INCREASED HEPATOTROPISM OF MUTANTS OF MHV, STRAIN JHM, SELECTED WITH MONOCLONAL ANTIBODIES

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INTRODUCTION

The mouse hepatitis viruses (MHV) make up a group of closely related murine coronaviruses which produce a wide spectrum of disease, depending upon the virus strain, the age and strain of the infected mouse, and the route of innoculation (Nelson, 1952; Gledhill and Neven, 1955). The determinants of the outcome of infection by MHV have been widely studied by many laboratories, with most groups concentrating their efforts on either the host response to the virus or on the viral genes responsible for virulence.

Work in our laboratories has concentrated on MHV, strain JHM (MHV-JHM), a highly neurotropic virus. Infection of weanling susceptible mice with this virus result in an encephalomyelitis in which neurons, astrocytes, and oligodendrocytes are infected (Powell and Lampert, 1975; Knobler et al, 1982). Destruction of oligodendrocytes results in demyelinated lesions in white matter of brain and spinal cord (Bailey et al, 1949; Lampert et al 1973; Weiner, 1973). Several viral genes have been implicated as determinants of virulence, amongst them the nucleocapsid (Fleming et al, 1983), E2 (Dalziel et al, 1986; Fleming et al, 1986), and polymerase (Lai et al, 1981) genes. The E2 gene is especially likely to be a major determinant of virulence since the E2 protein mediates MHV adsorption to its target cell, and it is the protein responsible for virus-induced cell fusion during the course of infection (Collins et al, 1982; Wege et al, 1984). Antibody directed against this protein is neutralizing (Collins et al, 1982). An approach to investigating the role of this protein in determining the biologic effects of MHV is to isolate a series of point mutations in the E2 gene which are no longer neutralizable by monoclonal antibodies directed against E2. The pathogenicity, cell and organ tropism of these "escape mutants" can then be compared with the parental virus. This approach has been used by several laboratories and generally confirms the importance of E2 as a viral determinant of virulence (Dalziel et al, 1986; Fleming et al, 1986). In this report we will describe our results analyzing several escape mutants of MHV-JHM.

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METHODS

Cells

The origin and growth of the 17Cl-1 and L2 cells used in this study have been described (Rothfels et al, 1959; Sturman and Takemoto, 1972; Robb and Bond, 1979). The P3 X 63 Ag8 clone 6531 myeloma, and hybridoma cell lines derived from this cell line, were grown in RPMI 1640 supplemented with 10% fetal bovine serum.

Virus

The origin and growth of the strains of MHV-JHM used in this study have been described. The virus used for most of the work described here is the strain used by Lampert et al (1973) and Robb and Bond (1979). It has been designated the Robb/Lampert virus in this study. This strain originated as isolate #718 of Hartley and Rowe and has been passaged 46 times in mouse brain prior to its being put into tissue culture. A second laboratory strain of MHV-JHM, designated as the Scripps strain in this manuscript, was obtained from Dr. Robert Knobler. This strain (Haspel et al, 1978; Collins et al, 1982) is derived from the virus originally described by Weiner et al (1973).

Monoclonal antibodies

The hybridoma lines secreting monoclonal antibodies 1.43.1 and 1.38.1 were isolated from spleen cells harvested from a Balb/c mouse immunized with the Robb/Lampert strain of MHV-JHM. A mouse was immunized by intraperitoneal innoculation with 1000 PFU of live virus and at 3 weeks post infection was boosted with 1,000 PFU, and then boosted a second time 2 months later with 10^7 PFU of MHV-JHM. Three days after boosting the spleen cells were harvested and fused with the mouse myeloma line P3 X 63 Ag8 clone 6531 using published procedures (Collins et al, 1982). Following fusion the cells were plated in 96 well plates. Selection for hybridoma cells was in HAT medium and after two weeks, culture supernatants were screened for the presence of anti-MHV antibodies by a radioimmunoassay (Leibowitz et al, 1983). Cells from antibody positive wells were cloned by limiting dilution, retested by radioimmunoassay and then grown up. Antibodies were concentrated from culture media by precipitation with saturated ammonium sulfate and were subsequently dissolved in a small volume of phosphate buffered saline (PBS), dialyzed against PBS and sterilized by filtration. Monoclonal antibody 5A-13.5 was kindly provided as an ascitic fluid by Dr. Michael Buchmeier of Scripps Clinic and Research Foundation. The isolation and characterization of this antibody has been described (Collins et al, 1982). It was isolated following immunization of a Balb/c mouse with the Scripps strain of MHV-JHM and recognizes the E2 protein.

