin basic protein may also perpetuate the disease, even when the virus is no longer demonstrable in CNS tissue. The features of this coronavirus JHM-associated CNS disease in rats reveal some similarities to multiple sclerosis in man and it is hoped that the analysis of the events which lead to this animal disease will be of relevance in our understanding of other relapsing, demyelinating processes.

## Acknowledgements

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