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CORONAVIRUSES

Ehud Lavi and Susan R. Weiss

Department of Microbiology
University of Pennsylvania
Philadelphia, PA 19104-6076

A. GENERAL INTRODUCTION

Coronaviruses form a group of pathogenic, enveloped positive stranded RNA viruses that infect many species of animals including man, mouse, chicken, turkey, cat, dog, pig and cow (1,2). The target organs and outcome of infection are dependent on the strain of virus, age and genetic composition of the animals as well as the dose and route of inoculation (1). Table 1 shows a list of coronaviruses and the diseases they cause.

Much of coronavirus pathogenesis research has centered on the use of murine coronaviruses as models for human chronic neurological diseases,including demyelination. Most of this review will concern mouse hepatitis virus (MHV) as a model for human virus-induced chronic neurological disease. Although some strains of MHV are primarily hepatotropic (and hence the name), several MHV strains can cause chronic neurological disease in

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rodents, either with or without concommitant hepatitis. These various MHV strains are highly related immunogically and in nucleotide sequences; however they have very different tropisms in vivo and differential affinities for central nervous system (CNS) cells in vitro. These strain differences may be exploited to study viral tropism as discussed below.

Human coronaviruses are primarily respiratory (1) and sometimes enteric viruses (4). These viruses are common in the human population and are estimated to be responsible for 15% of common colds (1). They have been divided into two antigenic groups (5). Viruses in one group, exemplified by OC43, are antigenically related to MHV and may be adapted to grow in suckling mouse brains. Viruses in the other group,exemplified

Table 1. MEMBERS OF THE CORONAVIRUS GENUS[1]

Common Name of Virus	Natural Host	Diseases
Avian infectious bronchitis virus (IBV)	Chicken	Tracheobronchitis, nephritis, oviduct hypoplasia
Bluecomb disease virus (TCV)	Turkey	Enteritis
Rat coronavirus (RCV)	Rat	Pneumonia, rhinotracheitis
Sialodacryoadentis (SDAV)	Rat	Sialodacryoadenitis, keratoconjunctivitis, rhinotracheitis
Transmissible gastroenteritis virus (TGEV)	Pig	Gastroenteritis

Hemagglutinating encephalomyelitis virus (HEV)	Pig	Encephalomyelitis, gastroenteritis ("vomiting and wasting disease")
Porcine virus CV777 (PCV)	Pig	Enteritis
Neonatal calf diarrhea coronavirus (BCV)	Bovine	Gastroenteritis
Human coronavirus (HCV)	Human	Common cold
Human enteric coronavirus (HECV)	Human	Gastroenteritis
Canine coronavirus (CCV)	Dog	Gastroenteritis
Feline infectious peritonitis virus (FIPV)	Cat	Meningoencephalitis, Peritonitis, pleuritis, pneumonia, wasting disease, vasculitis (immune complex disease), disseminated granulomatous disease
Feline enteric coronavirus (FEC)	Cat	Enteritis
Pleural effusion disease virus (RbCV)	Rabbit	Pleuritis, myocarditis, uveitis
Rabbit enteric coronavirus (RbECV)	Rabbit	Enteritis

[1] Adapted from Sturman and Holmes (3).

by 229E, are unrelated to MHV. It has been suggested that human coronaviruses may be involved in human demyelinating disease for the following reasons. 1) Murine coronaviruses are associated with demyelinating disease in rodents. 2) Human coronaviruses are common in the human population with most people having antibodies against these viruses (1). 3) Particles with coronavirus-like morphology have been seen in autopsy brain material from a MS patient (6). 4) Burks et al., (7) have isolated two coronaviruses, SD and SK by inoculation of CNS autopsy material from MS patients into the brains of weanling mice or into 17Cl-1 mouse fibroblasts. Subsequent experiments, however, have demonstrated that SD and SK are more closely related to murine coronaviruses than to human coronaviruses (8,9, J. Fleming, personal communication). Thus the question of a possible association of coronaviurses with demyelinating disease in humans is still an open one.

Coronaviruses were originally thought to be members of the myxovirus group because of their similar morphology. However, on more detailed examination of negative stained particles, coronaviruses were classified as a separate group (1). Coronavirus particles are enveloped, medium sized (80-160 nm), round and moderately pleiomorphic. They contain characteristic widely spaced club-shaped surface projections protruding from the membrane (20 nm long by 7 nm wide at the tip). It is these surface projections that give the virion a crown like image in the electron microscope; this morphology suggested the name coronaviurses. More recent biochemical and molecular biological

experiments have shown that coronaviruses have a unique strategy for replication. This will be discussed in detail below.

B. MOLECULAR BIOLOGY OF CORONAVIRUSES

1. Structure of the virion.

The coronavirus particle contains three structural proteins and one piece of positive stranded RNA with a length estimated to be 18-27 kilobases (kb) (10,11, see Figure 1.) The information in this section concerns MHV. Similar proteins have been described for other coronaviruses. The major structural protein is the nucleocapsid protein, a 50-60 K (K=1000 daltons) basic phosphoprotein that is complexed with the virion RNA to form a helical nucleocapsid structure. The other two structural proteins are glycoproteins. The E1 protein, a 23 K protein, is located primarily in the viral membrane with only a small portion accessible to proteases (12). The E1 protein is unusual for a viral glycoprotein in that it is glycosylated post translationally and the oligosaccharides are O-linked to serine and threonine residues. The other is the E2 protein that composes the coronavirus spikes and is responsible for cell

