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Characterization of the Structural Proteins of the Murine Coronavirus Strain A59 Using Monoclonal Antibodies (42532)

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Abstract. Monoclonal antibodies reacting with the A59 strain of mouse hepatitis virus (MHV-A59) were characterized and those specific to the E2 major envelope glycoprotein were studied in detail. Antibodies were tested for their ability to neutralize viral infectivity (N^+ characteristic) and prevent viral-induced cell-to-cell fusion (F^+ characteristic). All four possible combinations of activities reflecting E2 functions were found, i.e., N^+F^+, N^-F^-, N^+F^-, and N^-F^+. In addition, competitive binding studies with these monoclonal antibodies revealed two nonoverlapping antigenic regions. The first region, designated A, was recognized by antibodies which included each of the four functional types. Region B was identified by a single monoclonal antibody with N^-F^- activities. The existence of antibodies which only neutralize virus or only block viral-induced fusion implies that the structures on E2 which serve as targets for neutralization and which induce fusion are not identical. The critical determinants for neutralization and fusion must be closely related topographically on E2 since both N^+F^- and N^-F^+ antibodies recognize the same antigenic region.

Mouse hepatitis viruses (MHV) are members of the *coronaviridae,* a family of enveloped positive-stranded RNA viruses responsible for a variety of acute and chronic diseases in animals and man. MHV strains exhibit a marked organ tropism and are generally classified as either hepatotropic or neurotropic. The A59 strain of MHV (MHV-A59) is considered to be primarily hepatotropic; however, central nervous system (CNS) disease with little or no liver involvement can be induced in susceptible mice by intracranial (6, 18) and intranasal (16) inoculation. MHV-A59 induced CNS infections present as acute panencephalitis, followed in surviving mice by chronic demyelination. In this respect, the pathogenesis of MHV-A59 is similar to that of another neurotropic murine coronavirus, MHV-JHM, which is currently being studied by several laboratories as an animal model of human demyelinating diseases (1, 22, 24, 31).

MHV-A59 is currently the best characterized of the MHV strains with respect to its structural features, and serves as a general model for the description of coronavirus structural proteins (21, 25). Three structural proteins have been identified, including a 50,000-Da phosphorylated nucleocapsid protein which is associated with the genomic RNA. The virion envelope contains two glycoproteins. The E1 glycoprotein, 23,000 Da, probably serves as a matrix protein while the E2 glycoprotein, 180,000 Da, forms the characteristic projections on the external surface of the virion. Many of the biological properties of MHV, including the attachment to cells, the production of virus-induced cell fusion, and the induction of neutralizing antibody have been localized to the E2 protein, based on the ability of monoclonal antibodies specific to the neurotropic JHMV strain to inhibit these activities (3). Using monoclonal antibodies in competitive binding studies, Wege *et al.* (30) have described six distinct antigenic regions on E2. Their data indicate that two of these antigenic regions were associated with the tested biological activities of the antibodies, including *in vitro* neutralization and inhibition of virus-induced cell fusion and *in vivo* protection from lethal virus infection. They further indicate that one region was defined by antibodies which possessed all three activities, while the second region was defined by anti-

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bodies only able to neutralize virus *in vitro*. Similar results were reported by Talbot *et al.*, who were also investigating the structural basis of MHV-JHM E2 functions using monoclonal antibodies (28).

In the present report, we provide a detailed description of monoclonal antibodies to the hepatropic A59 strain of MHV and confirm that monoclonal antibodies specific to the E2 glycoprotein neutralize virus and prevent cell fusion. For the first time, data are presented to indicate that MHV-specific monoclonal antibodies capable of inhibiting cell fusion (F^+) can be differentiated functionally from monoclonal antibodies which neutralize virus (N^+). This indicates that the E2 sites responsible for the induction of cell fusion must be distinct from those inducing neutralizing antibody. Competitive binding studies further demonstrate that these antibodies define a single heterogeneous antigenic region on the MHV-A59 glycoprotein E2.

