EARLY AND LATE CNS-EFFECTS OF CORONA VIRUS INFECTION IN RATS[+]

K. Nagashima, H. Wege and V. ter Meulen

Institute of Virology and Immunobiology,
University of Würzburg
8700 Würzburg, G.F.R.

SUMMARY

The mouse hepatitis virus strain JHM was injected intracerebrally into newborn and weanling rats. Three types of diseases were observed:

1. Acute panencephalitis: Almost all suckling rats became moribund within 6 days. Histologically severe panencephalitis with demyelinating foci was noticed; the foci were similar to those found in mice. Virus was easily detectable in the oligodendroglial cells and neurons both by immunofluorescence and electron microscopy. Infectious virus could be isolated.

2. Subacute demyelinating encephalomyelitis (SDE): Three weeks after infection of weanling rats, about 35% of the animals developed paralysis. Neuropathologically, demyelination with a striking predilection for white matter was observed in the brain stem, optic nerve and spinal cord. Virus was detectable by electron microscopy in degenerating oligodendroglial cells only, which corresponded to the results obtained by the immunofluorescent techniques. Infectious virus could be recovered.

3. Chronic progressive paralysis: Inoculated weanling rats without SDE developed 6 to 8 months later a slowly progressing paralysis of the legs. Hydrocephalus and myelomalacia were present. Viral "footprints" could not be detected.

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J. Palo (ed.), *Myelination and Demyelination*

INTRODUCTION

The neuropathological finding of demyelination accompanying virus infections of the central nervous system (CNS) in human and animals is a common feature of acute and chronic diseases. So far, the pathogenetic mechanism by which such demyelination is induced is for most of these infections unknown. From a virological point of view it is of particular interest to study the virus-host relationship which is responsible for these changes. Such studies have been carried out with JHM virus infection in mice (2, 9, 16, 17). This murine corona virus causes an acute disseminated encephalomyelitis with destruction of the myelin, which results from infection of the myelin supporting oligodendroglial cells (9, 17). However, investigations in chronic viral infections of the CNS in small laboratory animals, with demyelination, have not been reported, since proper animal models are not available.

The present communication describes the neuropathological findings of three different CNS diseases obtained in rats after intracerebral JHM virus infection. Depending on the age of the animal an acute panencephalitis, a subacute demyelinating encephalomyelitis or a chronic progressive paralysis was observed. These experimentally induced diseases with a more subacute and chronic nature offer the possibility to analyze the virus and host factors, which play a role in the process of demyelination in persistent viral infections.

EXPERIMENTAL

Animals. Specific pathogen-free pregnant or weanling (20-25 days old) rats, strain CHBB/THOM, were purchased from Thomae, Biberach, Germany, and used for the experiments. The animals did not reveal neutralizing antibodies against JHM virus.

Virus. The original stock virus of JHM, a homogenate of suckling mouse brain, was kindly obtained from Dr. L.P. Weiner, Johns Hopkins University, Baltimore, U.S.A. The virus was passaged in our laboratory by intracerebral inoculation of suckling mice and was adapted also to grow in L-929 cells and Sacc(-)-cells. Both, 20% brain suspension or clarified supernatant of infected culture cells, were used as inoculum for the experiment. Depending on the experiment the virus preparation contained between 5×10^5 - 1×10^7 ID50/ml.

Animal inoculation. Weanling and newborn rats were inoculated into the left brain hemisphere. Newborn rats received 0.01 ml and weanling rats 0.03 ml of the desired virus suspension.

Immunofluorescence studies. Antiserum against JHM in mice was prepared by weekly intraperitoneal inoculations of brain suspension containing virus (0.5 ml/animal) over a period of six weeks. Animals were exsanguinated two weeks after the last injection. For the

detection of JHM antigen in CNS-tissue, cryostat sections were fixed for 10 min in acetone and stained with the specific antiserum applying the indirect immunofluorescent technique. FITC labeled anti-mouse globulin was obtained from Microbiological Ass., Maryland, U.S.A. Anti-JHM serum and FITC labeled anti-mouse globulin were used only after absorption with brain powder of control rat brain. Cryostat sections stained for viral antigens were further examined by hematoxylin-eosine staining to correlate the virological findings with the histological changes.

Virus isolation. The animals were dissected under aseptic conditions. After several washings in cold PBS with antibiotics specimens were homogenized in a glass douncer to give a 15% (w/v) suspension. Crude homogenates were adsorbed on monolayers of L-cells (0.3 ml/petridish 20 ccm) for one hour. The monolayers were washed and overlaid with 5 ml MEM containing 5% fetal calf serum and antibiotics. Cultures, which did not show a JHM-CPE after 48 h were passaged two times before a negative result was accepted.