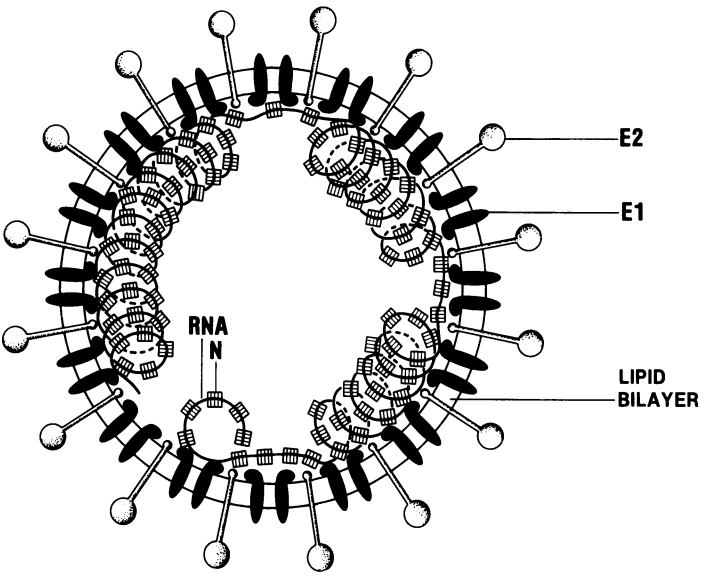

Figure 1. Schematic diagram of the mouse hepatitis virus particle. The three structural proteins are nucleocapsid (N), E1 (trans-membrane glycoprotein and E2 (peplomer glycoprotein). The N protein is complexed with genome RNA to form the helical nucleocapsid. Figure obtained from Dr. K. V. Holmes.

fusion and viral attachment. This protein is synthesized as a 120 K protein, and like most other viral glycoproteins, is glycosylated co-translationally with oligosaccharides N-linked to asparagine residues. This 180 K glycosylated precursor is cleaved by a cellular protease into two non-identical 90 K polypeptides at a late stage in maturation of the virus;

cleavage is necessary for cell-fusing activity (12). The E2 protein is thought to be important in determining viral tropism and disease; this will be discussed in detail below.

2. Genome and mRNA structure.

The coronavirus genome is a single piece of RNA of positive polarity, that is of the same polarity as messenger RNA. In analogy with the positive stranded RNA alpha viruses, the coronavirus genome RNA is likely to serve as mRNA for the viral polymerase; synthesis of this viral enzyme followed by transcription of viral mRNA are likely to be the first steps in viral replication. This is substantiated by the observation that naked coronavirus virion RNA is infectious (13). The coronavirus genome is the largest known RNA virus genome. Recent sequencing studies have shown that the genome of the avian coronavirus infectious bronchitis virus (IBV) is 27 kb (10). Current estimates are that the genome of MHV, as well, is much larger than the original 18 kb estimates; the polymerase gene alone is in excess of 16 kb (C. Pachuk et al., unpublished). During infection, MHV generates six subgenomic positive stranded putative mRNAs as well as genome sized RNA. These RNAs all overlap in sequence; they all contain the same 3' end sequences and extend various distances toward the 5' end (14,15,16). (See Figure 2). The genome RNA, as well as the six subgenomic RNA, resemble typical eukaryotic mRNAs in that they are polyadenylated at the 3' end and capped at the 5' end (14,15,16) The immediate 5' ends of each of the RNAs as well as the genome RNA contain an approximately 80 nucleotide leader

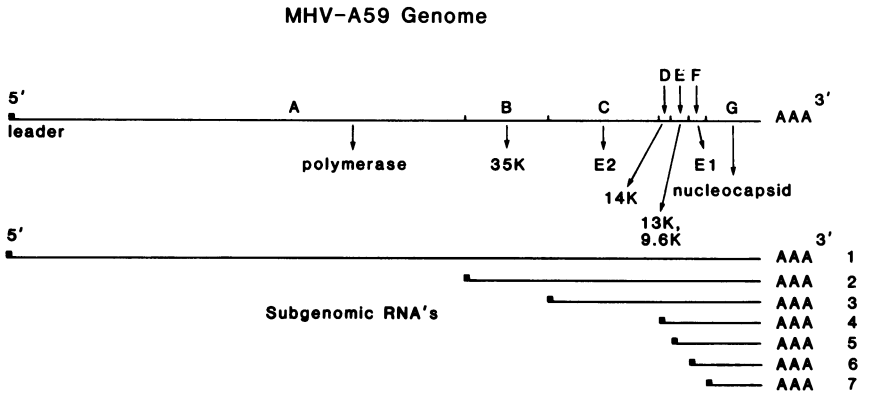

Figure 2. MHV A59 genome RNA and subgenomic intracellular mRNAs. The gene order was deduced from cell-free translation experiments, assuming that each RNA is translated from the 5' unique sequences, and from DNA sequencing data. The structural proteins are the nucleocapsid, E1 (small glycoprotein), and E2 (large peplomer glycoprotein). The nonstructural proteins include the 35K, 14K, 13K, 9.6K and polymermase proteins. The location of the polymerase gene is deduced from the facts that genome RNA is infectious and must therefore encode polymerase(s) and that eukaryotic translation usually proceeds from the 5' end of RNA. The leader sequence is shown by the bar at the 5' end of each mRNA.

sequence that may be involved in genesis of the mRNAs (16,17,18,19,20).

The genes of the MHV have been defined by the unique regions of each of the mRNAs. Each gene is defined as the region of the

mRNA (and corresponding genome portion) not overlapping with the next smaller mRNA. The structural genes have been mapped to mRNAs and corresponding genome regions by in vitro translation experiments. mRNAs 3, 6 and 7 encode E2 glycoprotein, E1 membrane glycoprotein and nucleocapsid proteins respectively (see Figure 2). These genome positions have been substantiated by sequencing of complementary DNA clones representing these mRNAs (19,20,21,22,23,23a).