Materials and Methods. *Viruses and cells.* The derivation and propagation of MHV strains A59 (MHV-A59), JHM-DL, JHM-DS, MHV-1, MHV-2, MHV-3, MHV-D, MHV-K, MHV-M, and MHV-Nuu and the New Jersey strain of vesicular stomatitis virus (VSV) have been previously described (7). Human coronavirus strain OC43 (HCV-OC43) was obtained from Dr. M. Cooney (University of Washington, Seattle, WA) and was propagated in a human rectal tumor cell line (HRT) obtained from Dr. D. Brian (University of Tennessee, Knoxville, TN). HCV strain 229E (HCV-229E) and the L132 cells in which it is propogated were both obtained from Dr. C. M. Johnson-Lussenburg (University of Ottawa, Ontario, Canada).

Preparation of monoclonal antibodies. Monoclonal antibodies to MHV-A59 were prepared as previously described (7). Briefly, immune spleen cells were obtained from C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) which had been inoculated ip with approximately 10^5 PFU serum-free MHV-A59 grown under serum-free conditions, followed by a secondary iv immunization 6 weeks later. Spleen cells were fused in serum-free medium with M5 cells, a horse serum-adapted cell line of SP2/0-Ag 14. The cell suspension was cultured in Dulbecco's minimum essential medium (DMEM) containing 1×10^{-4} *M* hypoxanthine, 4×10^{-7} *M* aminopterin, and 10^{-5} *M* thymidine (HAT (17)) in 96-well plates at 10^5 cells per well, supplemented with 10^5 feeder cells per well prepared from the spleens of nonimmune C57BL/6J mice. Within 2 weeks, wells were screened for the presence of antibody by enzyme-linked immunosorbent assay (screening ELISA; see below). Cells in positive wells were cloned by limiting dilution and subsequently expanded and reassayed.

Screening ELISA procedure. Solid-phase enzyme-linked immunosorbent assay (ELISA) procedures were used to screen the hybridoma supernatants for the presence of antiviral antibody and to identify monoclonal antibody immunoglobulin isotypes. A modification of procedures reported by Suter *et al.* (27) was employed as previously described (8) using virus-coated 96-well plates (Immulon II, Dynatech, Alexandria, VA) and staphylococcus protein A horseradish peroxidase (SPA-HRP; Zymed, San Francisco, CA). Postcoating and wash buffer (ELISA media) consisted of phosphate-buffered saline (PBS), pH 7.2, containing 0.2% Tween 20 and 0.1% bovine serum albumin (BSA, Fraction V, Sigma Chemical Co., St. Louis, MO). Isotyping was accomplished using monospecific antibodies available from American Qualex Inc. (La-Mirada, CA).

Radioimmunoprecipitation. Radioimmunoprecipitation (RIP) procedures, used for the determination of the specificity of the monoclonal antibodies for viral proteins, were carried out as described (7). Briefly, clarified lysates of [^{35}S]methionine-labeled, MHV-A59-infected DBT cells were used as antigen. Following incubation with the antibody, the proteins were precipitated using *Staphylococcus aureus* cowan strain I followed by electrophoretic analysis on 6–15% linear polyacrylamide gels as previously described (22).

Radioimmunoassays. The antigenic relationships among coronavirus strains were evaluated by solid-phase radioimmunoassay (RIA) using viral antigen and radioiodinated staphylococcal protein A (^{125}I-SPA) as previously described (7). Counts per minute (cpm) bound to homologous virus (MHV-A59) were compared with counts per minute bound to

the heterologous viruses. Data are presented according to the convention established by Gerhard *et al.* (9) as modified by Fleming *et al.* (7). The mean counts per minute bound to homologous virus was considered to represent 100% binding. Monoclonal antibody binding greater than 50% of this value was considered strongly positive; 25–50%, moderately positive; less than 25% but greater than twice background, weakly positive; and less than twice background, negative. Background binding was determined using a monoclonal antibody (7-16.17) specific for the murine histocompatibility determinant H-21^{P} (10). Block diagrams illustrating the binding patterns of each monoclonal antibody represented three to six individual assays, each employing triplicate samples.

