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# Acute and late disease induced by murine coronavirus, strain JHM, in a series of recombinant inbred strains between BALB/cHeA and STS/A mice

**Shigeru Kyuwa,[1] Kenjiro Yamaguchi,[1] Yutaka Toyoda,[1] Kosaku Fujiwara[2] and Jo Hilgers[3]**

[1]*Department of Animal Pathology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan,* [2]*Laboratory of Pathobiology, Nihon University School of Veterinary Medicine, Fujisawa 252, Japan and* [3]*Department of Obstetrics and Gynaecology, Academisch Ziekenhuis Vrije Universiteit, 1081 HV Amsterdam, The Netherlands*

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To examine the genetic control of acute and late disease induced by a murine coronavirus, strain JHM (JHMV), BALB/cHeA, STS/A, $F_1$ hybrids and 13 recombinant inbred (RI) strains between BALB/cHeA and STS/A mouse strains were inoculated intracerebrally with 100 pfu of JHMV. All the BALB/cHeA mice died within 2 weeks from acute encephalitis. In contrast, STS/A mice were shown to be partially resistant, with a mortality rate of 30%, longer survival times and lower rates of viral production. The mortality rates, survival times and viral titers of $F_1$ hybrids and the RI strains varied, suggesting involvement of multiple genes. STS/A, $F_1$ hybrid and RI mice surviving the acute infection occasionally developed severe paraparesis about 1 month post-infection. In these mice, vacuolar degeneration, astrocytosis, the absence of perivascular cuffing and minimal demyelination were found in the central nervous system. No infectious virus could be recovered from these mice. Although the paralysis of delayed onset was limited to STS/A, $F_1$ hybrid and eight of the 13 RI strains, the incidence varied significantly among the RI strains. These results may suggest that JHMV-induced late disease is also under multifactorial control. The pathogenesis of JHMV infection is discussed.

*Key words*: central nervous system; coronavirus; fatal encephalitis; genetic control; paralysis.

## Introduction

Murine coronavirus infections induce a variety of diseases in mice.[1] A number of informative studies using prototype viruses, including the JHM strain (JHMV),[2] have been reported. Similar to other viral infections, the fate and the process of primary coronavirus infection in mice are dependent on genetic factors of both host and virus. For instance, almost all the laboratory strains of mice except for SJL/J mice die after intracerebral (i.c.) infection with JHMV, due to acute encephalomyelitis.[3,4] A comparative study between JHMV-susceptible BALB/c and JHMV-resistant SJL/J mice indicated that susceptibility expressed in neuronal cells as well as macrophages was controlled by a single dominant gene.[4] Furthermore, the locus was linked to the

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*Svp-2* locus on the proximal end of mouse chromosome 7, using recombinant inbred (RI) strains between susceptible SWR/J and resistant SJL/J strains.[5] However, the resistance to acute JHMV infection may involve another gene, which probably regulates the immune response to JHMV.[3]

Mice surviving the acute phase of JHMV infection occasionally develop paralysis with demyelination in the central nervous system. This disease has been studied as an animal model of human demyelinating diseases such as multiple sclerosis. In contrast to the acute fatal infection, little is known about the host genetic factor(s) which control JHMV-induced paralysis with delayed onset. However, it is very important to clarify the role(s) of the factors because they may hold a key to understanding the pathogenesis of paralytic disease induced by this neurotropic virus.

On the other hand, viral genetic factors indubitably influence JHMV infection in mice. Although the wild-type virus induces acute fatal encephalomyelitis in mice, some mutants and variants of JHMV with different pathogenicities have been reported.[1,6] Temperature sensitive mutants,[7] plaque size mutants[8,9] and variants selected by resistance to neutralizing monoclonal antibodies (mAbs)[10,11] induce demyelination but not fatal encephalomyelitis. One variant, designated JHM-cc,[12] isolated from DBT cells persistently infected with JHM-x, induces vacuolar degeneration.[13]

We attempted to analyse the genetic control of the acute and late diseases after infection with a low dose of JHMV using BALB/cHeA, STS/A, $F_1$ hybrid and 13 RI strains between BALB/cHeA and STS/A mouse strains,[14] since the two parent strains of mice show different pathogenesis after i.c. infection.