Mapping of the MHV non-structural genes has been more difficult. Partial mapping has been accomplished by cell-free translation studies and some DNA sequence analysis. Sequence analysis has shown that the unique region of mRNA 5 contains two open reading frames (ORFs); the upstream ORF potentially encodes a basic 13K protein that may interact with RNA and the downstream one potentially encodes a 9.6K protein with an extremely long hydrophobic amino terminus suggestng it may interact with membranes (24,25). A protein of the size predicted from the downstream ORF may be detected specifically in infected but not uninfected cells while it has not yet been possible to detect the putative product of the upstream ORF in infected cells (24). Cell free translation of a synthetic RNA containing both ORFs suggests that the downstream protein may be preferentially translated (25); this protein has a more favorable context for initiation of protein synthesis as judged by the sequences surrounding the AUG initiation codon (26). Thus, we speculate that mRNA 5 may be bicistronic and be preferentially translated from the downstream ORF. Bicistronic mRNAs in which the downstream ORF is preferentially translated

have been described now in several animal virus systems (27, 28). Sequencing of mRNA 4 has demonstrated an ORF potentially encoding a protein of 15K with a hydrophobic amino terminus and basic carboxy terminal tail; a protein of this size has been detected in the infected cell (21,22,29). mRNA 2 directs the cell-free synthesis of a 35 K protein; a virus-specific polypeptide of similar size has been observed in infected cells as well and in the products of cell free translation of a pool of mRNAs 4 and 5 (21,22). Gene B, corresponding to mRNA 2, of MHV has not yet been sequenced and may encode other protein(s) as well as the 35K protein as the size of the gene is 3-4 Kb (C.J. Budzilowicz et al., unpublished). Gene A is presumed to encode the viral polymerase. This is based on the assumption that coronaviruses use a similar strategy of replication to the alphaviruses. In vitro translation of genome directs the synthesis of a 250K polypeptide that is processed in vitro into 229K and 28K polypeptides (21,30). These polypeptides have not yet been identified as part of the viral polymerase. Thus, a direct identification of the viral polymerase polypeptides is still lacking. Clearly, the raising of antisera against the non-structural coronavirus proteins will be a major step in their charaterization.

3. Mechanism of Transcription. The synthesis of coronavirus mRNAs has some features in common with synthesis of alphavirus RNA in that infected cells contain a full genome sized negative stranded RNA that presumably serves as a template for the synthesis of progeny genome RNA as well as subgenomic mRNAs (31). However, the mechanism of coronavirus RNA synthesis is

unique among RNA virus groups. The facts that the RNAs form a nested set, all overlapping with the 3' end of genome RNA (14.15.17) and contain a common 5' leader sequence (16,17,18) suggest that they are synthesized by a mechanism involving the joining of non-contiguous regions of the genome. Ultraviolet irradiation inactivation studies ruled out the possiblity that subgenomic RNAs are processed from a full genome length precursor RNA (32). This along with the fact that the coronavirus replication occurs completely in the cytoplasm of cells (33) demonstrates that these mRNA are not synthesized by the nuclear splicing techniques used in the genesis of eukaryotic mRNAs.

It is currently thought that leader RNA is transcribed from the negative stranded RNA and subsequently serves as a primer for the synthesis of virus specific RNA (17). There is a small region of homology (10-12 base pairs) between viral intergenic regions and the 3' end of the leader RNA (18,24,34). It is possible that the amount of homology between the leader and each intergenic region may be important in determining the frequency of transcription of each gene. Two models for priming are: 1) that leader is released from its template and then attaches to, perhaps in combination with the viral polymerase, to intergenic regions or; 2) that after transcription leader remains attached to the 3' end of the negative strand template while the RNA loops out to bring more downstream regions closer to the priming site. The former mechanism is favored because only one size replicative intermediate has been detected in the infected cells (35) and because free small leader related RNAs have been found

in the infected cell (36). Furthermore, recombination between two virus strains occurs at high frequency in vitro suggesting that the MHV transcription machinery can change templates (37).

4. Maturation During infection, nucleocapsid protein complexes with progency virion RNA to form a helical nucleocapsid. E1 protein is observed mostly in the perinculear area of infected cells and accumulates in the Golgi apparatus (12). E2 is dispersed throughout the cells from early times after infection and appears on the plasma membrane (12). The amount of 90K as opposed to 180K form of E1 protein present on the membrane correlates with the amount of fusion observed (38,39). It is thought that viral nucleocapsids bud into the endoplasmic reticulum at sites that have been modified by E1 glycoprotein. Virus is then transported to the plasma membrane through vessicles and extruded by the cells secretory mechanisms. The outcome of tissue culture infections usually results in syncytium formation followed by cell death, although persistent infections may also be readily established (see below).

C) Murine Coronavirus Pathogenesis

1) Neuropathology of MHV Infection in vivo.

Some strains of MHV such as MHV-2 are purely hepatotropic (40); JHM is mainly neurotropic (41); and A59 (42,42a), MHV-S (43) and MHV-3 (44) are both hepatotropic and neurotropic. Other organs, such as lung and intestines, may also be involved when infection occurs in suckling mice (45). The respiratory and gastrointestinal systems are considered to be the routes of entry of MHV into the animal (45,46). Several animal model

systems have been established for the study of MHV neuropathogenesis. These include JHM infection of weanling mice (41,47,48,49,50), JHM infections of suckling rats (51) and weanling rats (52), A59 infection of weanling mice (42,53,54) and suckling rats (55), and MHV-3 infection of weanling mice (44). With some differences in severity and viral spread, all the experimental models of MHV infection exhibit bi-phasic disease. Acute meningoencephalitis (with or without hepatitis) is the major pathological process in the first two weeks following incoculation with MHV. Subsequently, subacute and chronic diseases develop, which can be divided into two major categories: demyelination or vasculitis. Primary demyelination is found during the subacute and chronic stages of JHM and A59 chronic infections, whereas vasculitis is present in MHV-3 chronic infection.

Clinical manifestations of the acute phase in mice or rats may range from asymptomatic disease (46,51,52) to a severe illness characterized by ruffled fur, hunched position, loss of appetite, wasting, lethargy and finally death (41,42). Animals that survive the acute phase of the disease may progress into a chronic phase, chacterized by a paralytic disease affecting the hind limbs or all four limbs. The course of the chronic disease may be progressive leading to death, mostly associated with vasculitis (44,56) or monophasic with partial or complete recovery, and remitting-relapsing, mostly associated with demyelination (52). The severity of the disease is dependent on the strain, the dose and the route of inoculation of the virus as well as the species, the strain and the age of the infected

animal (46,57). Pathological findings can be detected even in the absence of clinical signs; however, in that case a more detailed examination of motor function may reveal subtle deficits (53).

a. Acute encephalitis.