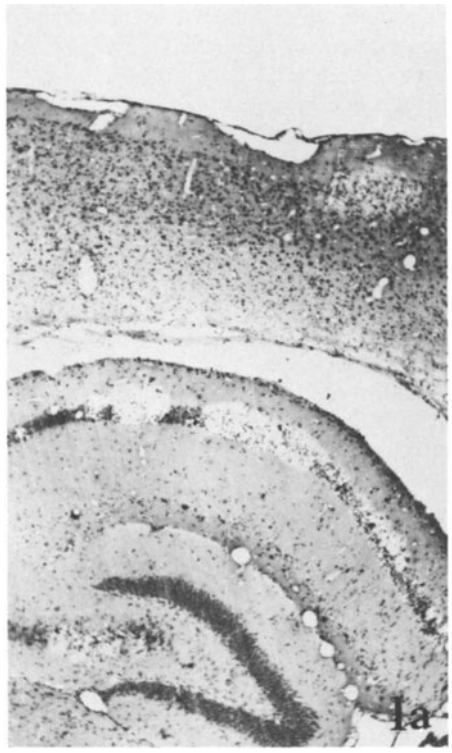
Histology and electron microscopy. For histological and ultrastructural examinations tissue blocks were taken from animals perfused with 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M phosphate buffer solution at 37°C. Paraffine embedded specimens were stained with hematoxylin-eosine (HE), Klüver-Barrera method for staining of myelin (KB) and Glees and Marsland's method for staining of axons. Blocks for electron microscopy were postfixed in 1% osmium tetroxide, stained with 2% uranyl acetate in 70% alcohol, dehydrated and embedded in epon. Thick sections, cut with a Reichert microtome, were stained with toluidine blue or paraphenylene diamine (PPD). Thin sections from selected blocks were cut with a diamond knife. The sections were stained with lead citrate and examined with a Zeiss 10B electron microscope.

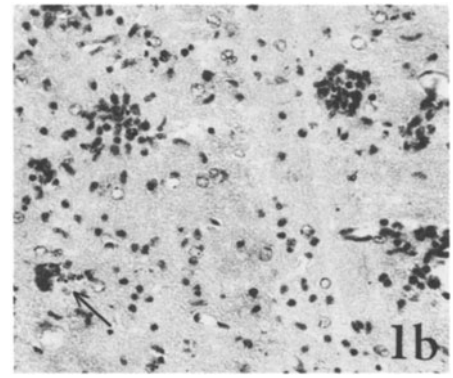
RESULTS

Acute panencephalitis. Intracerebral inoculation of JHM virus in newborn rats led to a hindleg paralysis within 3 to 4 days after virus infection (Table 1). The animals died one to two days after onset of the disease. Neuropathologically the following changes were observed. In the cerebral cortex and hippocampal gyrus (Fig. 1) foci of fresh necrosis were present. In these necrotic areas pyknotic neurons were found surrounded by nuclear debri and polymorphonuclear leukocytes. Glial nodules (Fig. 1b) were often encountered in the hippocampal gyrus and basal ganglia. Meningitis had developed in all cases and ependymitis was found in a few rats. Perivascular areas of the cortex and white matter were infiltrated by polymorphonuclear leukocytes and small lymphoid cells. A few multinucleated giant cells were found in the perivascular areas and meninges. No inclusion bodies were detected.

Table 1. Acute encephalitis in newborn rats. - The incubation period ranged from 3 to 7 days. Virus could be isolated from the brain material of all animals tested.

No. of animals infected (I.C.)	No. of diseased animals
12	5
9	7
15	13
38	32





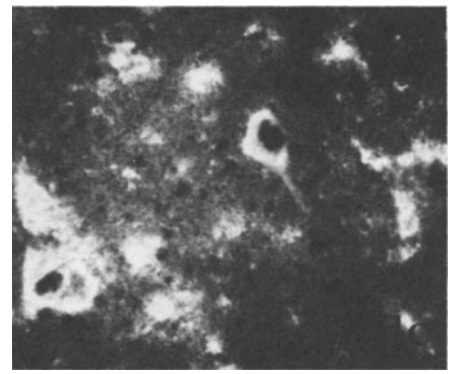


Fig. 1. Cerebrum of newborn rats 6 days after injection.
1a. Patchy circumscribed fresh necrosis found in hippocampal gyri. HE x 120.
1b. Scattered glia-mesenchymal nodules in basal ganglia. Note polymorphonuclear infiltration and multinucleated giant cell (↖). HE x 300.
1c. Immunofluorescent staining of brain stem. JHM antigen detectable in glial cells and large neurons. x 600.

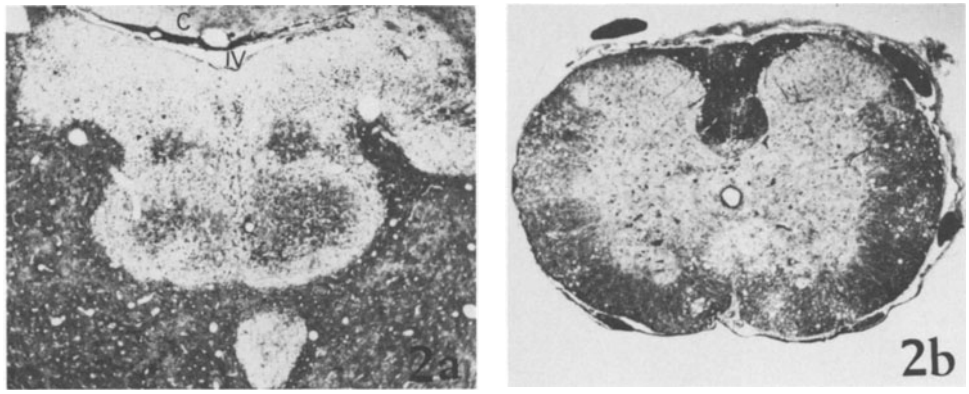


Fig. 2. Myelin stained preparations of pons and spinal cord of newborn rats 6 days after injection.
2a. Almost symmetrically distributed large circumscribed spongy lesions. Note the relatively well remaining neurons in lesions. C: cerebellar cortex, IV: fourth ventricle (vessels were dilated due to perfusion). x 60.
2b. Spongy state in the white matter of spinal cord. Advanced lesions were found deep in the anterior column. Note cell infiltration in the gray matter. x 60.