## Results

*JHMV-induced acute disease*

Female, 6–8-week-old mice of BALB/cHeA, STS/A, $F_1$ hybrid and RI strains were inoculated i.c. with 100 pfu of JHMV and monitored for acute disease for 2 weeks. A total of 588 mice were used in 21 experiments. The cumulative results of these experiments are summarized in Table 1. After i.c. infection with 100 pfu of JHMV, all mice showed signs of mild or severe encephalitis. Following the onset of the clinical signs, 100% of the BALB/cHeA mice and 30% of the STS/A mice died within 2 weeks. Both $F_1$ hybrids (BALB/cHeA×STS/A)$F_1$ ($CSF_1$) and (STS/A×BALB/cHeA)$F_1$ ($SCF_1$) died with intermediate rates during the acute phase. The mortality of the RI strains varied from 72–100%. Interestingly, two RI strains (CXS6 and CXS14) exhibited high mortality similar to the BALB/cHeA parental strain. STS/A mice were also less susceptible than BALB/cHeA mice with respect to survival times.

Virus titers in the brains from all strains were assayed on day 5 post-infection (p.i.), when virus titer peaks in both BALB/cHeA and STS/A mice. Although the difference in viral titers was limited among the strains tested, a lower rate of viral growth was observed in STS/A mice compared to BALB/cHeA mice. There was a slight positive correlation between the mortality and the viral titer ($r = 0.42$). There was also a slight negative correlation between survival times and the viral titers ($r = 0.53$).

Two each of BALB/cHeA, STS/A and both $F_1$ hybrid mice were sacrificed on day 5 p.i. for histological examinations. Immunoperoxidase staining revealed abundant viral antigens in the brains and spinal cords of BALB/cHeA mice [Fig. 1(a)]. In STS/A mice, less viral antigens were detected; however, the inflammatory response, especially perivascular cuffing, was more prominent than BALB/cHeA mice [Fig. 1(b)].

*JHMV-induced late disease*

The animals that survived acute infection were monitored for late disease till 2 months p.i. (Table 2). All of the STS/A mice that had survived acute infection recovered

**Table 1** Summary of acute JHMV infection in BALB/cHeA, STS/A, $F_1$ hybrid and CXS RI mice

| Mouse | Mortality[a] (no. dead/ no. tested) | (%) | Survival time[b] (day) | Virus titer[c] (log pfu/g) |
|---|---|---|---|---|
| BALB/cHeA | 29/29 | 100 | 5.7±1.1 | 6.7±0.1 |
| STS/A | 6/20 | 30 | 9.3±1.6 | 5.7±0.1 |
| $CSF_1$ | 20/32 | 63 | 8.8±1.7 | 6.1±0.1 |
| $SCF_1$ | 7/10 | 70 | 9.4±1.4 | NT[d] |
| CXS1 | 45/50 | 90 | 7.1±1.4 | 6.5±0.2 |
| CXS2 | 32/41 | 78 | 9.5±1.7 | 6.2±0.3 |
| CXS3 | 36/38 | 95 | 8.1±1.5 | 5.7±0.1 |
| CXS4 | 9/10 | 90 | 5.7±0.9 | NT |
| CXS5 | 49/61 | 80 | 6.7±2.2 | 6.1±0.1 |
| CXS6 | 30/30 | 100 | 8.2±2.1 | NT |
| CXS7 | 42/47 | 89 | 7.8±1.6 | 6.1±0.8 |
| CXS8 | 14/16 | 88 | 7.7±1.3 | 6.5±0.3 |
| CXS10 | 26/34 | 76 | 8.7±1.6 | 6.2±0.1 |
| CXS11 | 50/68 | 74 | 8.5±1.3 | 6.1±0.1 |
| CXS12 | 32/41 | 78 | 8.8±1.7 | 5.6±0.4 |
| CXS13 | 21/29 | 72 | 8.4±1.2 | 6.4±0.1 |
| CXS14 | 32/32 | 100 | 6.5±1.1 | 6.1±0.4 |