The acute phase of disease is different among infections with the various strains of MHV. This provides an opportunity to study the interaction of virus with specific populations of cells of the CNS. Molecular biological analysis of the different strains of MHV and the use of recombinant, mutant, and antigenic variant viruses will make it possible to determine which genes and gene products are responsible for biological properties of the viruses including neurotropism.

The neurotropic JHM strain is highly virulent. It kills mice more rapidly and with lower doses of virus than does any other MHV strain. When injected intracerebrally or intranasally into weanling mice, JHM causes a panencephalitis which involves the telencephalon, diencephalon, brain stem, cerebellum and spinal cord (41,50,58,59). The high mortality rate following JHM infection in mice is attributed to the CNS disease. Hepatic involvement in JHM infection is minimal and usually constitutes only a few foci of inflammatory cells. In suckling rats, JHM replication is found mainly in the cerebellar and hippocampal neurons (60,61). In weanling rats intracerebral inoculation of JHM produces only demyelinating disease without neuronal involvement in only 30% of the animals (52).

In contrast to the pantropic property of JHM, MHV-A59 in weanling mice produces a non lethal CNS disease involving only a

selected population of neurons during the acute phase. The surrounding glial cells and meninges are involved in these areas as well. When a high dose of the virus is used, hepatitis occurs in addition to mild encephalitis and mortality is then directly related to the severity of the hepatitis (46). Following intracerebral inoculation with A59, the basal ganglia region of the brain shows the most intense staining for viral antigens (62). When mice are inoculated intranasally, virus is propagated through the olfactory pathways and subsequently reaches and remains restricted to the limbic system (62a). The kinetics of appearance of viral antigen in the brain mimics the propagation of a physiological signal in the limbic system. This suggests an interneuronal transport of the virus. Interneuronal transport has also been implicated in the spread of other viruses such as rabies, herpes simplex and poliovirus. These findings suggest that the receptor for the virus may be a molecule shared by neuronal cells of the limbic system, possibly a neurotransmitter receptor. A precedent for such an association has been shown for reovirus with the β-adrenergic receptor and rabies virus with the acetylcholine receptor (63,64).

b. Demyelinating disease

Demyelination is one of the most important hallmarks of MHV infection. Therefore, MHV infection has been used as model system for human demyelinating diseases such as multiple sclerosis (MS) and acute disseminated encephalemylitis (ADEM). Demyelination has been described in mice infected with either JHM (41,47) or A59 (42) (see Figures 3 and 4) and in rats

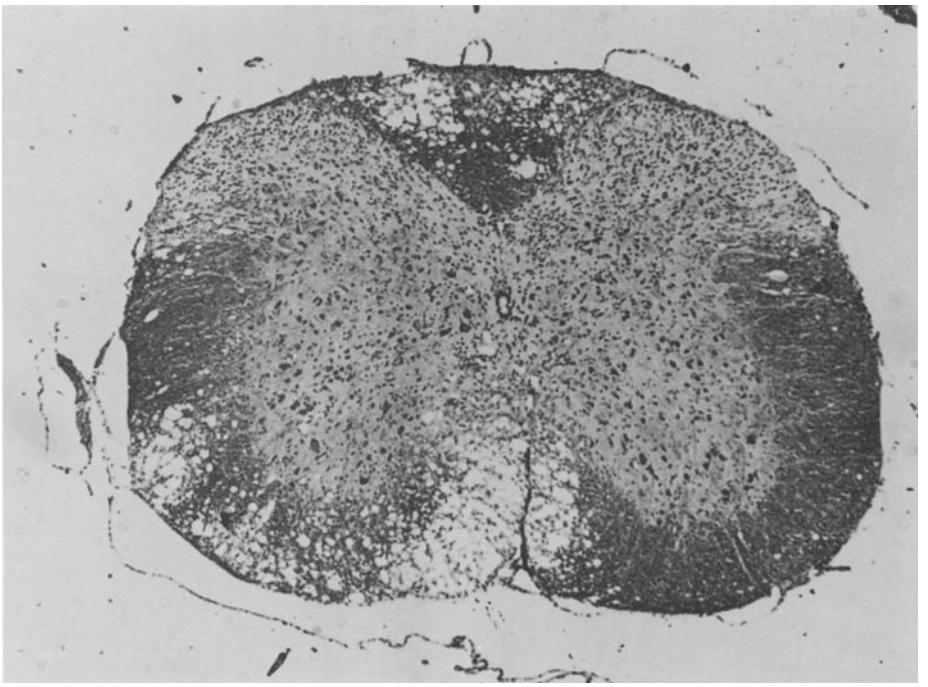

Figure 3. Transverse section of mouse spinal cord 60 days after intracerebral inoculation with 1 LD_{50} of MHV-A59, showing extensive white matter lesions. Hematoxylin-eosin x 80.

infected with JHM (51,52). An ultrastructural study was performed on intraperitoneally inoculated weanling mice with JHM virus (47). The pathology consisted of acute encephalomyelitis with patchy demyelinative lesions in the brain stem and spinal cord. Coronavirus particles were found in cells that were identified as oligodendrocytes by the connections of their plasma membranes with myelin lamelae. Following the degeneration of oligodendrocytes the myelin sheaths disintegrated or