In the brain stem many sharply circumscribed lesions were found, which consisted of a spongy and rarified ground substance (Fig. 2a). The neurons in these areas were relatively unaltered although staining with HE was very pale. Myelin sheaths were completely destroyed and in addition to some extent axons. However, in some areas axons were relatively well preserved in contrast to the severe destruction of myelin sheaths.

In the gray matter of the spinal cord glial nodules and a few mononuclear cell infiltrations were detectable whereas the white matter revealed lesions of demyelination unrelated to specific tracts (Fig. 2b). The pronounced white matter lesions lacked myelin sheaths completely and were filled with large amounts of macrophages. The early lesions of demyelination showed the beginning of a spongy degeneration. Spinal ganglia and peripheral nerves were histologically normal.

By immunofluorescence technique JHM antigen could be demonstrated in neurons and glial cells. In the cerebrum, the pyramidal cells of hippocampal gyrus were constantly affected. In the cerebral white matter antigen-positive small cells were arranged in parallel to the myelin sheaths. The Purkinje cells of the cerebellum were stained positive up to their dendrites. Large neurons as well as glial cells in the brain stem showed positive immunofluorescence (Fig. 1c). In the spinal cord antigens were

found both in the gray and white matter. It is of particular interest that in the white matter of the spinal cord the antigen present in cells was arranged in a triangular shape pointing to the center of the spinal cord. This suggests that the virus infection proceeds from the periphery to the center. Viral antigen could also be found in some meningeal cells, however, ependymal cells, nerve roots, spinal ganglia and peripheral nerves were negative.

Electron microscopy revealed virus particles in the neurons, astrocytes and oligodendroglial cells. In the cytoplasmic vacuoles of large neurons in brain stem many virus particles were found (Fig. 3). Some were seen in the vesicles near the Golgi apparatus (Fig. 3). The particles were also detectable outside the neuron

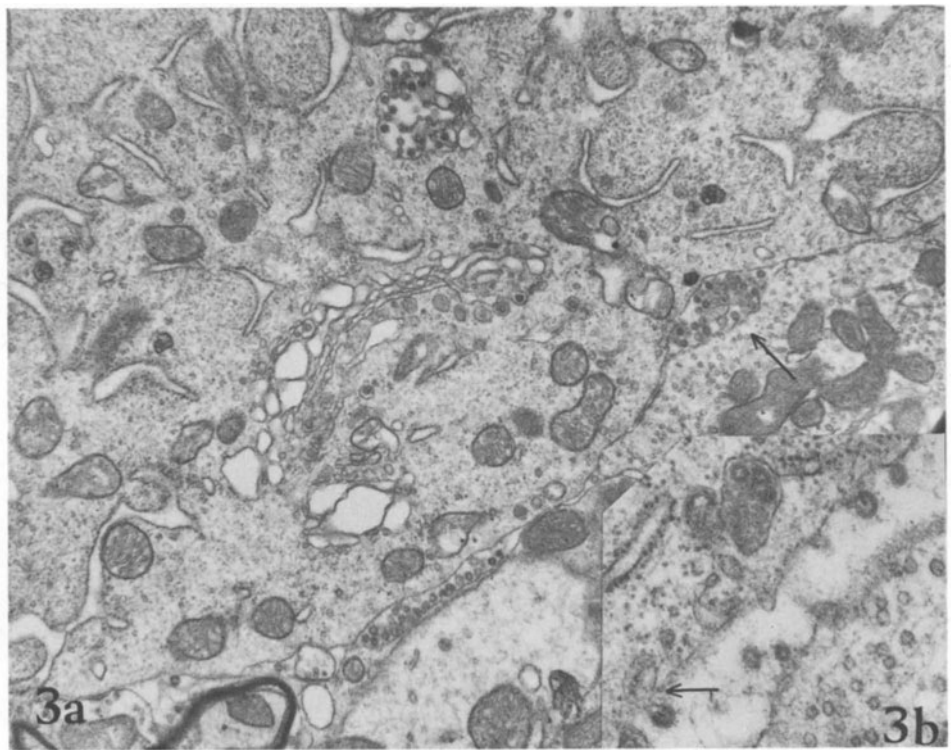


Fig. 3. Neuron in brain stem of newborn rats 5 days after injection.
3a. Virus particles observed in the vacuoles and vesicles around Golgi apparatus as well as in the synaptic cleft (↖). x 20,000.
3b. Neuronal cytoplasm on left upper side and synapse on right lower side. Virus particles found in the vacuoles and synaptic cleft. One vacuole adjacent of cell membrane releasing a particle into the cleft (↖). x 40,000.

in the synaptic cleft (Fig. 3a). A vacuole just beneath the cell membrane contained many virus particles (Fig. 3b) and in another vacuole attached to the cell membrane it seemed that a particle was released into the synaptic cleft (Fig. 3b). The virus particle had a diameter of 60-80 mμ. In the early lesions of the spinal white matter scattered degenerating cells containing virus particles were observed (Fig. 4a). These cells were considered to be oligodendroglia because of their location within the white matter and lack of glial fibrils. Moreover, in the cytoplasmic processes extending along the myelin sheaths viruses budding into the vacuole were detectable (Fig. 4b).

