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## Antigenic variation among murine coronaviruses: Evidence for polymorphism on the peplomer glycoprotein, E2

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### Summary

A panel of 28 monoclonal antibodies (MAB) against the structural proteins of murine hepatitis virus-4, strain JHM (MHV-4) was used in three antigen binding assays to determine the extent of antigenic homology among six strains of murine coronaviruses. The antigenic determinants studied were highly conserved on the E1 glycoproteins and nucleocapsid (N) proteins of all strains tested. In contrast, antigenic polymorphism was observed among the E2 glycoproteins. Of three previously described antigenic determinants against which neutralizing antibodies are directed, only one, termed A(E2), was conserved on all strains. Antigenic site B(E2) was found only on the strongly neurotropic MHV-4 and site C(E2) was present on the virulent MHV-4 and MHV-3 (hepatotropic) strains, but absent on the weakly pathogenic MHV-A59, MHV-1 and MHV-S strains. Four non-neutralizing antibodies against at least one topographically distinct antigenic determinant, which we previously designated D(E2), gave binding patterns consistent with two distinct sites. One of these was present on all MHV strains tested and the other was present on all strains except MHV-S. These non-neutralizing antigenic sites were redesignated E(E2) and D(E2) respectively.

coronavirus, antigen, glycoprotein, monoclonal antibody, epitope, polymorphism

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## Introduction

Murine hepatitis viruses (MHV) are coronaviruses which cause a variety of diseases such as hepatitis, gastroenteritis and encephalomyelitis (Siddell et al., 1983a, 1983b; Wege et al., 1982) in the natural host. Despite these variations in pathogenesis, the viruses are closely related in structure and in their mode of replication. Viral RNA is complexed with N protein ( $M_r = 56-60\,000$ ) and the envelope is studded with petal-shaped peplomers composed of a major glycoprotein, E2 ( $M_r$  180–200 000) made up of two nonidentical 90 000 dalton subunits. A second glycoprotein, E1 ( $M_r = 23-25\,000$ ), spans the membrane and interacts internally with the viral RNA. E1 may determine the intracellular site of virus maturation (Siddell et al., 1982; Sturman and Holmes, 1983).

RNA homologies between strains of MHV with different virulence characteristics and organ tropisms have been analyzed. Oligonucleotide fingerprinting analysis of the highly virulent hepatotropic strain MHV-3 and the weakly pathogenic MHV-A59 strain revealed structural differences in the genes coding for the RNA polymerase and the E2 glycoprotein (Lai et al., 1981). In similar analyses with other strains, each MHV isolate yielded a unique oligonucleotide pattern. The neurotropic MHV-4 strain showed the highest degree of difference among the strains tested, which also included MHV-2, 3 and A59 (Wege et al., 1981; Weiss and Leibowitz, 1983). Hybridization studies with cDNA clones of the viral RNAs revealed extensive nucleic acid homologies between MHV strains (Cheley et al., 1981; Weiss and Leibowitz, 1983). More recently sequence analyses of the N gene indicated an overall 94% homology between the strongly neurotropic MHV-4 and the weakly neurotropic MHV-A59 (Armstrong et al., 1983; Skinner and Siddell, 1983).

Antigenic homologies were initially probed in cross-neutralization assays (Hierholzer et al., 1979; Taguchi et al., 1982; Wege et al., 1981) and neutralization kinetics (Childs et al., 1983). These studies showed that MHV strains could be distinguished, but that significant levels of cross-reactivity occurred. Strains MHV-2 and 4 were closely related, as were strains MHV-1, 3, S and A59. There was no correlation between serological typing and virulence. These neutralization assays likely measured antigenic homologies at the level of the E2 glycoprotein, which is the target for neutralizing antibodies (Collins et al., 1982; Fleming et al., 1983). Tryptic maps of the N proteins from five MHV strains also showed a strong level of similarity (Cheley et al., 1981). Recently, antigenic relationships among eleven MHV isolates were probed in radioimmunoassays with monoclonal antibodies to MHV-4 (Fleming et al., 1983). Extensive antigenic variation of the E2 glycoprotein was observed but a correlation was drawn between less extensive variations in the N protein and pathogenicity. E1 glycoprotein was markedly conserved among the MHV isolates tested.