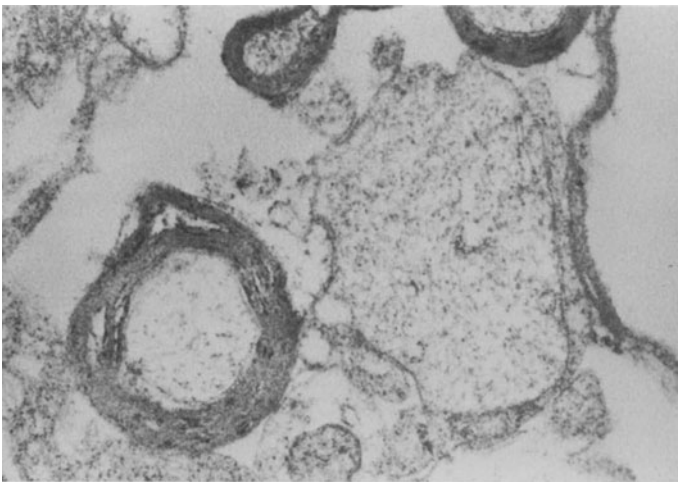

Figure 4. Electronmicrograph of spinal cord white matter from a mouse 60 days after inoculation with 1 LD_{50} of MHV-A59. A transverse section of demyelinated axon is seen next to a normal myelinated axon. x7000.

were stripped of intact axons by cytoplasmic "tongues" of polymorphonuclear leukocytes that intruded between myelin lamelae. This study was the first to demonstrate an affinity of JHM virus for oligodendrocytes and that acute demyelination occurs subsequently to the degeneration of the infected oligodendrocytes (47). Another ultrastructural study (48) showed that in regions of demyelination, oligodendrocytes exhibited a propensity to proliferate abberant membrane. Myelin degeneration was accompanied by membrane vesiculation and by the stripping action of macrophages.

During the acute phase of the MHV disease, demyelination is part of a more extensive encephalitic process, whether focal as

in A59 infection or widespread as in JHM infection. The chronic demyelinating disease consists of primary demyelination without neuronal damage. It is not clear whether demyelination during the chronic stage is also due to a cytolytic effect on oligodendrocytes, or whether immune mediated pathology also contributes to the process of demyelination.

Following MHV-induced demyelination, electron-microscope (EM) studies (42,65,66) showed that remyelination occurs. The new myelin sheath is characterized by reduction in thickness and decreased internodal distance. The ferric ion- ferrocyanide staining appears to be normal in the nodes of remyelinated axons implying that at least some aspects of the remyelinated axons are normally functioning (67). Remyelinated axons occasionally undergo a second process of demyelination (65).

A recent study revealed that in spinal cords of MHV-A59 infected mice with active demyelination and inflammation, an increase in myelin basic protein (MBP) specific mRNA occured at 2-3 weeks after virus inoculation. This was before remyelination could be detected morphologically. The increase in MBP specific mRNA was found at the edge of the demyelinating area and extended into the surrounding normally appearing white matter (54). These results suggest that glial cells in mice react to demyelination with widespread synthesis of MBP mRNA which may be triggered by a diffusable factor released in the demyelinated area.

The process of remyelination could result either from surviving oligodendrocytes increasing their rate of myelin metabolism, and thus extending their territory, or from the

generation of new oligodendrocytes. EM autoradiographic studies with ^{3}H-labeled thymidine demonstrated that the cells associated with remyelination after JHM infection are newly generated oligodendroglia (68).

c. Vasculitis

A different CNS tropism was found following infection with MHV3. Although MHV-3 exhibits 95% nucleotide sequence homology with the demyelinating MHV strain, A59, MHV-3 disease has a different pathogenesis. It produces fatal hepatitis in susceptible mice (C57BL/6), and CNS disease in semi-susceptible mice (C3H and A2G). Following intraperitoneal inoculation of MHV-3, mice develop a chronic persistent paralytic disease which begins 2-12 weeks afer inoculation and progresses for 2-12 months until death. Infectious virus can be recovered from infected brains at any time after infection (44). The CNS involvement is restricted to the CNS blood vessels, meninges, choroid plexus and ependymal cells. CNS lesions consist of vasculitis and meningoependymitis. There is no neuronal damage or primary demyelination. Vasculitis is characterized by perivascular infiltrations of polymorphonuclear and mononuclear cells, and fibrinoid necrosis of blood vessle walls. Viral antigens and bound immunoglobulins are found by immuno-fluorescene in the blood vessel walls, suggesting the presence of immune complexes. Systemic vasculitis is suggested by the findings of amyloid deposits in the spleens and livers of infected mice and viral antigens and immunoglobulin deposits in blood vessels in spleens, livers, and kidneys (44).

2. Virus-cell interaction of MHVs

To understand the pathogenesis of CNS diseases produced by MHVs, two major issues, involving virus-cell interaction, must be considered. 1) Differential cell tropism of MHV strains results in producing different pathological damage. 2) The virus has the ability to persist in CNS cells.

a. Neural cell tropism of MHV

In vitro studies with mouse cultures showed that JHM can cause a cytolytic infection in neurons, astrocytes and oligodendrocytes. This is folowed by a persistent infection in glial cells (69,70,71). EM studies demonstrated the JHM can be found in the mouse CNS in vivo in ependymal cells, astrocytes, oligodendrocytes, neurons, endothelial cells and cells of hematogenous origin (48). In vitro studies with JHM in rat glial cells gave contradicting results. In a study of JHM infection of glial cells derived from Wistar rats, oligodendrocytes but not astrocytes were infected (72). In another study, using glial cultures derived from Lewis rats, astrocytes were more susceptible to JHM infection than oligodendrocytes (73).

MHV-A59 in mouse glial cells cultures is capable of infecting both astrocytes and oligodendrocytes in vitro (74,75). In vivo infection of A59 in weanling mice includes glial cells infection as well as selected neuronal infection (62,62a,76).

MHV-3 grows in vitro in rat astrocytes but not in rat oligodendrocytes (72). MHV-3 in mice grows in both astrocytes and oligodendrocytes (71). Another study concluded that MHV-3

in mouse cultures grows in neurons and ependymal cells but not in glial cells,neither astrocytes nor oligondrocytes (77). The same study showed that some oligodendrocytes were infected.