[a] Mortality was determined at 2 weeks p.i.
[b] Mean survival days±SD of dead mice.
[c] Mean virus titer of the brains±SD from 3 mice at day 5 p.i.
[d] Not tested.

clinically by 3 or 4 weeks p.i. Subsequently, one-third of the STS/A survivors began to show severe hind leg paralysis beginning about 1 month p.i., and then died by 2 months p.i. Some of the $F_1$ hybrids and of some RI strains (CXS2, CXS3, CXS4, CXS5, CXS8, CXS11, CXS12 and CXS13) also showed hind leg paralysis. However, the incidence of paralysis varied greatly among the RI strains (8–100%). In addition, some mice recovered from the paralysis, in contrast to the parent STS/A mice. Mice of CXS1, CXS7 and CXS10 strains showed no evidence of paralysis. Some mice which were clinically normal during the initial 2 months were monitored for an extended

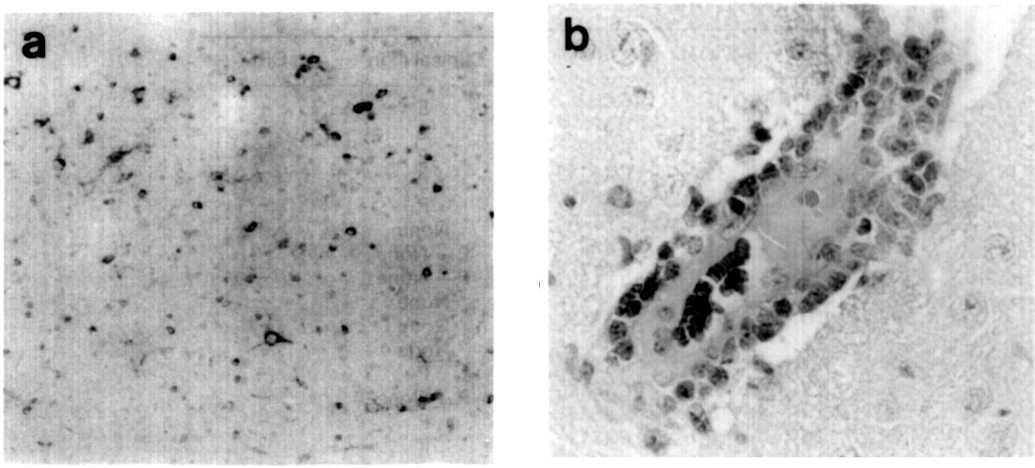


**Fig. 1.** Acute JHMV infection in the brains after i.c. inoculation with 100 pfu of JHMV. (a) Acute necrotic panencephalitis in BALB/cHeA mouse. Note abundant viral antigen in neurons and glia, and nucleus caudatus. 5 days post-infection. Immunoperoxidase staining ×105. (b) Perivascular cuffing observed in the cerebrum of STS/A mouse. 5 days post-infection. Hematoxylin and eosin ×420.

**Table 2** Summary of subacute JHMV disease in BALB/cHeA, STS/A, $F_1$ hybrid and CXS RI mice

| Mouse | No. observed | No. paralysed | (%) | No. dead | Mean day of death |
|---|---|---|---|---|---|
| BALB/cHeA | — | — | — | — | — |
| STS/A | 14 | 5 | 36 | 5 | 47.2 |
| $CSF_1$ | 12 | 4 | 33 | 4 | 33.8 |
| $SCF_1$ | 3 | 2 | 67 | 2 | 36.5 |
| CXS1 | 5 | 0 | 0 | 0 | — |
| CXS2 | 9 | 1 | 11 | 0 | — |
| CXS3 | 2 | 1 | 50 | 0 | — |
| CXS4 | 1 | 1 | 100 | 1 | 26.0 |
| CXS5 | 12 | 1 | 8 | 0 | — |
| CXS6 | — | — | — | — | — |
| CXS7 | 5 | 0 | 0 | 0 | — |
| CXS8 | 2 | 2 | 100 | 2 | 31.0 |
| CXS10 | 8 | 0 | 0 | 0 | — |
| CXS11 | 18 | 7 | 39 | 2 | 31.5 |
| CXS12 | 9 | 3 | 33 | 0 | — |
| CXS13 | 8 | 6 | 75 | 5 | 34.4 |
| CXS14 | — | — | — | — | — |