Isolation of escape mutants

Parental MHV-JHM, about 10^6 PFU, was incubated for 30 minutes on ice with 2.0 ml of undiluted saturated ammonium sulfate concentrated monoclonal antibody or with a similar volume of a 1:10 dilution of ascitic fluid. The virus-antibody mixture was then innoculated onto 17CL-1 or L-2 cells and unneutralized virus allowed to adsorb to the cells for 60 minutes at room temperature. The cells were then fed with DME containing 2% FBS and incubated at 37°C until cytopathic effect was prominent. The cultures were then harvested by one cycle of freeze-thawing and disrupted by sonication. Following clarification by low speed centrifugation, the virus yield was determined by plaque assay, and a second round of selection by antibody was performed in a manner similar to that described

above. A hundred PFU of this second passage was then incubated on ice for 30 min with 0.2 ml of monoclonal antibody and any non-neutralizable escape mutants present were clonally isolated by two cycles of plaque purification. Small stocks of these putative mutants were then grown up in 17CL-1 cells and tested for their ability to escape neutralization with the monoclonal antibody used for selection, and with other neutralizing monoclonals.

Radioimmunoprecipation

Cytoplasmic lysates of MHV-JHM infected cultures which had been labeled with ^{35}S-methionine were prepared as described previously (Leibowitz et al, 1982). These lysates were reacted with monoclonal antibodies for 30 minutes in a buffer containing 10 mM phosphate, pH 7.4, 0.5 M NaCl, 0.5% NP-40, 0.1% SDS and 1% approtinin (Sigma), on ice. The antibody-antigen complexes were then precipitated with rabbit anti-mouse Ig adsorbed to Staphlococcus aureus. The precipitated material was washed extensively in the buffer described above. The precipitated material was released from the Staph. aureus by heating to 70°C in SDS polyacrylamide sample buffer and subsequently analyzed by polyacrylamide gel electrophoresis (Laemmli, 1970).

Histology

Animals to be sacrificed were anesthetized with ether and fixed by perfusion in buffered 4% paraformaldehyde. Tissue was embedded in B5 resin or paraffin, cut at 4 microns and stained with hematoxalin and eosin by standard histologic methods.

RESULTS

Characterization of antibodies

Our panel of monoclonal antibodies contained four antibodies which recognized the MHV-JHM E2 protein by radioimmunoprecipation (Figure 1). None of these antibodies reacted with E2 in a western blot assay (data not shown). Two of these antibodies, 1.38.1 and 1.43.2, were capable of neutralizing viral infectivity and were used to select escape mutants. The nomenclature used for the escape mutants generated during the course of these experiments is shown in Table 1.