Virological studies revealed infectious virus present in the area of the brain and spinal cord which showed histological changes. From all animals tested infectious virus could be isolated. Histological studies of extraneural tissue revealed foci of necrosis in the liver of some animals.

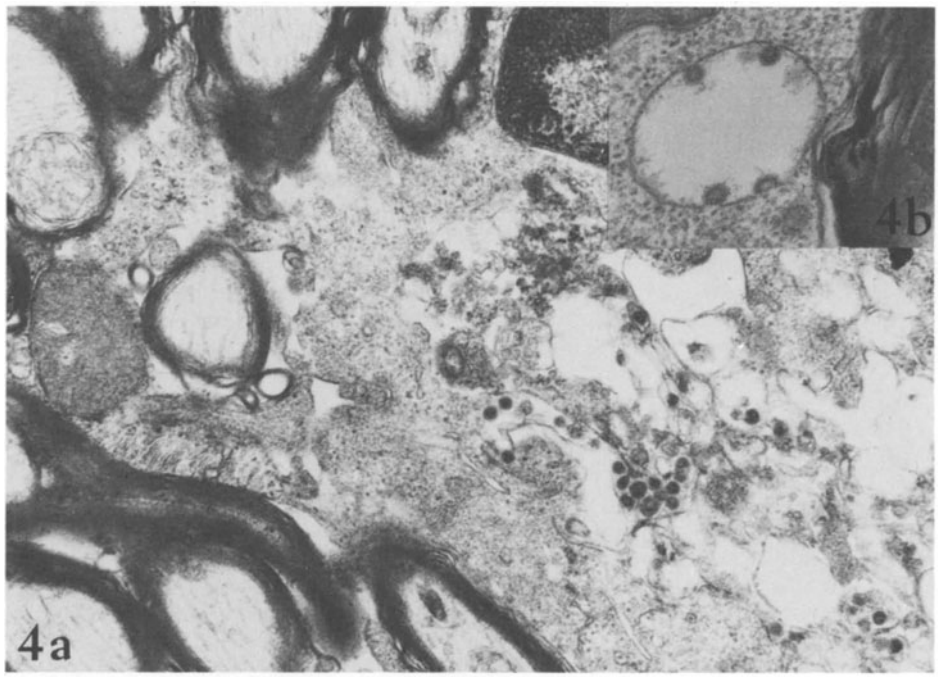


Fig. 4. Degenerating cell in the spinal white matter of a weanling rat 5 days after injection.
4a. Virus particles found in the vacuoles. x 28,000.
4b. A vacuole in the cytoplasm adjacent of myelin sheath. Note budding process. x 44,000.

Table 2. Subacute demyelinating encephalitis in weanling rats. - The incubation period ranged from 2 to 10 weeks with an average of 3 weeks. Virus could be isolated from the brain material up to 45 days after inoculation.

No. of animals infected (I.C.)	No. of diseased animals
12	6
10	4
32	12
45	15

Subacute demyelinating encephalomyelitis. A total of 99 weanling rats were intracerebrally inoculated with JHM virus (Table 2). Thirty-five rats developed hindleg paralysis between the 14th to the 24th day after inoculation, two rats showed the first symptoms on 45th and 66th day respectively, and the other animals remained clinically well. The incubation period ranged from 2 to 10 weeks after inoculation with an average incubating period of 3 weeks (Table 2). Clinically the rats showed signs of a spastic paralysis with hunched backs and a ruffled fur. Infectious JHM virus could be isolated from brain and spinal cord specimens up to 45 days after virus inoculation. A precise account of this subacute encephalomyelitis will be published elsewhere (13). The results are briefly summarized here: Histologically, acute lesions such as fresh necrosis or polymorphonuclear infiltration, observed in the suckling rats, were absent. The prominent lesions were present in the brain stem, optic chiasma and spinal cord. The lesions showed a more striking predilection for the white matter. The large circumscribed patchy lesions in the brain stem were partly similar to those of suckling rats with regard to complete loss of the myelin sheath. The neurons and axons, however, were far better preserved than those in acute cases. Moreover, neither polymorphonuclear infiltration nor multinucleated giant cells were observed. Lesions in the spinal cord were strictly confined to the white matter. Early or more pronounced demyelinating areas were found without connection to specific tracts (Fig. 5a).

In the advanced areas many gitter cells were detectable (Fig. 5b). In the early areas spongy stages were noticed, which consisted of the two types of vacuoles, one surrounded by myelin sheath and the other containing amorphous flocculent material (Fig. 5c).

Like in the acute panencephalitis the spinal ganglia and peripheral nerve were histologically normal.

The liver of a few animals revealed scattered nodules of small, round cells; foci of necrosis were absent.