MHV-4 is a useful animal model for virus-induced primary demyelination (Haspel et al., 1978; Knobler et al., 1981a, 1982; Lampert et al., 1973; Nagashima et al., 1978; Sorensen et al., 1980; Stohlman and Weiner, 1981; Weiner, 1973). Rapidly fatal encephalitis normally masks the more slowly developing demyelinating disease in mice (Knobler et al., 1981b; Stohlman and Weiner, 1981; Weiner, 1973). How-

ever, infection with mutants or variants of MHV-4 results in survival with accompanying primary demyelination, due to infection of the oligodendrocyte (Haspel et al., 1978; Stohlman et al., 1982; Knobler et al., 1982). Monoclonal antibodies (MAb) to MHV-4 structural proteins were raised and characterized as described previously (Collins et al., 1982). A minimum of four topographically distinct antigenic determinants on E2 and two on E1 were mapped by competition binding assays (Talbot et al., 1984a). At least two distinct antigenic determinants were mapped on the N protein (Talbot et al., 1985). Antibodies to sites A, B and C(E2) were capable of neutralizing viral infectivity in vitro and passive antibody transfer experiments showed that neutralizing antibodies to sites A and B(E2) but not C(E2) could protect mice from a lethal intracerebral virus challenge. Such antibodies modulated disease from fatal encephalitis to sublethal demyelination (Buchmeier et al., 1984). In the present study, we have used a panel of eighteen MAb to MHV-4 generated in our laboratory and an independently derived panel of ten additional MAb to E2 raised by Fleming et al. (1983) to analyze antigenic relationships among six MHV strains differing in virulence and organ tropism. These were MHV-4 (JHM), both the wild-type (*wt*) strain (Cheever et al., 1949) and the *ts8* mutant (Haspel et al., 1978); MHV-3 (Dick et al., 1956); MHV-A59 (Manaker et al., 1961); MHV-1 (Gledhill and Andrewes, 1951); and MHV-S (Rowe et al., 1963).

## Materials and Methods

### *Virus and cell culture*

Murine hepatitis virus strains A59, 1 and S were purchased from the American Type Culture Collection. Dr. Julian Leibowitz kindly provided strain MHV-3, as well as DBT cells. The origin and cultivation of the *wt* and *ts8* strains of MHV-4 was described previously (Haspel et al., 1978). All viruses were plaque purified two or three times, propagated and assayed on L-2.4 or DBT cells.

### *Monoclonal antibodies*

The generation and characterization of MAb to the structural proteins of MHV-4 was described previously (Collins et al., 1982; Buchmeier et al., 1984; Talbot et al., 1984a). In addition to our panel we obtained an independently generated panel of ten MAb to the E2 glycoprotein of MHV-JHM kindly supplied by Dr. John Fleming, USC Medical School, Los Angeles (Fleming et al., 1983).

### *Antigen preparation*

For indirect immunofluorescence, L-2.4 cells grown on coverslips were infected with each MHV strain at a MOI of 0.01–0.5 and fixed in cold acetone when extensive syncytia were observed (16–21 h after infection). Antigenic preparations for dot immunoblotting and enzyme immunoassays (EIA) consisted of microsomal fractions from MHV infected or uninfected control cells, as described previously (Talbot et al., 1984a).

### *Antigen binding assays*

Indirect immunofluorescence was performed as described previously (Collins et

al., 1982), using monoclonal hybridoma cell culture fluids undiluted or after a 10-fold concentration in a macrosolute concentrator, type B-15 (Amicon Corp., Danvers, MA). The degree of reactivity was scored qualitatively on a scale of 0 to 4 by comparison with positive and negative controls. Enzyme immunoassays were as described previously (Talbot et al., 1984a) except that 200-fold concentrates of tissue culture fluids were used (40-fold initial concentration by precipitation with 50% (w/w) saturated ammonium sulfate followed by 5-fold additional concentration as described above). Viral antigens were in the form of subcellular fractions, hence it was necessary to calibrate the amount of protein used for each virus strain. This was accomplished by determining, in preliminary titrations, the quantity of antigen necessary to saturate the binding of an optimal dilution of a mixture of five MAb to the highly conserved N protein as previously described (Talbot et al., 1984a). Dot immunoblotting assays were performed as previously detailed (Talbot et al., 1984b) in a Bio-Dot microfiltration apparatus (Bio-Rad Laboratories, Richmond, Calif.). An excess of viral antigen (as determined by enzyme immunoassay) was bound to nitrocellulose and reacted with six serial 5-fold dilutions of each MAb culture fluid. Bound immunoglobulin was detected by incubation of the 96 well nitrocellulose replica in a 150 mm Petri dish with  $^{125}\text{I}$ -labeled, affinity-purified goat antibody to mouse immunoglobulin (H + L chain specificity). After autoradiography, radioactivity bound on each dot was quantitated by gamma counting, and comparisons were made in the linear portion of the antibody binding curves. As with enzyme immunoassays, a positive reaction was scored as greater than twice background binding, a plus/minus reaction as twice background binding and a negative reaction as less than twice background binding.