[a] Mice surviving acute infection were monitored from 2 weeks to 2 months p.i.

period. None of these mice showed evidence of paralysis during this period (data not shown).

At various times p.i., we attempted to isolate infectious virus from the brains of surviving mice with various clinical status (Table 3). Infectious virus could only be isolated from the brains of mice which showed signs of mild encephalitis at day 11 p.i. No infectious virus was recovered from the brains of mice showing paralysis.

The brains and spinal cords of mice showing evidence of paralysis were studied histologically. Vacuolar degeneration was observed in the brainstem and spinal cord

**Table 3** Summary of virus isolation from the brains of infected mice

| No. | Days p.i. | Strain | Clinical sign[a] | CPE[b] |
|---|---|---|---|---|
| 1 | 11 | CXS2 | E | + |
| 2 | 11 | $CSF_1$ | E | + |
| 3 | 19 | CXS7 | E | − |
| 4 | 38 | CXS11 | P | − |
| 5 | 45 | CXS12 | P | − |
| 6 | 61 | STS/A | None | − |
| 7 | 61 | CXS2 | P → None | − |
| 8 | 61 | CXS3 | P → None | − |
| 9 | 61 | CXS7 | None | − |
| 10 | 61 | CXS11 | None | − |
| 11 | 61 | CXS11 | None | − |
| 12 | 78 | CXS11 | P | − |
| 13 | 78 | CXS11 | P → None | − |
| 14 | 115 | CXS11 | P | − |
| 15 | 115 | CXS11 | P | − |

[a] E, encephalitis; P, paralysis; P → None, healthy mice which have been recovered from paralysis.

[b] 10% homogenates of brains were inoculated on DBT cells. CPE was observed carefully by 48 h p.i.

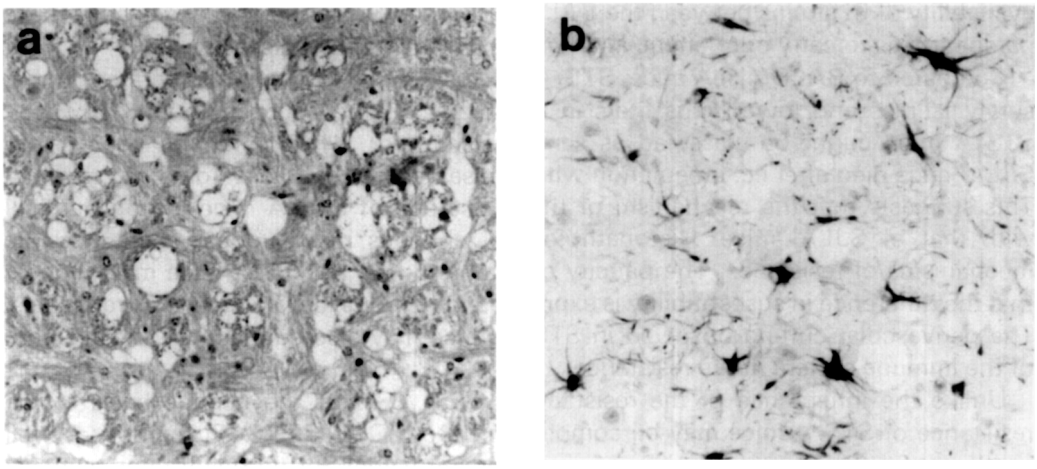


**Fig. 2.** Histopathological changes in brains of paralysed STS/A mice following i.c. inoculation with 100 pfu of JHMV. (a) Marked vacuolar degeneration in the pons. 50 days post-infection. Hematoxylin and eosin ×159. (b) Involvement of astrocytosis in vacuolar degeneration. 50 days post-infection. Immunoperoxidase staining ×159.