Neutralization and fusion inhibition assays. The ability of individual monoclonal antibodies to neutralize MHV-A59 virus was determined by a microneutralization assay in which serial dilutions of antibody were added to the wells of a 96-well microtiter plate (Linbro; Flow Labs, McLean, VA) containing virus diluted to 20 $TCID_{50}$ (50% tissue culture infectious doses) (2). After 1 hr incubation at 37°C, DBT cells were added to each well and incubated for an additional 24 hr. For complement-dependent neutralization, guinea pig complement (Miles Laboratories, Elkhart, IN: Lot No. 0054) was added to wells containing virus and antibody and incubated for 1 hr prior to the addition of DBT cells. Complement was not toxic to the cells or to the virus at dilutions greater than 1:2 of the final volume in the wells. Antibodies with a neutralizing titer greater than 1:10 were considered positive for neutralization. Neutralization titer was expressed as the highest dilution of antibody which prevented cytopathic effects (CPE) in 50% of the wells.

To determine whether individual monoclonal antibodies inhibited virus-induced cell fusion, confluent monolayers of DBT cells in 96-well plates were infected with MHV-A59 virus (1×10^6 PFU/ml), used undiluted for a multiplicity of infection greater than 5. After 1 hr incubation at 37°C, supernatants containing virus were gently aspirated and replaced with DMEM for an additional 2 hr. The cells were then washed two times with DMEM before the addition of monoclonal antibodies (20 μl/well), used as undiluted tissue culture supernatants. Wells were examined for the presence of fused cells at 24 hr post-infection and scored positive (F^+) for fusion prevention if fused cells were not observed and negative (F^-) if fused cells were observed.

Competitive binding. Competitive ELISAs were performed using biotinylated and unlabeled competitor monoclonal antibodies prepared as described (8). Briefly, immunoglobulins were isolated from serum-free hybridoma supernatants by affinity chromatography using protein A Sepharose (Pharmacia, Piscataway, NJ) as described by Ey *et al.* (5) and adjusted to 1 mg protein/ml, determined by the Bradford method (Bio-Rad, Richmond, CA). Half of each purified monoclonal was then stored at −70°C for use as the unlabeled competitor, while the other half was biotinylated using biotin-*N*-hydroxysuccimide ester (Calbiochem–Behring, La Jolla, CA). In addition to the anti-E2 monoclonal antibodies, one anti-N and one anti-E1 monoclonal antibody were purified and biotinylated to serve as controls. One monoclonal anti-E2 antibody (A.2.1) was not biotinylated due to the loss of the cell line secreting it. Therefore, A.2.1 was used in competitive binding assays as a tissue culture supernatant.

Topographical relationships among the E2 binding sites recognized by the anti-E2 monoclonal antibodies were determined by competitive ELISA as previously described (8). Varying concentrations of unlabeled competitor antibody (10–10,000 ng/well) were added to virus-coated wells in a 96-well plate and incubated at room temperature for 2 hr. Without washing, 25 μl of the biotinylated antibody (10–1,000 ng/well) was added for a second 2-hr incubation. Plates were then washed six times with ELISA media, after which 100 μl streptavidin–horseradish peroxidase conjugate (Bethesda Research Labs, No. 9534SA) was added at a dilution of 1:1000 for 30 min. The plates were again washed six times, 150 μl *O*-phenylenediamine (OPD; Sigma Chemical Co.) peroxide substrate solution was added, and color was allowed to develop for 30 min prior to termination of the reaction.

Each individual unlabeled monoclonal antibody was first evaluated for its ability to in-

hibit the binding of the biotinylated preparation (homologous competition) to provide a standard of comparison for determining its ability to compete with the remaining anti-E2 monoclonal panel. Percentage competition was determined by the following formula:

$$\frac{OD_{\text{no competitor}} - OD_{\text{competitor}}}{OD_{\text{no competitor}}} \times 100 = \text{percentage competition.}$$

Scoring of the competitive interactions was accomplished according to the following criteria: (i) strong positive: interactions occurring with competition greater than 50% over two or greater $\log_{10}$ dilutions of the unlabeled competitor, (ii) moderately positive: interactions occurring with competition greater than 50% only at the highest concentration (10,000 ng) of unlabeled competitor, and (iii) negative: interactions occurring with competition less than 50% at the highest concentration of unlabeled competitor.