## Results

### *Antigenic relationships among murine coronaviruses*

Indirect immunofluorescence on acetone-fixed cells was initially used to screen hybridoma antibodies and proved to be a helpful tool to delineate antigenic relationships among MHV strains (Fig. 1). Intense fluorescence staining was seen in multinucleated giant cells infected by strains of MHV in which a particular antigenic site was conserved. In this example, the variation of the neutralization sites, previously assigned to the E2 glycoprotein (Talbot et al., 1984a), is shown for three MHV strains. Epitope A(E2), recognized by MAb 5B19.2 was conserved among these and all other MHV strains tested. In contrast, epitope B(E2), recognized by MAb 5A13.5, was unique to MHV-4. Epitope C(E2) recognized by MAb 4B11.6 was observed on MHV-4 and MHV-3 but not on the other strains tested. Staining of control uninfected cells with these MAb was always at background negative levels.

A second antigen-binding assay was developed to confirm antigenic relationships initially determined by indirect immunofluorescence. We have previously used the dot blotting assay to analyze detergent sensitivities of different viral epitopes (Talbot et al., 1984b). In the present study, this assay was modified into a solid-phase radioimmunoassay using nitrocellulose as an efficient antigen-binding matrix. The

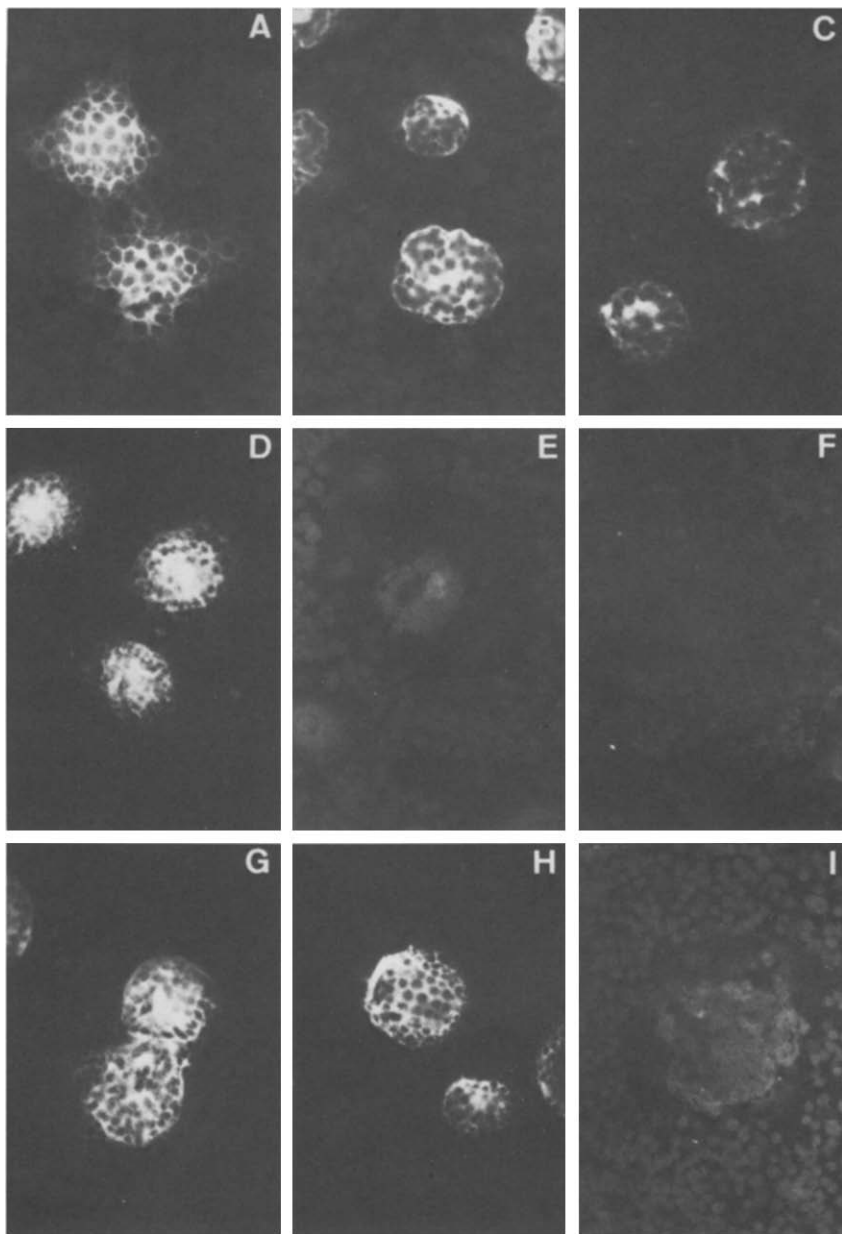


Fig. 1. Antigenic variation of neutralization sites on MHV E2 glycoproteins. Monolayers of L-2.4 cells were infected with MHV-4 (panels A, D, G), MHV-3 (panels B, E, H) or MHV-A59 (panels C, F, I) at a MOI of 0.01–0.02 for 16 h. Acetone-fixed cells were