[Fig. 2(a)]. Astrocytosis was also observed in the lesions [Fig. 2(b)]. However, there was little infiltration by mononuclear cells. Trace amounts of viral antigens were detected. The degree of demyelination also appeared to be slight. Although the number of samples was limited, no obvious difference in the histopathological changes dependent on mouse strain was observed in mice manifesting paralysis. These histological changes appeared to be distinct from that of mice infected with a JHMV variant (2.2-V-1) with a major demyelination inducing determinant.[15,16]

## Discussion

Several strains of murine coronavirus have facilitated studies of natural resistance to viral infections. Bang *et al.* found that a single recessive gene encodes the resistance of PRI mice to mouse hepatitis virus type 2 infection.[17] Alternatively, a genetic study indicated that resistance to mouse hepatitis virus type 3 infection involves at least two major genes: one for the acute disease and the other, H-2-linked, for the chronic disease.[18] The genetic resistance to JHMV infection has also been studied by several groups but remains controversial. Stohlman *et al.* found that only SJL/J mice are resistant to acute fatal JHMV infection among 19 strains tested[3,19] and suggest that two genes are involved in the resistance to acute fatal JHMV infection.[3] However, using a lower dose of virus, Knobler *et al.* suggest that a single gene controls the resistance to acute JHMV infection in SJL/J mice.[4] Furthermore, the mechanism of the resistance seems to be discrepant. Although Wilson and Dales find no critical difference in the early events from adsorption to genome activation between JHMV-resistant SJL/J and JHMV-susceptible CD.1 glial cultures,[20] Holmes and colleagues have proposed that the resistance of SJL/J mice is perhaps due to the lack of functional virus receptor on the plasma membrane.[21]

To examine the genetic control of diseases induce by the JHMV strain maintained in this laboratory, some preliminary experiments were performed. As reported earlier,[3,4] BALB/cHeA, C57BL/6, C57BL/10 and DBA/2 mice died due to acute encephaiitis after i.c. infection with 100 pfu of JHMV. However, 30% of STS/A and 100% of SJL/J mice survived the acute phase of infection. In addition, some survivors of the STS/A strain subsequently manifested hind leg paralysis (data not shown). Due to the

availability of RI strains between the BALB/cHeA and STS/A strains,[14] we chose these parent strains to carry out genetic analysis of JHMV-induced disease.

Compared to BALB/cHeA mice, STS/A mice exhibited lower mortality rates, longer survival times and lower virus titers in the brains during acute infection. However, STS/A mice might be classified as semi-resistant for JHMV infection because no SJL/J mice died after i.c. inoculation with the same dose of JHMV (data not shown). This suggests that the mechanism of the resistance of STS/A mice is not identical with that of SJL/J mice. Histopathological changes may suggest some possible mechanisms of resistance. The paucity of viral antigens in STS/A mice may suggest that the difference in susceptibility is expressed in neuronal cells where virus replicates. The perivascular cuffing observed in STS/A mice may also suggest the involvement of the immune system in the resistance.

Unlike the inheritance of the resistance of SJL/J mice, the genetic basis of the resistance of STS/A mice may be complicated. Both $CSF_1$ and $SCF_1$ hybrids, as well as almost all the RI strains, showed intermediate values for mortality rates, survival times and virus titers in the brain. These data suggest that the resistance is a multiple trait and not due to one major gene effect in this combination of mouse strains. The hypothesis is consistent with the multiple differences of histopathological changes between STS/A and BALB/cHeA mice during the acute phase.