Results. *Characterization of monoclonal antibodies to MHV-A59.* Nineteen monoclonal antibodies were obtained from three separate fusions in which the supernatants showed binding to MHV-A59 by ELISA. Following cloning by limiting dilution, the specificity of each antibody was identified by immunoprecipitation of radiolabeled infected cell lysates, followed by analysis on 6–15% polyacrylamide gels. Figure 1 presents a representative analysis of 9 of the 19 monoclonal antibodies. Seven monoclonal antibodies precipitated the major envelope glycoprotein (E2), another 7 reacted with the nucleocapsid protein (N), and the remaining 5 precipitated the minor envelope glycoprotein (E1) (see also Table I).

Analysis of antigenic relatedness among coronavirus strains. Evaluation of the reactivity of monoclonal antibodies against serologically distinct viral strains is not only useful in studying the antigenic relatedness, but also in defining antigenic regions on viral structural proteins (7, 9). The MHV-A59 monoclonal antibodies were tested for binding to a panel of murine and human coronaviruses (Fig. 2). Although similarities in binding patterns

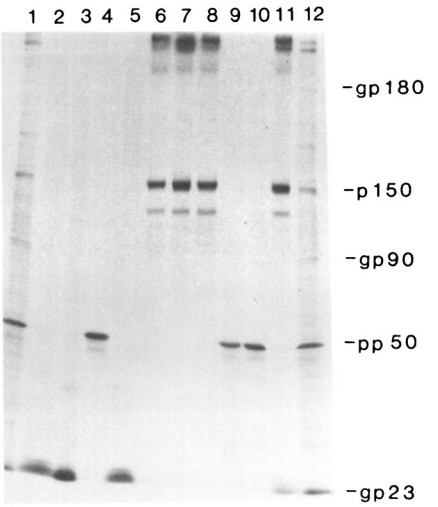

FIG. 1. Immunoprecipitation of MHV-A59 viral proteins from [^{35}S]methionine-labeled infected cell lysates by anti-MHV-A59 monoclonal antibodies. Lanes 1 and 12 represent whole-cell lysates which were not immunoprecipitated and show gp 180, p 150, gp 90 (E2), pp 50 (N), and gp 23 (E1). Lane 11 represents viral proteins precipitated with hyperimmune serum. Lanes 2, 3, and 5 contain immunoprecipitates identifying the anti-E1 specificity of monoclonal antibodies A.1.1, A.1.8, and A.1.11, respectively. Similarly, lanes 4, 9, and 10 identify the anti-N specificity of A.1.10, A.2.6, and A.3.1, respectively. Lanes 6, 7, and 8 identify the anti-E-2 monoclonal antibodies A.1.12, A.2.1, and A.2.3.

across the MHV panel exist between antibodies specific to the three viral structural proteins, Fig. 2 also illustrates the individuality of both the viruses and the antibodies. The E2 molecules recognized by our panel of monoclonal antibodies were markedly conserved among the MHV strains tested, especially MHV-A59, MHV-K, MHV-D, and MHV-2 (Fig. 2A). Less conservation was evident among MHV-3, MHV-Nuu, JHMV (DL and DS), MHV-M, and MHV-1. One monoclonal antibody, A.1.4 (specific for E2 antigenic region B; see below), recognized determinants on the human coronavirus OC43. Three of the monoclonal antibodies, A.1.12, A.2.1, and A.3.10 recognized the same murine coronavirus strains (MHV-A59, MHV-K, MHV-D, and MHV-3), suggesting that they are very similar. They are not, however, identical since minor binding differences exist among them (Fig.