Immunofluorescent studies revealed JHM antigen in the white matter of the cerebrum, brain stem and spinal cord (Fig. 5d). The neurons of the cerebral cortex, brain stem and spinal cord remained

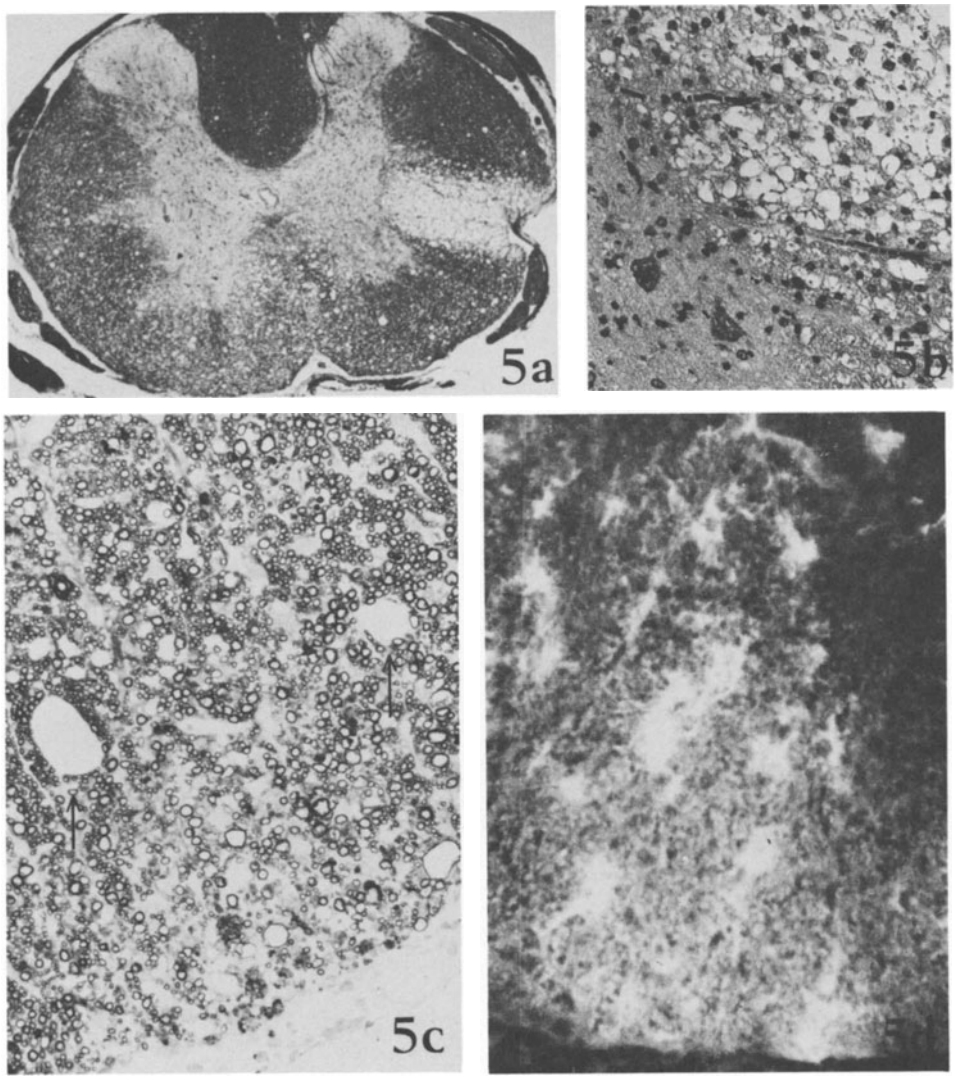


Fig. 5. Subacute demyelinating encephalomyelitis.
5a. Well circumscribed advanced demyelinating lesion found in the left lateral column. Early spongy state found in other white matter. KB x 60.
5b. Advanced demyelinating area near gray matter. Many gitter cells found in the lesions. Note small round cell infiltrations in the gray matter. HE x 300.
5c. Early spongy area in the white matter. Note lipid-loaded macrophage near the arachnoidea. PPD x 600.
5d. Immunofluorescent staining of the spinal white matter, only oligodendroglial cells contain JHM antigen. x 900.

negative. In the spinal cord, viral antigen was only found in the early spongy areas and not in or around advanced demyelinating lesions.

By electron microscopy, the virus particles were detected only in the hypertrophically degenerating cells in the early spongy state of spinal white matter, which seemed to correspond to the vacuoles containing amorphous material in the histological preparations. The cells were highly degenerated but particles were still found within the vacuoles (Fig. 6a). Around the vacuoles there was a large amount of microtubules, characteristic for oligodendroglia (Fig. 6b).