A recent study indicated that a mutant JHMV designated JHM-cc induced paralysis associated with vacolar degeneration but not demyelination,[13] although demyelination has generally been ascribed as the cause of paralysis following murine coronavirus infections in the central nervous system of mice.[7–11] The late disease observed in this study largely agrees with these results. The virus used in this study does not have a major demyelination-inducing determinant characterized by mAb J.7.2.[15] Similarly, JHM-cc virus probably lacks the determinant because it is derived from the virus used in this study.[12] Furthermore, murine coronavirus strain A59, which also induces vacuolar degeneration in the spinal cord,[22] did not react with this mAb.[23] These data suggest that some genetic factor of virus affects paralysis with vacuolar degeneration. One possibility is the deletions in the S gene found among these virus strains.[24] However, some secondary response(s) to the viral component rather than viral cytopathic effect may be responsible for the vacuolar degeneration, since it does not coincide with viral replication.

Vacuolar degeneration in the central nervous system is not unique in coronavirus infections, but is also observed in other virus infections,[25,26] poisoning and metabolic defects,[27] and is often associated with astrocytosis.[25,26] Furthermore, there has been accumulating evidence which accords prominence to the role of astrocytes in neurodegenerative diseases such as scrapie and Alzheimer's.[28,29] Although only traces of viral antigen were detected in the lesions of paralysed mice in this study, Perlman and Ries suggested that astrocyte is a potential target cell in which JHMV establishes a latent or low level persistent infection.[30] Therefore, it may be important to know what happens during the process of recovery from acute encephalitis in those mice doomed to paralysis, especially interaction between virus and astrocyte.

In the genetic study of late disease, $F_1$ hybrid mice exhibited paralysis with almost the same incidence (40%) as STS/A mice (36%). This suggests that the paralytic disease induced by this virus may be inherited as a dominant trait. Although the number of mice tested was not large enough to make this conclusion, the large variation in the incidence of paralysis among RI strains might suggest that paralytic disease is also inherited as a multiple trait.

Our data suggest that JHMV-induced acute and late disease is inherited as a multiple trait. Nonetheless, differential incidence of gene expression, possibly due to

environmental influences and variable penetrance of the gene, cannot be ruled out. Therefore, it may be worth analysing the genetics of JHMV-induced disease using the hypothesis that a single gene, or one major gene, controls the disease. We have tentatively designated the gene as Pj-1 and divided the mouse strains into the strains showing sublethal infection with paralysis of delayed onset, represented by STS/A mice, and the strains inducing fatal encephalitis, represented by BALB/cHeA mice. Mice exhibiting more than a 30% incidence were considered to constitute a STS/A type strain. The mouse strain which died from acute encephalitis was considered to constitute a BALB/cHeA type. Comparing with the strain distribution pattern (SDP) of 92 genes which have determined thus far, we found a good correlation between the SDP of the Pj-1 and the SDP of some markers on mouse chromosome 7 (Table 4). Since no recombinant was observed between the Pj-1 and Ly-15, the Pj-1 gene may be located near the Ly-15 locus. The Ly-15 encodes one of immunologically important molecules known as lymphocyte function-associated antigen-1 (LFA-1).[33] LFA-1 is believed to bind to ICAM-1 and facilitate T cell recognition. In fact, Davignon *et al.* have shown that this molecule is involved in cytotoxic T cell response,[34] which is believed to be a major effector mechanism in JHMV clearance from the brain.[35,36] Since infection of neurons resulting in a necrotizing fatal encephalomyelitis is a major cause of death due to JHMV infection, it is possible that this molecule is involved, not only in the prevention of death by limiting virus replication, but also in the development of late disease.

Finally, STS/A mice was semi-resistant to JHMV and showed a biphasic CNS disease following JHMV infection. The same phenomenon was reported in mouse hepatitis type 3 infection.[18] Semi-susceptibility of the host seems to be necessary for the development of late disease. This may suggest that host's antiviral response(s) not only protect mice from a necrotizing encephalitis but also trigger late disease. Alternatively, increased incidence of late disease was observed following transfer of partial immunity to JHMV.[37] Taken together, JHMV-induced late disease may occur under a subtle balance between the virus and the immune system. Polygenic inheritance may be suitable to interpret such a complex situation.