TABLE I. CHARACTERISTICS OF MONOCLONAL ANTIBODIES TO MHV-A59

Protein specificity[a]	Monoclonal antibody	Isotype[b]	Neutralization[c]		Fusion inhibition[d]
			Without C′ (titer)	With C′	
E2	A.1.3	IgG3	–	–	–
	A.1.4	IgG2a	–	–	–
	A.1.9	IgG3	–	–	–
	A.1.12	IgG2b	–	–	–
	A.2.1	IgG2a	+ (1:160)	+ (1:160)	+
	A.2.3	IgG3	+ (1:128)	+ (1:128)	–
	A.3.10	IgG2a	–	–	+
N	A.1.10	IgG2b	–	–	–
	A.2.6	IgG2a	–	–	–
	A.2.16	IgG2b	NT	NT	NT
	A.2.17	IgG2a	NT	NT	NT
	A.3.1	IgG2a	NT	NT	NT
	A.3.7	IgG2a	NT	NT	NT
	A.3.11	IgG2a	NT	NT	NT
E1	A.1.1	IgG3	–	+ (1:80)	NT
	A.1.2	IgG2b	–	+ (1:40)	NT
	A.1.6	IgG2b	–	+ (1:40)	–
	A.1.8	IgG2a	–	+ (1:60)	NT
	A.1.11	IgG2b	–	–	NT

[a] The viral protein specificity of each Mab was determined by immunoprecipitation of [^{35}S]methionine-labeled MHV-A59-infected DBT cell lysates, followed by analysis on 6–15% polyacrylamide gels.

[b] Monoclonal antibody heavy-chain isotypes were identified in an ELISA using monospecific antiisotype reagents.

[c] Monoclonal antibodies were tested for their ability to neutralize MHV-A59 in the presence and absence of guinea pig complement in a microneutralization assay using DBT cells in 96-well plates. Monoclonal antibodies were scored positive for neutralization if CPE was not observed.

[d] Monoclonal antibodies were added to monolayers of MHV-A59-infected DBT cells, which were observed for the presence of fused cells at 24 hr postinfection. Monoclonal antibodies were scored positive for fusion inhibition if fused cells were absent.

2A). In addition, the heavy-chain immunoglobulin isotype of A.1.12 is IgG2b, while that of A.2.1 and A.3.10 is IgG2a (Table I).

Marked antigenic conservation was also observed in the N protein of the coronaviruses tested (Fig. 2B). However, MHV-D, MHV-JHM, MHV-M, and MHV-1 showed less cross-reactivity among the anti-N monoclonal antibodies, suggesting some degree of divergence. One monoclonal antibody (A.2.17) also showed significant binding to HCV-OC43. Four monoclonal antibodies (A.1.10, A.2.17, A.3.7, and A.3.11) showed almost identical binding patterns across the MHV panel, again suggesting that they are very similar.

The anti-E1 monoclonal antibodies identified the highest degree of antigenic variation (Fig. 2C). It is interesting to note that JHM-DL and JHM-DS, which are plaque morphology variants of MHV-JHM, were distinguishable by these anti-E1 monoclonals. Thus, A.1.2 and A.1.8 showed strong binding to JHM-DL, while none of the antibodies recognized JHM-DS. JHM-DS and JHM-DL were previously shown to differ only at N when anti-MHV-JHM-DL monoclonal antibodies were tested for binding to a similar panel of viruses (7). None of the anti-E1 antibodies reacted with either human coronavirus.

Neutralization of virus with anti-A59 monoclonal antibodies. To determine whether the monoclonal antibodies specific to MHV-A59 E2 neutralized virus, as has been reported for anti-E2 MHV-JHM monoclonal antibodies (2, 7), each antibody was mixed with virus for 1 hr prior to the addition of indicator cells. Table I shows that two of the seven anti-E2 monoclonals, A.2.1 and A.2.3, neutralized MHV-A59 (N^+ characteristic: N^+). Monoclonal antibodies A.1.3, A.1.4, A.1.9, A.1.12, and A.3.10 did not neutralize virus (N^- characteristic: N^-). In addition, two anti-N monoclonal