Selection and characterization of escape mutants

Our initial series of mutants were selected from the Robb/Lampert strain of MHV-JHM with monoclonal antibodies 1.38.1, 1.43.2, and 5A-13.5 as described in Materials and Methods. At least three independently isolated mutants were selected with each monoclonal. The unique identity of the monoclonals used to select these mutants, and the mutants themselves, was demonstrated in a cross neutralization test (Table 2). Monoclonal antibodies 4B11.6 and 5B170.3 (provided by Dr. Michael Buchmeier), as well as the selecting monoclonals 1.38.1, 1.43.2, and 5A-13.5, were tested as to their capacity to neutralize the escape mutants. Mutants selected with monoclonal antibody 1.38.1 still contained the epitope recognized by the other neutralizing monoclonals although they are no longer neutralizable by 1.38.1. Mutants selected with monoclonal antibody 1.43.2 are no longer recognized by most of the other monoclonal antibodies used in this study, as well as by the selecting antibody, 1.43.2. This is presumably due to allosteric effects of a mutation in the region recognized by 1.43.2. A similar phenomenon has been described by others (Fleming et al, 1986). Mutants selected

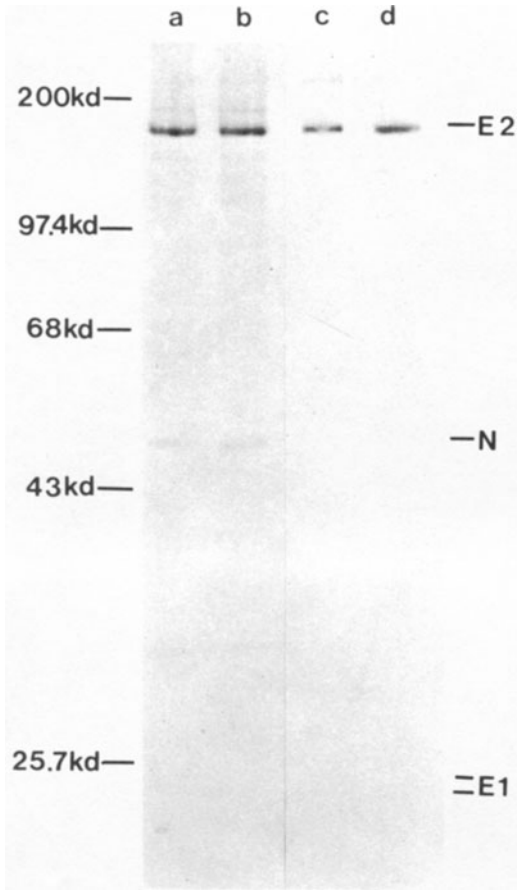

Fig. 1. Radioimmunoprecipitation of MHV-infected cell lysates with monoclonal antibodies. ^{35}S-methionine labeled cytoplasmic extracts were prepared from MHV-JHM infected L-2 cells. 50 ul of lysate was reacted with 50 ul of monoclonal antibodies 1.14.4 (lane a), 1.35.1 (lane b), 1.38.1 (lane c), or 1.43.2 (lane d) and the immunoprecipitates analyzed on an 8% polyacrylamide gel. The positions of molecular weight markers and MHV-specific intracellular proteins are indicated on the side of the autoradiograph.

with antibody 5A-13.5 could not be neutralized by the selecting antibody but still were recognized by the other monoclonals. It should be noted that all the antibodies we tested which were originally raised against the Scripps strain of MHV-JHM also recognized the Robb/Lampert strain.

The mutants described above were then tested for their biologic activity in mice. Mice were injected intracerebrally with ten fold dilutions of virus and observed. All of the escape mutants derived from the Robb/Lampert virus were almost as virulent as the parental virus. The LD_{50} and median time of death of infected mice were only slightly altered from that observed with the parental Robb/Lampert virus (Table 3). This was true for mutants selected with antibody 5A-13.5, a monoclonal antibody previously demonstrated to select severely attenuated mutants from the Scripps strain of MHV-JHM (Dalziel et al, 1986), as well as for mutants selected with antibodies 1.38.1 and 1.43.2.