## Materials and methods

*Mice.* BALB/cHeA, STS/A, $CSF_1$, $SCF_1$ and 13 RI strains between BALB/cHeA and STS/A[14] were bred and maintained in our laboratory. The breeding colonies were kept in a lamina flow-

**Table 4** Inheritance of the Pj-1 and other markers of mouse chromosome 7 in CXS RI strains of mice

| | CXS | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Locus | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 10 | 11 | 12 | 13 | 14 | References |
| Mpt-1 | S | S | S | S | S | S | C | C | S | C | S | S | S | 31 |
| Gpi-1 | S | S | S | S | S | S × | C | C | S | C × | S | S | S | 14 |
| Prt-4,5 | S | S | S | S | S | C | C | C × | S | S | S | | S × | 32 |
| Pj-1 | — | — | S | S | — | C | — | S | — | S | S | S | C | |
| Ly-15 | C | C | S | S | S | C | C | S | C | S | S | S | C | 33 |

The symbols C and S indicate the allels inherited from BALB/cHeA and STS/A mice, respectively. (—) Indicates not to be unclassified; ( ) indicates not to be determined. Gene loci are aligned from the centromere to the telomere. × Denotes a region of recombination.

ventilated rack system and were routinely checked serologically for the absence of murine hepatitis virus (MHV).[38] Female, 6–8-week-old mice were used throughout the experiments.

*Virus.* JHMV was propagated and plaque assayed on DBT cells as described previously.[39] A single pool of virus was divided into aliquots and stored at −70°C until use. The virus used in this study does not have virus neutralizing determinants characterized by mAbs J.7.2 and J.2.2 (data not shown).[10,23] Mice were inoculated i.c. with 100 pfu of JHMV in a volume of 0.02 ml.

*Titration and isolation of infectious virus.* To determine the virus titer, brains were removed aseptically. Ten per cent homogenates of the brains were made in chilled Eagle's minimum essential medium (MEM). After centrifugation at 600×*g* for 10 min, 0.2 ml of serial 10-fold dilutions of the supernatants was inoculated on monolayers of DBT cells in duplicate. After adsorption for 60 min, cultures were overlaid with MEM containing 1.5% pre-screened newborn calf serum, 5% tryptose phosphate broth and 0.8% agar. After incubation for 2 days at 37°C, plaques were visualized with neutral red.

For virus isolation, the supernatants of 10% homogenates were inoculated on monolayers of DBT cells in 60 mm dishes. After adsorption for 60 min, cultures were washed once and then fed with 5 ml of MEM supplemented with 1.5% newborn calf serum and 5% tryptose phosphate broth. Cytopathic effect was carefully checked visually under microscope after 48 h.

*Clinical observation.* The clinical status of infected mice was assessed by the methods of Fleming *et al.*[15] Briefly, mice were considered normal if they appeared alert and could turn over within 2 s after being placed on their back. Mice that had a waddling gait and were unable to rapidly turn over were scored as having mild paralysis. Mice with frank hind leg immobility were scored as having severe paralysis. Animals with hyperirritability or myoclonus were judged to have mild encephalitic signs. Seizure, persistent turning or static, hunched posture were considered to be signs of severe encephalitis.

*Histology.* The brains and spinal cords were embedded in paraffin and stained with hematoxylin and eosin, and luxol fast blue as previously described.[36] Sections were stained by an avidin-biotin immunoperoxidase procedure using mAb to the JHMV N protein[23] and anti-glial fibrillary acidic protein antibody (Paesel, Frankfurt).

*Statistics.* Coefficient of correlation was calculated by a scientific calculator (Casio, Tokyo) by the following formula:

$$r = (n\sum xy - \sum x \sum y)/[n\sum x^2 - (\sum x)^2]\{n\sum y^2 - (\sum y)^2]^{1/2}$$

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