stained with undiluted hybridoma culture fluids of MAb 5B19.2 (panels A–C), 5A13.5 (panels D–F) or 4B11.6 (panels G–I), which are directed against the three neutralization sites A, B and C(E2), respectively. Fluorescence staining of syncytia induced by the three MHV strains is evident with MAb 5B19.2 whereas MAb 5A13.5 only stained MHV-4 infected cells and MAb 4B11.6 only stained MHV-4 and MHV-3 infected cells. Magnification is 250 $\times$ .

assay was very sensitive and quantitative (Jahn et al., 1984; Talbot et al., 1984b), and also provided a visual representation of results. Membrane-enriched fractions from virus infected cells were used as a source of viral antigens. Indirect immunofluorescence showed extensive antigenic conservation of the N protein among all MHV strains tested, hence a pool of five MAb against N was used to standardize the protein concentration of each viral antigen preparation. A quantity of viral antigen

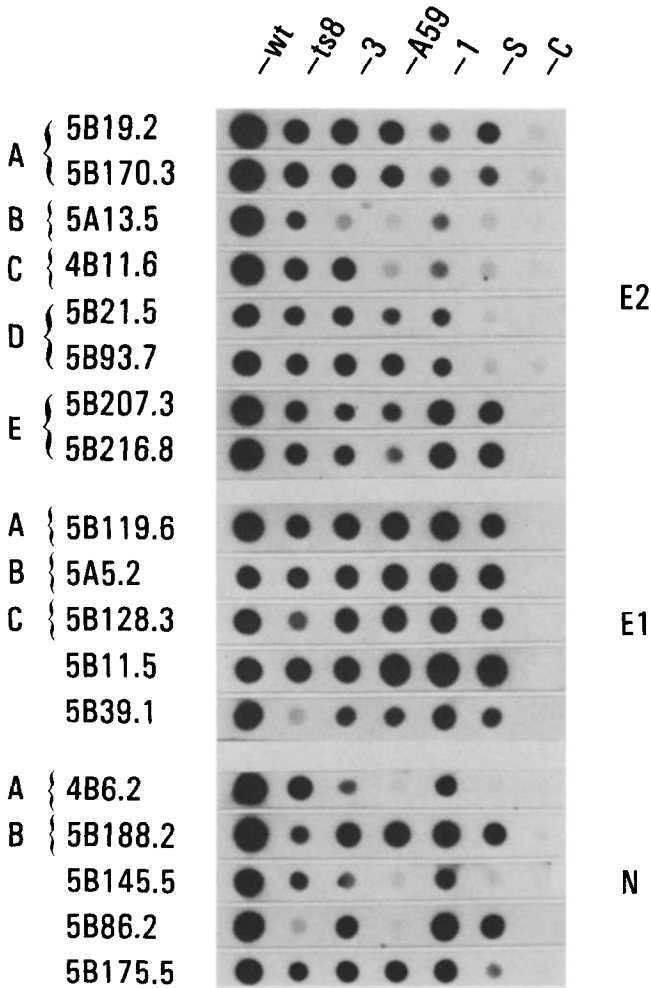


Fig. 2. Antigenic relationships among murine coronaviruses determined by dot immunoblotting. An excess of viral antigen was applied to nitrocellulose paper as dots and labeled with MAb culture fluids (dilutions 1:5-1:125). Antigen preparations from control uninfected cells (C) or cells infected with either of six MHV strains were used, which were MHV-4 (*wt* and *ts8*), MHV-3 (3), MHV-A59 (A59), MHV-1 (1) or MHV-S (S). The polypeptide specificity of the MAb is indicated on the right and the hybridoma designation, as well as the epitope specificity are on the left. Binding of each MAb to viral antigen was detected with radiolabeled goat anti-mouse immunoglobulin.