It is difficult to make a statement about the relevance of in vitro studies to the understanding of MHV pathogenesis because the in vitro studies are not always consistent with the biological properties of the virus. The conflicting results may stem from the use of different virus stocks, different strains of animals, and different methods for the preparation and maintenance of the cultures. The most consistant data is that both mouse and rat oligodendrocytes are susceptible to the demyelinating strains, A59 and JHM.

b. MHV persistence in vitro and in vivo

Viral persistence has been studied with A59, JHM and MHV3 in both mouse and rat model systems. Much of the work has been carried out in an attempt to determine whether viruses persist during the chronic disease state, and if so whether it is in an infectious form and whether there is a relationship between viral persistence and demyelination. In most of these studies, after the initial acute stages of infection it is difficult if not impossible to recover infectious virus from the CNS of MHV infected mice or rats. The three examples of recovery of infectious virus long after infection are: 1) Knobler et. al. (66) were able to recover very low levels of a temperature sensitive mutant (ts8) of JHM from the CNS of chronically demyelinated mice one year after inoculation; 2) Infectious virus was occasionally recovered from rats chronically infected with JHM (2); and 3) MHV3 can be recovered from mice that

undergo chronic CNS disease (44,56). This suggests that virus does persist at a low level in an infectious form during the chronic neurological disease state.

In the JHM chronic infection of rats (60,78) and A59 induced chronic demyelination of mice (76), virus specific RNA has been detected by in situ hybridization and dot blots using virus specific radiolabeled probes. In the A59 mouse model during chronic demyelinating stages of disease viral nucleic acids were detected only in the white matter suggesting that glial cells are the site of viral persistence. However, in the JHM rat model, nucleic acids were detected in neurons as well, suggesting they also may be sites of viral persistence (60,78). Viral antigen was also detectible in the CNS rat model (78).

Although the result of infection of 17Cl-1 or L2 murine fibroblast cell lines in vitro is lytic, persistent productive infections of cultured cell lines can be readily established with MHV. Persistent infections have been described with A59 in 17Cl-1 cells (79), JHM in DBT cells (40), 17Cl-1 cells (80), Neuroblastoma cells (80,81) SAC(-) cells (82), in RN-2 rat Schwannoma cells, mouse oligodendroglioma (G26-20) and rat HTC hepatoma cell lines (83,83a). These infections are probably not mediated by interferon or defective interfering particles. In some cases temperature sensitive mutants, small plaque variants, and variants, less pathogenic in animals, have been isolated suggesting perhaps that persistence is a result of selection in vivo of a less pathogenic variant. Several groups have studied persistent infection of MHV in neural cells in in vitro cultures derived from mouse or rat CNS tissue. A59 causes a persistent

productive infection of both mixed glial cultures as well as enriched oligodendrocyte and enriched astrocyte cultures. derived from mouse or rat CNS tissue (71,74,75). These cultures which produce moderate levels of virus undergo little cytopathic effect. Thus, virus can persist as long as the cultures are viable, up to 45 days in one study (74). Persistence of virus *in vitro* in glial cells cultures suggests a mechanism to maintain virus during infection *in vivo*. JHM infection of glial cells *in vitro* causes a chronic infection in cells from both susceptible and resistant mice (69,71) Bueshausen et al., (72) have shown that JHM persists in rat oligodendrocytes while causing an abortive infection in astrocytes. Furthermore, in this study the susceptibility of oligodendrocytes decreases as the cells mature reflecting the age acquired resistance to the disease *in vivo*. These workers suggest that persistence in oligodendrocytes is related to the demyelinating disease. Massa et al., (73) have found that JHM infects rat astrocytes as the primary target and infects oligodendrocytes only occasionally by fusion with infected astrocytes. The workers hypothesize that the astrocyte is the primary site of persistence *in vivo*.

From these *in vitro* and *in vivo* experiments, it is clear that viral RNA and antigens and probably infectious virus can persist in the CNS. The site(s) of persistence may vary depending on viral and host strain. Two important questions concerning persistence remain to be answered. 1) What are the molecular mechansims involved in persistence? 2) How does persistence pertain to demyelination? Persistence in fibroblasts

cell lines seem to be related to selection of variants that are less pathogenic in tissue culture; however, molecular descriptions of these variants is at present lacking. There has been little if any characterization of virus released by persistently infected glial cells. In the A59 infections of glial cells *in vitro*, the virus released is neither temperature sensitive nor small plaque variant (74). In the infections of animals there has been little characterization of virus isolated from persistently infected mice. There are several suggestions that persistence may be related to alterations in E2 glycoprotein. In *in vitro* cultures a less neuropathogenic mutant of A59 grew less than wild type and exhibited changes in E2 protein synthesis (75). Furthermore JHM viruses isolated from the acutely infected rats exhibited changes in size of E2 mRNA and protein (84). It remains to be determined whether this is related to persistence. On the second question, it is not known whether a lytic infection leads to a destruction of oligodendrocytes and thus demyelination or that persistent infection of glial cells causes alterations in MHC surface antigen expression that leads to an immune-mediated demyelination (85,86). This will be discussed further below.

3) The control of pathogenesis by viral genes and proteins

In several model systems of viral pathogenesis (reovirus, rabies and poliovirus), certain biological properties of the viruses have been mapped to viral genes (87,88,89). The question of which viral genes control MHV pathogenesis is currently being studied using approaches including the production and characterization of 1) viral mutants 2) antigenic

variant viruses selected with monoclonal antibodies to specific viral proteins and 3) recombinants between two different strains of MHV. In the future, in vitro mutagenesis of cloned DNAs will be useful to study genetic control of MHV pathogenesis.