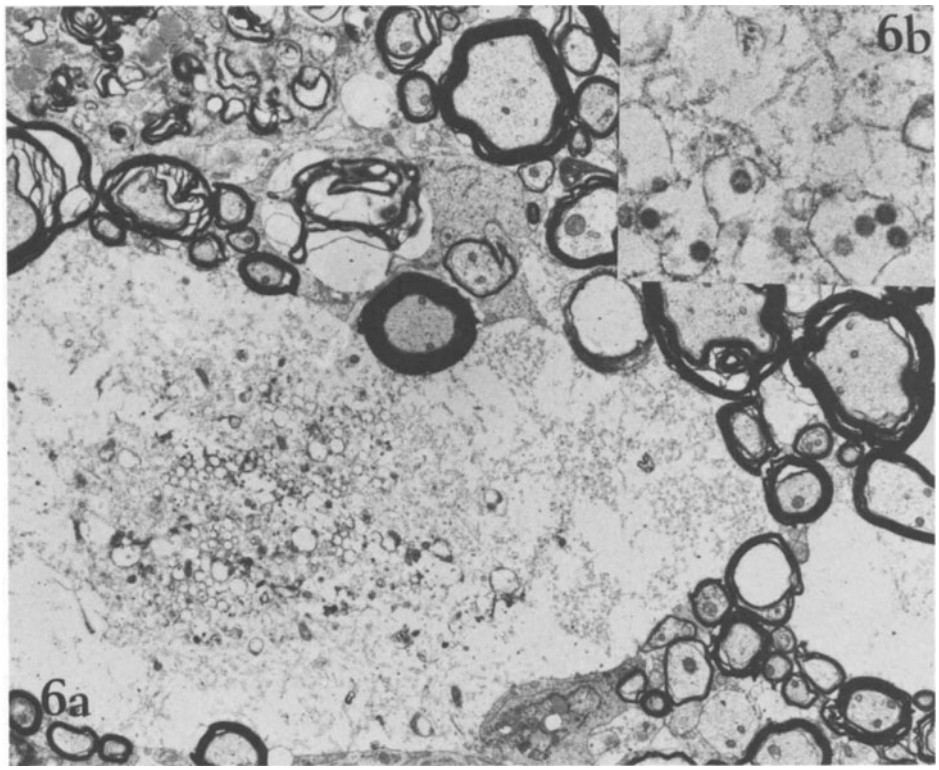


Fig. 6. Hyperthropically degenerating cell in the spinal white matter, corresponding to "marked" cell in Fig. 5c.
6a. Vacuoles containing virus particles. Abundant microtubules are seen. Myelin sheaths are degenerating but axons are still preserved. Macrophage containing myelin debris found on the left upper corner. x 5,000.
6b. Higher magnification of Fig. 6a. Virus particles and microtubules. x 44,000.

Table 3. Chronic progressive paralysis in weanling rats. - The incubation period ranged from 6 to 8 months. No infectious virus or viral antigen was detected in brain tissue.

No. of animals infected (I.C.)	No. of diseased animals
17	4
24	6

Chronic progressive paralysis. Weanling rats, which did not exhibit subacute demyelinating encephalitis, developed 6 to 8 months later a slowly progressive paralysis of the legs (Table 3). Four rats were neuropathologically examined. Hydrocephalus was found in all rats and in three of them also myelomalacia. Hydrocephalus consisted of an enlargement of the lateral ventricles as well as of the fourth aqueduct (Fig. 7). The aqueductus Sylvii was not closed. The leptomeninges showed diffuse fibrous thickening. Fibrosis was most prominent around the cerebrospinal junction. Cerebral cortices became thin and the white matter was partly degenerated with gliosis along the ventricles. No inflammatory changes were found throughout the central nervous system. In the cerebro-cerebellar cortices and spinal cord scattered calcospherite deposition was found. Myelomalacia consisted of the rarefactive degeneration partly involving

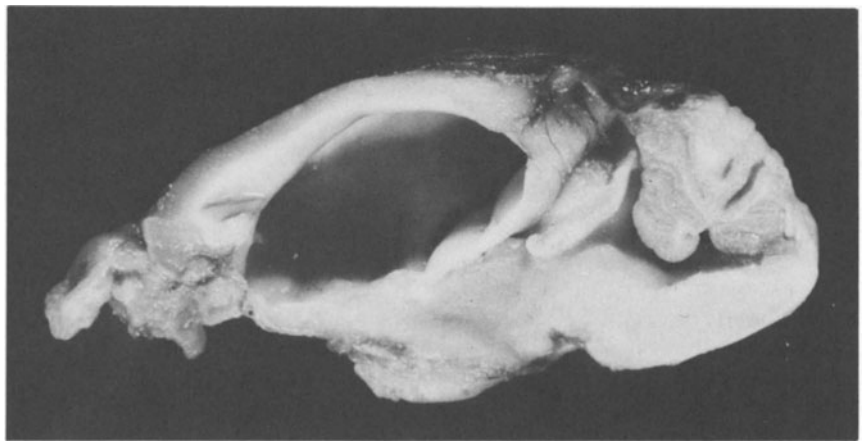


Fig. 7. Sagittal section of the brain from an animal with chronic progressive paralysis. Lateral ventricle as well as fourth ventricle are dilated. Note the thickened meninges. x 5.

the gray matter (Fig. 8a). It was most prominent in the upper cervical cord and gradually less marked down to the thoracic cord. The central canal was neither obstructed nor dilated. In less affected areas, gray matter was in a spongy state (Fig. 8c). In these areas neurons remained relatively unaltered. No ballooning nerve cells were found. The wall of the anterior spinal artery was thickened but the lumen was not closed (Fig. 8b). Severe arachnoid fibrosis with partial calcification was observed (Fig. 8d). The liver of two rats revealed few nodules of inflammatory cells.