Table 1. Antibodies and Viruses

Monoclonal Antibody	Wide Type JHM	Mutant
1.38.1	Robb/Lampert	RL/A.4-2/1.38.1
1.38.1	Scripps	Scripps/C.1-1/138.1
1.43.2	Robb/Lampert	RL/B.3-2/1.43.2
1.43.2	Scripps	Scripps/D.1-4/1.43.2
5A-13.5	Robb/Lampert	RL/E.1-7/5A-13.5

Table 2. Cross Neutralization of Monoclonal Antibody Selected Mutants

Antibody	Escape Mutant		
	RL/E.1-7/5A13.5	RL/A.4-2/1.38.1	RL/B.3-2/1.43.2
4B11.6	+	+	-
5A13.5	-	+	-
5B19.2 or 5B170.3	+	+	+
1.38.1	+	-	-
1.43.2	+	+	-

+ = More than 95% of the input virus was neutralized
- = No neutralization

Histologic examination of brain and spinal cord taken from animals infected with these mutants revealed no significant differences from that observed with the parental virus. The pathology was similar to that previously described (Bailey et al, 1949; Lampert et al, 1973; Weiner, 1973) and consisted of an encephalomyelitis involving both gray and white matter with accompanying demyelinating lesions in the spinal cord. This is illustrated for one of these mutants, RL/A.4.2/1.38.1, in Figure 2. Examination of the histology of the liver revealed some differences. The Robb/Lampert parental virus has a very minimal apparent hepatotropism. At best only one or two small lesions could be observed per low power field (Figure 3, panel A). The escape mutants appeared to be somewhat more hepatotropic. This was most marked with mutants selected with monoclone 5A-13.5 (Figure 3, panel B). A summary of the histologic changes observed is presented in Table 4.

Ten percent homogenates of CNS and liver from infected animals sacrificed at varying times post infection were titrated for virus content in the presence and absence of the antibody used to select each mutant, in order to insure that the biologic effects we were observing were not due to revertants selected in vivo. In all cases the virus

Table 3. Relative Virulence of Mutants

Virus	LD_{50}	Median Time of Death
Robb/Lampert	2	5 days
RL/A.4-2/1.38.1	10	7 days
RL/B.3-2/1.43.2	6	7 days
RL/E.1-7/5A-13.5	3	6 days
Scripps	<1	5 days
Scripps/C.1-1/1.38.1	<1	7 days
Scripps/D.1-4/1.43.2	6	7 days

Table 4. Summary of Histologic Changes In Infected Mice

Virus	Pathologic Lesions		
	White Matter	Gray Matter	Liver
Robb/Lampert	2-3+	3-4+	1+
RL/A.4.2/1.38.1	2+	3+	1-2+
RL/B.3.2/1.43.2	2+	3+	1-2+
RL/E.1-7/5A-13.5	2-3+	3+	2+
Scripps	3+	3+	1-2+
Scripps/C.1-1/1.38.1	3+	1-2+	2-3+
Scripps/D.1-4/1.43.2	2-3+	2-3+	3+

recovered was indistinguishable from the mutant innoculated (data not shown). Virus titers in the CNS did not vary appreciably from virus to virus, and were approximately 10^6 per gram of tissue. The situation regarding the amount of virus present in the liver was somewhat different. As much as ten-fold more virus could be recovered from mice infected with mutant T/E.1-7/5A-13.5 (1.5×10^3 PFU/g), than from mice infected with the parental strain (2×10^2 PFU/g). The amount of virus recovered from escape mutants derived using monoclonal antibodies 1.38.1 and 1.43.2 was somewhat less, between 5×10^2 and 1×10^3 PFU/g.

Since we could not produce an attenuated escape mutant from the Robb/Lampert strain of MHV-JHM we considered the possibility that the different origin and passage history of this virus, as compared to other laboratory strains, might account for this. Therefore, we selected a