Successful manipulation of MHV by either mutation (90,91,92) or antigenic variation (93,94), resulted in selective attenutation of the ability of the virus to produce an acute fatal encephalomyelitis, without affecting the ability to demyelinate. This is probably related to the ability of the virus to infect neurons. This suggests that the two pathological properties of acute encephalitis and chronic demyelination can be separated by manipulations of genes or proteins and one (encephalitis) can be readily altered.

a. MHV mutants

At present, temparature-sensitive mutants of JHM or A59, which are attenuated in the acute phase of disease have been described (90,91,92). A delicate dissection of genetic control of pathogenesis will be mapping of biological properties to a single amino acid, potentially possible by sequencing of genome RNA of mutants with single point mutations. Such precedent have been described in rabies virus by nucleic acid sequence analysis of antigenic variants (89).

b. Antigenic variants

Two laboratories have reported results suggesting that the E2 glycoprotein is important in MHV pathogenesis (93,94). Antigenic variants resistent to monoclonal antibodies against certain epitopes of the JHM E2 envelope glycoprotein were found to have produced milder acute encephalitis without affecting

chronic demyelination. Variants selected for resistence to monoclonal antibodies against other epitopes of E2 did not show difference in pathogenesis; neither did variants selected with monoclonal antibodies against other structural proteins (E1 and N). A partial protection against JHM induced acute encephalitis but not against demyelination was reported using an adoptive transfer of E2 monoclonal antibodies (95). Thus, the E2 glycoprotein contains a specific determinant that controls the outcome of the interaction of the virus with its host during the acute phase. This epitope may be important for the acute infection in neurons by MHV. It is also likely that the difference between JHM and A59 in their selectivity of neuronal infection is determined by this epitope.

c. Recombinant viruses

Since a high rate of recombination was found in mixed infections of susceptible cells with JHM and A59, it allowed the selection of a panel of recombinant viruses (37,96). The biological properties of pathogenesis *in vivo* and growth *in vitro* could be mapped to sites on the genome. At a lethal dose, JHM produces in C57BL/6 mice panencephalitis and mild hepatitis while A59 produces focal encephalitis and severe hepatitis (41,42,53,62,62a). A59 grows to higher titers than JHM *in vitro*. The 3' part of the viral genome (genes C-G), containing the genes for structural proteins, was found to control the biological properties of plaque morphology and replication *in vitro*, and organ tropism and pattern of pathology *in vivo*.

4). Immune response to MHV infection

The characterization of the immune response to MHV infection in vivo raises major questions. What are the cell mediated and humoral defense mechanisms in response to MHV infection? How is MHV persistence established in the presence of competent immune response? Does the virus have immunosuppressive properties which contribute to the establishment of persistence? Are immune mediated mechanisms palying a role in the pathogenesis of MHV disease? The answers to these questions are only partially known.

a. Antibody response

Mice or rats inoculated with MHVs develop neutralizing antibodies against the virus which persist during the lifetime of the animal (42,49). Thus chronic paralytic disease, associated with either demyelination (JHM and A59) or vasculitis (MHV-3), occurs in the presence of neutralizing antibodies. Antibody titers or immunization with either polyclonal or anti-E2-monoclonal antibodies provide protection against reinfection with MHV (41,95,97). Protection is against the acute hepatitis or encephalitis but not against demyelination.

b. T helper/inducer cells in MHV disease

An intracerebral inoculation of JHM-specific cloned helper/inducer T lymphocytes (extracted from JHM-infected mice) protected mice from JHM-induced lethal disease (98). These cells reacted in an MHC class II restricted manner. This did not result in altered antiviral immunoglobulin synthesis or in the suppression of viral replication in the CNS. It is possible that the localized administration of helper/inducer T

lymphocytes prevented neuronal infection but not other aspects of the disease. Since infectious virus titers usually drop after a few weeks following infection with JHM or A59 it is conceivable that clearance of the virus from the CNS implicates another immune effector mechanism, possibly a virus-specific CTL response. This has yet to be demonstrated in an MHV infection.

c. NK and inteferon activity in MHV infections.

Natural killer (NK) cells may play a role in host defence mechanisms against viral infections as demonstrated in LCM and herpes infections (99,100), and was found to be correlated with augmentation of interferon titers (99). In contrast to these reports, MHV infection induces NK activity with or without augmentation in interferon levels. In addition there is an inverse correlation between host resistance and both the interferon and NK response of mice to MHV-infection (101,102). This data suggests that NK activity does not play a major role in host survival following MHV infection.

d. VK activity

Spleen cells from uninfected normal mice selectivity lysed BALB/c 3T3 A59 infected but not uninfected fibroblasts (103). Lysis occured within 3 hours and histocompatibility between effector and target cells was not required. There is evidence that the effectors cells have the characteristics of B lymphocytes (104). The role of this phenomenon, called VK activity, in vivo is not clear. It seems however, that it does not have a significant role in resistence against the virus since spleen cells from susceptible mice exhibit more VK activity than spleen cells from resistent mice.

e. MHC induction

Infection of glial cells derived from C57BL/6 mice with MHV-A59 in vitro induced expression of MHC class I but not Ia antigens on the surface of oligodendrocytes and astrocytes (85). The same was true of glial cells derived from previously infected mice. Normal neural cells do not express these antigens. The use of UV inactivated supernatant from infected astrocytes, oligodendrocytes, other CNS cell cultures and 17Cl-1 mouse fibrobalsts suggested that a soluble factor (or factors) made by astrocytes was involved in the induction (85). While inteferons were ruled out as the "H-2 inducing factor" in MHV infections, the identity of the factor is at present unknown.

Hybridrization experiments of RNA isolated from MHV-A59 infected brains with a complementary DNA probe for H-2 class I RNA demonstrated increased levels of the messenger RNA for H-2 protein in the brains of infected mice (104a). This suggests that induction of MHC class 1 antigens occurs in vivo.

In MHV-3 infection of mice, MHC class I antigen expression was found in neuronal cells located in lesions of infected mice (105).

Treatment of glial cultures derived from Lewis rats with JHM resulted in induction of la antigen expresion in 10% of astrocytes (86). This could be induced by either infectious virus or UV inactivated virus. Inducibility of Ia could be blocked by neutralizing antibodies against the virus but not with non-neutralizing antibodies and not with antibodies against interferon. Transfer of supernatant from a culture that was induced to express Ia by UV inactivated virus failed to induce

Ia in another culture. These results suggest that induction of Ia is related to a direct effect of viral particles and different in mechanism from MHV-A59 induction of class I antigens.