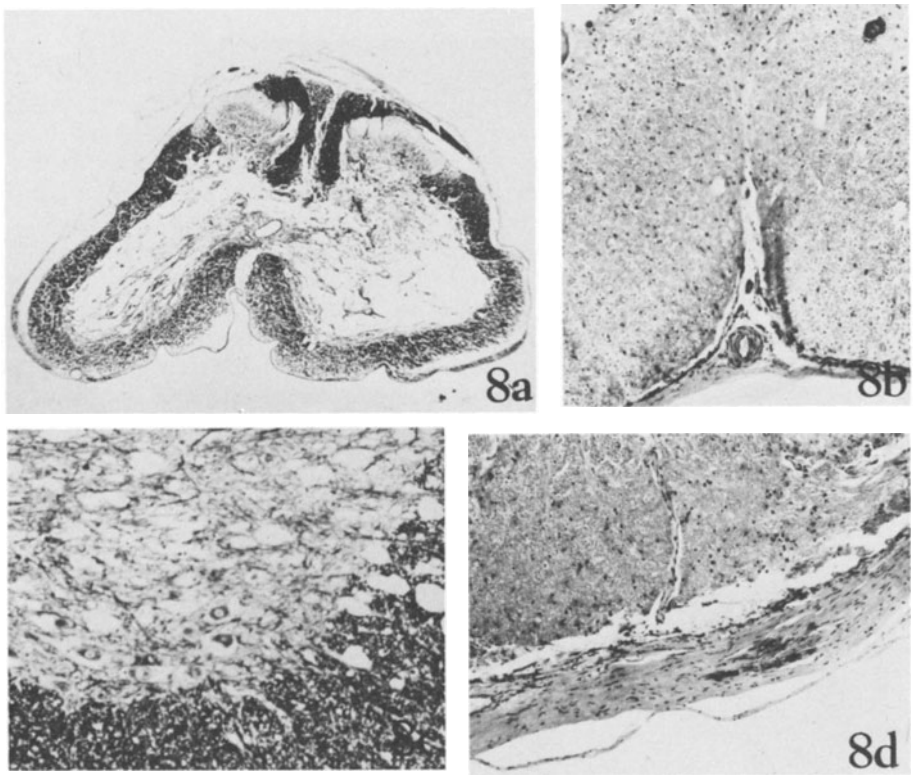


Fig. 8. Spinal cord of a case of chronic progressive paralysis.
8a. Gray matter of cervical cord showing advanced rarefactive degeneration. KB x 60.
8b. Thickened wall of the anterior spinal artery. Calcospherite deposition found on the right upper corner. HE x 150.
8c. Early spongy state of the gray matter, partly extending to the white matter. Note the well preserved anterior horn cells. KB x 150.
8d. Fibrously thickened arachnoid of the spinal cord. Note the calcification (the cleft between the cord and arachnoid is an artifact). HE x 150.

Attempts to isolate JHM virus were unsuccessful despite the fact that methods of isolation were applied which yielded infectious virus in subacute sclerosing panencephalitis. Moreover, JHM virus antigen could not be demonstrated in brain or spinal cord material by conventional immunofluorescent techniques.

DISCUSSION

In our experiments with JHM infections in rats three types of diseases were observed: acute panencephalitis, subacute demyelinating encephalitis and chronic progressive paralysis. The lesions of acute panencephalitis produced in newborn rats are comparable to those in mice. Studies carried out in mice have shown that JHM virus, independent of the age of the inoculated animals, affects the gray and white matter (2, 9, 16, 17). The neuropathology consists of an acute encephalomyelitis with patchy demyelinating lesions in the brain stems and spinal cord. Like in newborn rats, neurons and oligodendroglial cells are infected by JHM virus as it has been demonstrated by immunofluorescent techniques (17). The demyelination occurring in mice is interpreted as a result of oligodendroglial cell destruction by JHM virus infection (9, 17). Similar virus-host relationships probably account for the neuropathological changes in JHM infected newborn rats.

In contrast to the findings reported in mice, inoculated weanling rats revealed a different neuropathological picture. Subacute encephalomyelitis was observed, which showed the most striking changes in the white matter. Demyelination was very marked without destruction of axons. Electron microscopic examination of this subacute form showed that virus particles were found only in the hypertrophically degenerated cells in the white matter.

The co-presence of microtubules in these cells may support the contention that the cells are oligodendroglia by origin. These degenerated oligodendroglial cells were only found in the slightly spongy areas of the white matter, where the myelin sheaths and axons were spared and only few macrophages had infiltrated. The demyelination may be caused by the death of oligodendroglia in this subacute form, a mechanism that was suggested from experiments with mice (9).

It is noteworthy that the CNS diseases in rats by JHM virus are apparently influenced by host factors. In mice, regardless of the age of the animals, JHM virus always causes an acute encephalomyelitis. In weanling rats, however, a subacute encephalomyelitis or a chronic paralysis is observed, suggesting that the host's defense mechanisms play a role in the development of these diseases.

Chronic progressive paralysis in JHM virus infection has so far not been reported. Herndon et al. (6) examined mice without any clinical signs at 16 months after inoculation and reported that small foci of active myelin degeneration were found by electron

microscopy. In our experiments in rats, hydrocephalus and myelomalacia were found with corresponding clinical symptoms. No inflammatory signs were noticed. The most frequent cause of virus-induced hydrocephalus is acute aqueductal stenosis caused by viral ependymitis (5, 7, 14). In our cases no aqueductal stenosis was found. Instead, marked meningeal fibrosis was observed, resembling the picture of hydrocephalus caused by polyoma virus inoculation (10).