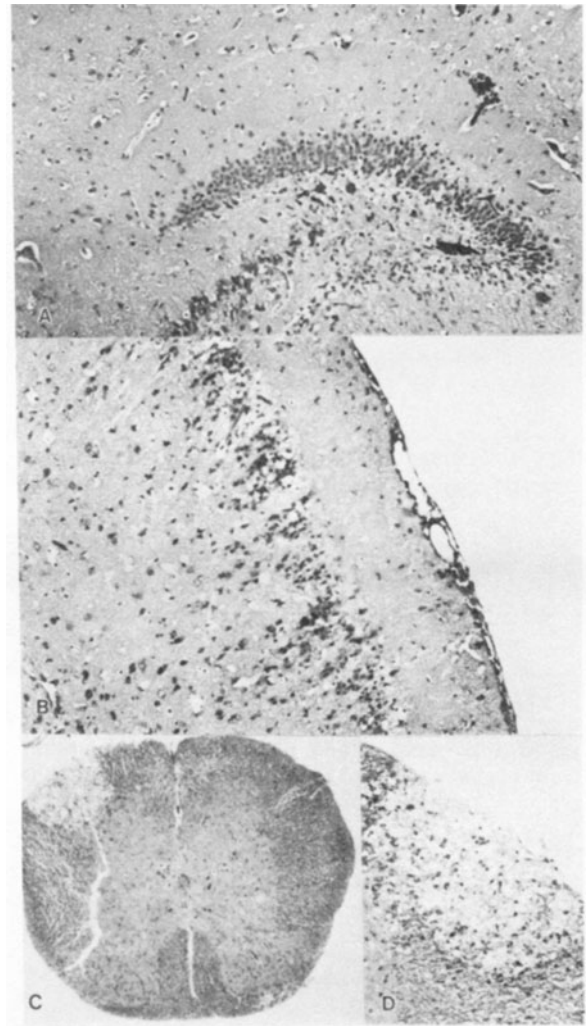

Fig. 2. Histology of CNS from mice infected with escape mutant RL/A.4.2/1.38.1. Mice were infected with 100 pfu intracerebrally and sacrificed when moribund on days 5-7 post infection. Panel A, hippocampus, low power; Panel B, hippocampus, high power; Panel C, spinal cord, low power; Panel D, high power view of demyelinated lesion in spinal cord.

second series of escape mutants with monoclones 1.38.1 and 1.43.2 using the Scripps strain of MHV-JHM as the parent virus. The LD_{50}s of these mutants and the parental virus were determined by innoculating mice intracerebrally in a similar manner as described above. As shown in Table 3, these escape mutants, typified by Scripps/C.1-1/1.38.1 and Scripps/D.1-4/1.43.2, were either as virulent as the parental virus or only marginally attenuated. Histologic examination of the CNS of these mutants and the parental virus demonstrated that the lesions they produced were similar to those observed with the Robb/Lampert strain (Table 4). This was also reflected in the amount of virus recovered from CNS