In conclusion, MHC class I antigen expression can be induced by A59, JHM and MHV-3. Expression is observed in a variety of susceptible mice and rats. MHC class II antigen induction is also observed under certain conditions. The importance of this phenomenon in the pathogenesis of MHV diseases needs further investigation.

f. Delayed type hypersensitivity (DTH) to myelin basic protein (MBP) in JHM induced demyelination.

JHM induced subacute demyelination (SDE) in Lewis rats is thought to involve an immune mediated mechanism (106). Spleen, thymus and peripheral blood lymphocytes from demyelinated rats were found to be stimulated by MBP in vitro (as in experimental allergic encephalomyelitis, EAE). When the stimulated lymphocytes were used for adoptive transfer to naive Lewis rats, demyelination developed. This study shows that JHM infection in rats induces cell mediated autoimmunity against MBP. Further studies are needed to prove that sensitization to MBP specifically is part of the mechanism of MHV induced demyelination.

5) Host genetic control of MHV disease.

a. Strain related susceptibility and resistance

As in other experimental viral infections (107) MHV infection exhibits a strain related resistance and susceptibility to disease. This provides a tool to study

genetic control of predisposition to viral disease. Strains of mice susceptible to JHM infection include Balb/c, C3H and C57BL/6 mice. Adult SJL mice are resistent. Resistance and susceptibility to disease is mirrored by resistance and susceptibility to growth in peritoneal macrophages of these mice (108). Studies conducted on the analysis of genetic control of susceptibility used recombinant-inbred mice from susceptible and resistant strains have conflicting results. One group of studies suggested that resistance to disease by JHM or A59 is inherited as a single autosomal recessive trait, independent of the MHC, which maps to the proximal end of murine chromosome 7 (109,110). Another study suggested that susceptibility to MHV-2 is inherited by an autosomal dominant gene (111). Another study proposed a two gene model of resistance and susceptibility to JHM (111a).

Studies with MHV-3 revealed that strain A mice are completely resistant to this strain of virus while C57BL/6, DBA/2, BALB/c and NZB mice are fully susceptible and die of acute hepatitis. C3H mice and A2G mice are semi-susceptible and develop a persistent infection including a paralytic neurologic disease. This is mirrored by growth of the virus in macrophage (112,113), hepatocytes (114) and embryonic fibroblast (115) cultures derived from these strains. Genetic analysis indicated that acute and chronic diseases are under the influence of at least two major genes or gene complexes which are different for each disease state and that in addition resistance to paralysis is H-2 linked (116).

Recently Boyle et al. (117) have shown that genetic resistance to MHV-A59 infection correlated with the absence of virus-binding activity on target tissues. They suggest resistance is due to the absence of a viral receptor. Using a binding assay to measure binding of virus to membranes from hepatocytes and enterocytes, these workers have identified a 100K-110K protein as a possible receptor for MHV-A59. This molecule is present in cells from susceptible C57BL/6 mice and semi- susceptible C3H mice but not in cells from resistent SJL/J mice. This study should lead the way to characterize the function of this putative viral receptor and its presence in various cell types and thus shed light on tropism as well as genetic resistance.

b. Age related resistance to MHV infection

Acquisition of resistance to viral infection with increasing age has been described for numerous viruses (118,119). Various mouse strains possess age related susceptibility and resistance to MHV infection and age related disease pattern. Susceptible mice possess age related resistence to A59 (Balb/c, C57BL/6) and MHV-S (ICR) (120, Lavi unpublished); however susceptible mice do not possess age related resistence to JHM, MHV-3 and MHV-2 (121). Semi susceptible (C3/H) and resistent mice (A/J) do express age related resistance to MHV-3 (122). Resistant mice (SJL) also show age related resistence to JHM (123). Rat oligodendrocytes exhibit development of resistence to JHM infection *in vitro* as they mature (72).

Maturation state of the cells of the target organs or changes in viral receptors or maturation or changes of the

immune system could account for this phenonemenon. Several studies investigating the mechanism of age related resistance to MHV infection suggest that it is a multifactorial immune related phenomenon which may vary with different strains of MHV. Spleen cells and peritoneal exudate cells from resistant 12-week-old SJL mice can transfer resistance to 6-week-old susceptible recipients (123). Removal of the adherent cells from either spleen cells or peritoneal cells ablated protection. Adherent cells were protective even after depletion of Ia and Thy-1 bearing cells. Neither the identify of the cells responsible for age dependent resistance nor the mechanism of protection could be determined. However, protection did not correlate with the ability of peritoneal cells to support viral replication since peritoneal cells from neither susceptible nor resistant ages would support JHM replication in vitro (123). Studies using MHV-3 infection suggested that both T cells and an adherent spleen cell population are required for resistance against MHV-3 infection (124).

Immunosuppressive treatments such as cyclophosphamide and corticosteroids can overcome age related resistance in MHV-S, MHV-3 and A59 (120,125,126, Lavi unpublished). Treatment with anti-interferon serum can overcome age related resistance only in wealing resistant mice but not in adult mice infected with MHV-3 (122).

D. Future Directions of Coronavirus Pathogenesis Research

Coronavirus research has progressed quickly over the last ten years in terms of both pathogenesis and molecular biology.

There are now well described model systems for infection both *in vivo* and *in vitro*. The development of monoclonal antibodies against the MHV E2 protein has allowed us to begin to define the epitopes important in the production of viral encephalitis. The entire infectious bronchitis virus genome and large portions of the mouse hepatitis virus genome have been sequenced. The next major step may be the construction of infectious recombinant DNA copies of the viral genome. Over the next few years, it should be possible to use molecular biology to analyze viral tropism and pathogenesis. More specifically, the use of recombinant, variant and mutant viruses will be important in the molecular biological analysis of viral genes important in cell tropism and demyelination.

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