determined by enzyme immunoassay to be in excess of that required for maximal antibody binding was applied on the nitrocellulose solid support. Serial dilutions of MAb culture fluids were then reacted and detected with a radiolabeled second antibody. After autoradiography, each dot was cut out and counted for  $^{125}\text{I}$  radioactivity. Comparisons between antibody binding on viral antigens from different MHV strains were established in the linear portion of the binding curves and the results obtained are shown in Fig. 2. Binding of each MAb to control antigen dots was negligible (400–500 cpm), whereas specific binding on viral antigen dots reached levels of up to 13 000 cpm. Background binding on MHV-1 was often higher than on the other MHV strains (Fig. 2, MAb 5A13.5 and 4B11.6) and this background binding was not diluted out with decreasing concentration of antibody. In agreement with the results of Fleming et al. (1983), extensive conservation of the E1 glycoprotein was evident and similar levels of antibody binding were seen on each MHV strain, with the exception of the *ts8* mutant of MHV-4. Antigenic variation was observed in the N protein, particularly with MHV-A59 and MHV-S. However, the most extensive polymorphism was seen on the E2 glycoprotein. As was shown by indirect immunofluorescence, site A(E2) was conserved among all MHV strains tested, whereas site B(E2) was specific for MHV-4 and site C(E2) was specific for MHV-4 and MHV-3. Four non-neutralizing MAb directed against at least one antigenic site provisionally designated D(E2) (Talbot et al., 1984a) gave two distinct binding patterns. MAb 5B21.5 and 5B93.7 reacted with all strains tested except MHV-S, whereas MAb 5B207.3 and 5B216.8 reacted with all of the strains we tested. Thus, these two pairs of antibodies likely recognize at least two different structures on the E2 glycoprotein, which we have redefined D(E2) and E(E2), respectively.

In order to confirm the antigenic polymorphism observed on the E2 glycoprotein, we obtained a panel of MAb against E2 generated independently by Fleming et al. (1983), and assayed these antibodies for strain-specific binding in the dot immunoblotting assay described above. Results of such an experiment are shown in Fig. 3. Polymorphism similar in extent to that described above was also observed with these antibodies. Moreover, similarities in binding patterns emerged between these two panels of MAb. MAb J.1.2, J.2.2 and J.7.2 were similar to our neutralizing MAb 4B11.6 and J.7.18 was similar to our 5A13.5. All of these antibodies, except J.1.2 neutralized virus *in vitro* (Fleming et al., 1983; Collins et al., 1982). Non-neutralizing antibodies J.7.1 and J.1.16 showed a similar binding pattern to that of antibodies against nonneutralizing site E (5B207.3 and 5B216.8). The binding pattern of antibodies to antigenic site D was not observed with the Fleming panel of antibodies. Conversely, the specificity patterns of antibodies J.2.5 and J.2.6, as well as that of J.7.5 and J.7.6 were unique to the panel obtained from Fleming.

A third antigen-binding assay was also used to delineate antigenic relationships. An enzyme immunoassay (EIA) which we have previously described (Talbot et al., 1984a) was applied to the present study. We found EIA to be less sensitive than either the immunofluorescence or dot blotting assays when MAb culture fluids were used. Thus, it was necessary to concentrate these fluids to be used in EIA. Nevertheless, only ten of eighteen MAb could reliably be used in the assay. The cumulative results of the EIA are combined with results of the two other antigen-

binding assays and presented in Table 1. In dot immunoblotting assays and EIA, a positive reaction was scored as greater than twice background binding. A plus or minus reaction corresponded to approximately twice background binding and a negative reaction to less than twice background binding. The results of all three antigen binding assays generally agreed, with a few exceptions. For example, EIA results were sometimes negative but dot blotting and immunofluorescence results scored as positive, such as MAb 5B19.2 and 5B170.3 with MHV-1 and S, MAb 5B119.6 and 5B11.5 for MHV-1 and MAb 5B188.2 for MHV-4 *ts8*. Similarly, dot blotting results could be negative while immunofluorescence was positive, such as MAb 5B39.1 and 5B175.5 for MHV-4 *ts8*, MAb 5B145.5 for MHV-A59 and S and MAb 4B6.2 for MHV-A59. In the latter case, EIA results were also positive. Such discrepancies likely result from differences in assay sensitivity and/or antigen stability.