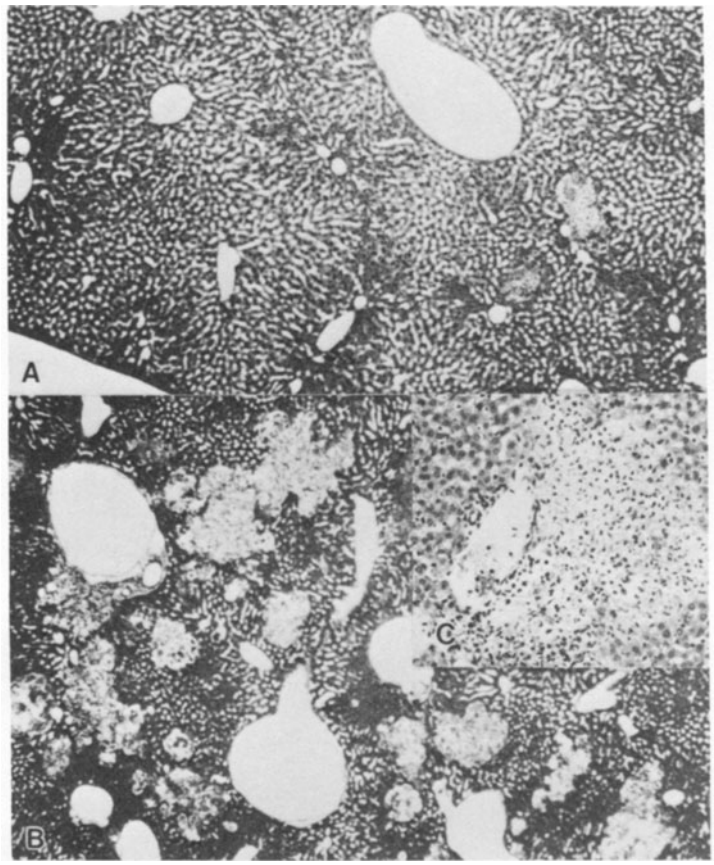

Fig. 3. Histology of livers from mice infected with either wild type RL virus, Panel A, or escape mutant RL/E.1.7/5A-13.5, Panels B and C. Mice were infected with wild type or mutant MHV-JHM by intracerebral injection and sacrificed at 5 days post infection.

homogenates (data not shown). The parental Scripps virus appeared to be as hepatotropic as the escape mutants of the Robb/Lampert strain. This was reflected both in the amount of virus recovered (10^4 PFU/g) and in the observed histology. When livers from animals infected with the escape mutants Scripps/C.1-1/1.38.1 and Scripps/D.1-4/1.43.2 were examined an increased extent and number of necrotic lesions were observed, especially for the latter mutant. This was only partially reflected in increased recovery of virus from these animals. Only a two-fold increase in titer was observed (2×10^4). The virus recovered from all tissues was titrated in the presence and absence of the antibody used to select each mutant to insure that the effects we were observing were not due to in vivo selected revertants. No revertants were detected.

DISCUSSION

A major goal for understanding the pathogenesis of MHV induced disease is to determine which viral genes are responsible for the varied

biologic effects produced by this group of viruses. For MHV-JHM recent efforts in this regard have concentrated on the E2 gene. The E2 gene encodes the peplomer protein which mediates virus adsorption to the host cell and cell-cell fusion (Collins et al, 1982; Wege et al, 1984). It is therefore highly likely to be an important determinant of virulence for this virus.

Our efforts to attenuate MHV-JHM by selecting antigenic variants from the Robb/Lampert strain of MHV-JHM with three different monoclonal antibodies were unsuccessful. The mutants selected were all almost as virulent as the wild-type virus from which they were derived. These results are somewhat at variance with the experience of others. Dalziel et al (1986), and Fleming et al (1986), succeeded in isolating attenuated escape mutants of MHV-JHM. The parental virus from which they selected theses mutants is derived from a different isolate from that of the Robb/Lampert strain. We attribute these divergent results to differences in the parental viruses used in these studies. This is especially likely to be the case since the antibody 5A-13.5 was used to select 1,000-fold attenuated mutants of the Scripps strain of MHV-JHM (LD_{50} >1,800 PFU), while antigenic variants selected from the Robb/Lampert strain with this antibody were only slightly decreased in virulence. This serves to point out the potential pitfalls in attributing virulence to a single epitope on a viral protein or even to a single gene. Previous work has implicated several genes in addition to E2 as determinants of virulence. Both the nucleocapsid (Fleming et al, 1983) and polymerase (Lai et al, 1981) genes have been suggested to be important in this regard. We cannot determine at this time the genetic basis for the biologic differences observed between the two different parental strains of MHV-JHM used in this study.

This is not to discount the role of E2 in the pathogenesis of MHV-induced disease. Several of the mutants selected in this study, although not attenuated, had an apparently increased ability to grow and produce lesions in liver. This was most obviously true for mutants of the Robb/Lampert virus, since the parent virus only produced minimal lesions in the livers of infected mice. The organ tropism and pathogenicity of a given virus is undoubtly a multigenic effect. Clearly much further work needs to be done to dissect how the various MHV gene products interact with the host to produce disease.

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