Since the meningeal or arachnoid fibrosis is the common cause of hydrocephalus of human infants (4) we suggest that in our cases the thickened meninges gradually obstructed the lateral apertulas of medulla oblongata, resulting in chronic hydrocephalus. Prior to meningeal fibrosis, diffuse meningitis might have been present, which was caused by JHM virus inoculation. Myelomalacia found in our rats represented not necrotic but spongy degenerations, almost confined to gray matter. Marked arachnoid fibrosis was observed. The histology of this myelomalacia, however, is quite different from those found in spinal adhesive arachnoiditis (3, 11, 15), because neither cavitations and slit-like defects nor marginal white matter involvement were observed in our cases. Although the walls of anterior spinal arteries were thickened, the pathology of insufficient arterial blood supply for spinal cord is dissimilar to this myelomalacia.

It must be taken into consideration that cultured L cells used for JHM virus propagation contain endogenous C type virus (13). Andrews and Gardner (1) reported a lower motor neuron degeneration associated with oncorna virus infection in mice. The spongy degeneration of gray matter is similar to our myelomalacia but the predilection for the spinal segment is different; C type virus affects the lumbosacral cord, while myelomalacia was found in the upper cervical cord. Moreover, the cytoplasmic vacuolization in neurons was not observed in the remaining neurons in myelomalacia. The actual cause of myelomalacia in this chronic progressive paralysis of rats remains to be discovered.

The neuropathological description of the three diseases associated with JHM virus in the rat suggests the importance of host factors in the development of CNS changes. It is evident that the mechanisms responsible for the three diseases differ from each other. Detailed virological and immunological studies are necessary to unravel the pathogenicity of these diseases. Hopefully, the subacute encephalomyelitis and the chronic paralysis will provide an animal model to understand CNS changes induced by persistent viral infections.

REFERENCES

1. Andrews, J.M. and Gardner, M.B., Lower motor neuron degeneration associated with type C, RNA virus infection in mice: neuropathological features, J. Neuropath. Exp. Neurol. 33 (1974) 285-307.

2. Bailey, O.T., Pappenheimer, A.M., Cheever, F.S. and Daniels, J.B., A murine virus (JHM) causing disseminated encephalomyelitis with extensive destruction of myelin. II. Pathology, J. Exp. Med. 90 (1949) 195-212.
3. Dohrmann, G.J., Cervical spinal cord in experimental hydrocephalus, J. Neurosurg. 37 (1972) 538-542.
4. Fried, R.L., Hydrocephalus - Special Pathology. Developmental Neuropathology, Springer, Wien (1975) pp. 214-229.
5. Friedman, H.M., Gilden, D.H., Lief, F.S., Rorke, L.B., Santoli, D. and Koprowski, H., Hydrocephalus produced by the 6/94 virus: A parainfluenza type I isolated from multiple sclerosis brain tissue, Arch. Neurol. 32 (1975) 408-413.
6. Herndon, R.M., Griffin, D.E., McCormick, U. and Weiner, L.P., Mouse hepatitis virus-induced recurrent demyelination. A preliminary report, Arch. Neurol. 32 (1975) 32-35.
7. Johnson, R.T., Johnson, K.P. and Edmonds, J.C., Virus induced hydrocephalus: Development of aqueductal stenosis in hamsters after mumps infection, Science 157 (1967) 1066-1067.
8. Kersting, G. and Pette, E., Zur Pathohistologie und Pathogenese der experimentellen JHM-Virus Encephalomyelitis des Affen, Dtsch. Z. Nervenheilk. 174 (1956) 238-304.
9. Lampert, P.W., Sims, J.K. and Kinazeff, A.J., Mechanism of demyelination in JHM virus encephalomyelitis. Electron microscopic studies, Acta neuropath. (Berl.) 24 (1973) 76-85.
10. Li, C.P. and Jahnes, W.G., Hydrocephalus in suckling mice inoculated with SE polyoma virus, Virology 9 (1959) 489-492.
11. McLaurin, R.L., Bailey, O.T., Schurr, P.H. and Ingraham, F.D., Myelomalacia and multiple cavitations of spinal cord secondary to adhesive arachnoiditis. An experimental study, Arch. Pathol. 57 (1954) 138-146.
12. Nagashima, K., Wege, H. and ter Meulen, V., Corona virus induced subacute demyelinating encephalomyelitis in rats, in preparation.
13. Nagashima, K., Wege, H. and ter Meulen, V., Corona virus in cultured cell: ultrastructural and immunofluorescent studies on L-cell infected with JHM strain, in preparation.
14. Nielsen, S.L. and Baringer, J.R., Reovirus induced aqueductal stenosis in hamsters. Phase contrast and electron microscopic studies, Lab. Invest. 27 (1972) 531-537.
15. Solov'ev, V.N., Changes in the spinal cord in so-called spinal ossifying arachnoiditis, Arkh. Pathol. 35 (1973) 48-54.
16. Waksman, B.H. and Adams, R.D., Infectious leukoencephalitis. A critical comparison of certain experimental and naturally occurring viral leukoencephalitides with experimental allergic encephalomyelitis, J. Neuropath. Exp. Neurol. 21 (1962) 491-518.
17. Weiner, L.P., Pathogenesis of demyelination induced by a mouse hepatitis virus (JHM virus), Arch. Neurol. 28 (1973) 298-303.