It is apparent from Table 1 that nearly complete conservation of E1 glycoprotein antigens exists among the six MHV strains tested. Some variation was shown on the N protein in agreement with Fleming et al. (1983). Antigenic site A(N) is apparently lost on MHV-S and a distinct determinant recognized by MAb 5B175.5 is lost on MHV-A59. Extensive antigenic variation is evident on the E2 glycoprotein. The three different antigen binding assays confirm conservation of sites A and E(E2) but strain variation was observed in sites B, C and D(E2). Site B(E2) was only found on the neurotropic strain MHV-4, whereas site C(E2) was present on the two virulent

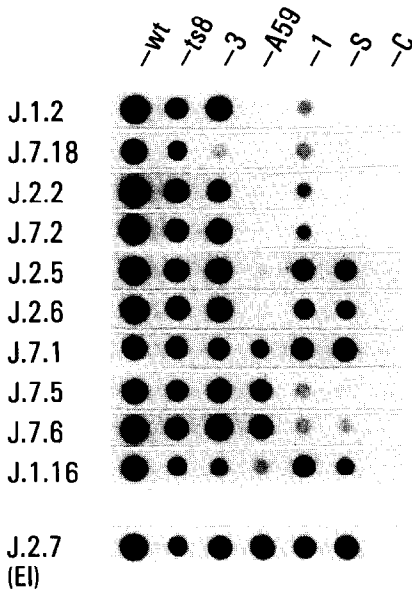


Fig. 3. Antigenic variation of the E2 glycoprotein confirmed with a separately generated panel of MAb (Fleming et al., 1983). The experiment was performed as described in the legend to Fig. 2. The hybridoma designation is on the left. All these MAb were specific to E2, except J.2.7 which is directed to E1.

TABLE 1

## SUMMARY OF ANTIGEN BINDING ASSAYS WITH MONOCLONAL ANTIBODIES TO MHV-4

Hybridoma	Epitope	MHV Strain											
		wt		ts8		3		A59		1		S	
		1	2	3	1	2	3	1	2	3	1	2	3
5B19.2	A(E2)	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●
5B170.3	A(E2)	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●
5A13.5	B(E2)	●●●	●●●	○●○	○●○	○●○	○●○	○●○	○●○	○●○	○●○	○●○	○●○
4B11.6	C(E2)	●●●	●●●	●●●	●●●	○●○	○●○	○●○	○●○	○●○	○●○	○●○	○●○
5B21.5	D(E2)	●●	●●	●●	●●	●●	●●	●●	●●	○●	○●	○●	○●
5B93.7	D(E2)	●●	●●	●●	●●	●●	●●	●●	●●	○●	○●	○●	○●
5B207.3	E(E2)	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●
5B216.8	E(E2)	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●
5B119.6	A(E1)	●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●	○●○	○●○	○●○	○●○
5A5.2	B(E1)	●●	●●	●●	●●	●●	●●	●●	●●	●●	●●	●●	●●
5B128.3	C(E1)	●●	●●	●●	●●	●●	●●	●●	●●	●●	●●	●●	●●
5B11.5		●●●	●●●	●●●	●●●	●●●	●●●	●●●	●●●	○●○	○●○	○●○	○●○
5B39.1		●●	○●	○●	○●	○●	○●	○●	○●	○●	○●	○●	○●
4B6.2	A(N)	●●●	●●●	○●○	○●○	○●○	○●○	○●○	○●○	○●○	○●○	○●○	○●○
5B188.2	B(N)	●●●	○●○	○●○	○●○	○●○	○●○	○●○	○●○	○●○	○●○	○●○	○●○
5B145.5		●●	●●	○●	○●	○●	○●	○●	○●	○●	○●	○●	○●
5B86.2		●●	○●	○●	○●	○●	○●	○●	○●	○●	○●	○●	○●
5B175.5		●●	○●	○●	○●	○●	○●	○●	○●	○●	○●	○●	○●

● : +    ○ : ±    ○ : -  
 1 : immunofluorescence    2 : dot immunoblotting  
 3 : enzyme immunoassay

strains MHV-4 and -3 but lost on the weakly pathogenic MHV strains A59, 1 and S. Finally, site D(E2) was found on all MHV strains tested, with the exception of MHV-S.

## Discussion

28 monoclonal antibodies against the JHM strain of murine hepatitis virus (MHV-4) were used in three different antigen binding assays to delineate antigenic relationships among the structural proteins of six MHV strains. In agreement with studies of Fleming and colleagues (Fleming et al., 1983), we found extensive conservation of antigenic determinants on the internal viral protein N, and particularly on the transmembrane glycoprotein, E1. Using eighteen MAb against the peplomer glycoprotein, E2 and raised in two laboratories, we found significant antigenic polymorphism on this glycoprotein. In contrast to the report of Fleming and colleagues (Fleming et al., 1983), we found that antigenic variation of the E2 glycoprotein was a better correlate of pathogenicity of a given virus strain than was variation in the N protein. Variation on E2 has been correlated with previously mapped antigenic determinants (Talbot et al., 1984a). Neutralizing antibodies 5A13.5 and 4B11.6 are directed against two topographically distinct antigenic determinants designated B and C(E2), respectively (Talbot et al., 1984a). In separate studies, detection of these two determinants was found to be dependent on the native conformation of the molecule (Talbot et al., 1984b). In the present study, we found minimal conservation of these two antigenic sites in the MHV strains tested: site

B(E2) was only recognized on the MHV-4 strain, which is known to be strongly neurotropic (Siddell et al., 1983; Wege et al., 1982) and site C(E2) was preserved on the two virulent strains MHV-4 and MHV-3 but absent on the weakly pathogenic strains MHV-A59, MHV-1 and MHV-S.

A third neutralization site on the MHV-4 E2 glycoprotein, designated A(E2), was conserved among all MHV strains tested. Separate studies have shown that this site is stable to sodium dodecyl sulfate (SDS) denaturation suggesting that it may consist of a primary sequence of amino acids (Talbot et al., 1984b) and that the frequency of mutation at this site reflected as antibody resistance is very low (data not shown). Conservation of this determinant and its resistance to antibody selection may indicate a structure that is essential for productive infection. Conservation of site A(E2) may account for the reported cross-neutralization between different MHV strains (Childs et al., 1983; Hierholzer et al., 1979; Taguchi et al., 1982; Wege et al., 1981). In other studies (Talbot et al., 1985) we have shown that mice infected with MHV-4, A-59, MHV-1 and MHV-S all respond by producing antibody to site A(E2). Finally, non-neutralizing sites D and E(E2), which are also stable to SDS denaturation (Talbot et al., 1984b), are extensively conserved among MHV strains, with the exception of MHV-S which has lost site D(E2).

In contrast to the small plaque variant of MHV-4 generated by Stohlman and colleagues (Stohlman et al., 1982), the *ts8* mutant generated by Haspel and colleagues (Haspel et al., 1978) appears to be antigenically indistinguishable from the wild-type MHV-4 strain. In our study using three different antigen binding assays, we assumed that a positive reaction in any one of the assays was an indication of antigenic conservation and that only in those instances where a negative reaction was obtained in all assays could we conclude a loss of antigenic determinants. In several instances, the importance of using more than one assay is stressed since often a positive reaction was found in only one or two assays.

Our studies use two independently derived panels totaling 28 MAbs against the structural proteins of MHV-4 to analyze antigenic relationships among murine hepatitis viruses. In part these studies confirm and extend the studies of Fleming et al. (1983) which showed extensive conservation of antigenic determinants on MHV E1 and N proteins and minimal conservation of epitopes on the E2 glycoprotein. This report differs from the previously reported studies in that we found a better correlation between antigenic variation, organ tropism and virulence at the level of the E2 glycoprotein rather than the previously reported correlation in N. This may reflect the larger number of E2 antibodies employed in the present study. We have also confirmed the identity of each MHV as a separate strain with a distinctive mosaic of antigenic markers. More detailed structural studies on E2, its functional properties, and interaction(s) with cellular receptors will be helpful to gain a better understanding of the molecular basis for coronavirus tropism and virulence.

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