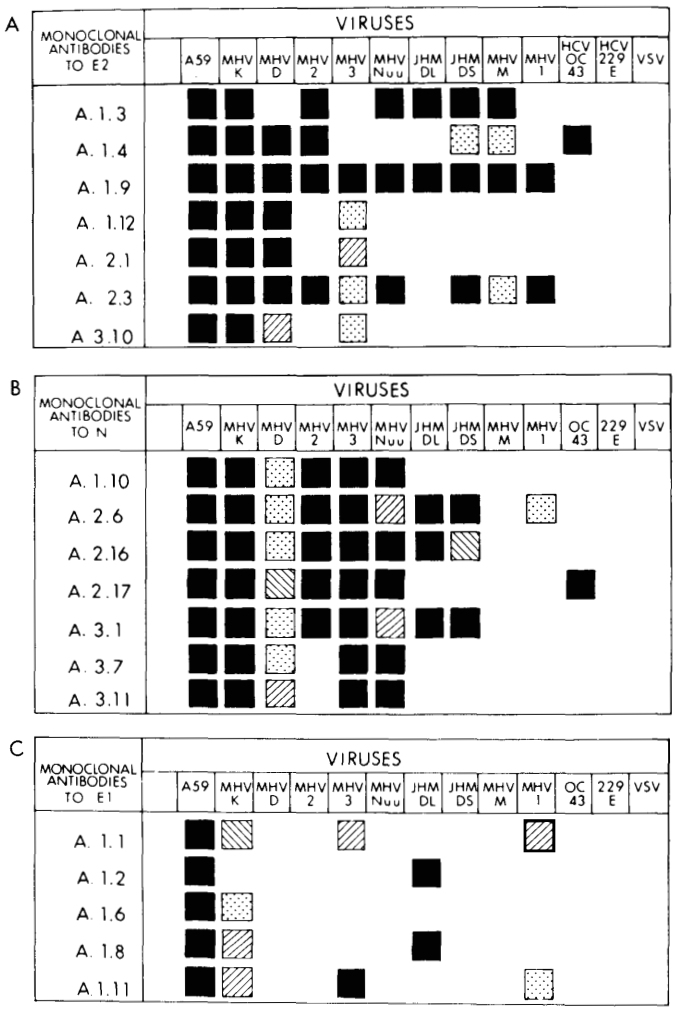

FIG. 2. Antigenic relatedness of the structural proteins of murine and human coronaviruses. Reactivities of anti-MHV-A59 monoclonal antibodies were determined by solid-phase RIA in which binding is expressed as a percentage of mean counts per minute relative to homologous virus (MHV-A59). The blocks represent greater than 50% (■), 25–50% (▨), twice assay background to 25% (□), and less than twice assay background counts per minute (□: no square). All determinations represent an average of at least three assays. (A) Reactivities of anti-E2 MHV-A59 monoclonal antibodies. (B) Reactivities of anti-N monoclonal antibodies. (C) Reactivities of anti-E1 monoclonal antibodies.

antibodies (A.1.10 and A.2.6) showed no neutralization. Four out of five monoclonal antibodies specific for the minor envelope glycoprotein, E1, neutralized MHV-A59 in the presence of complement (Table I). Table I also indicates the immunoglobulin heavy-chain isotype of each monoclonal antibody.

Inhibition of cell fusion with anti-MHV-A59 monoclonal antibodies. The ability of the MHV-A59 anti-E2 monoclonal antibodies to inhibit the fusion of cells infected at a high multiplicity of infection was also evaluated. Table I shows that two monoclonal antibodies, A.2.1 and A.3.10, used as undiluted tissue culture supernatants, completely inhibited cell fusion (F^+ characteristic: F^+). These two monoclonal antibodies can be differentiated by their ability to neutralize virus; i.e., A.2.1 neutralizes MHV-A59 (N^+F^+), while A.3.10 does not (N^-F^+). These differences are not likely to be due to relative binding since A.2.1 and A.3.10 showed comparable titers by

ELISA and RIA (data not shown). This suggests that the sites on E2 responsible for inducing neutralizing antibody and for mediating cell fusion represent separate antigenic determinants. In addition, the neutralization and fusion inhibition studies allowed us to classify the remaining anti-E2 monoclonal antibodies as N^+F^-(A.2.3) and N^-F^-(A.1.3, A.1.4, A.1.9, and A.1.12).

Competitive ELISAs. To evaluate the topographical relationships of the N^+F^+, N^+F^-, N^-F^+, and N^-F^- determinants, competitive binding studies were conducted. Figure 3 summarizes our results in block diagram form. Homologous competition occurred at 50% over at least two $\log_{10}$ dilutions and was scored as strong positive (represented by the solid squares). Heterologous competitions subdivided E2 into two distinct nonoverlapping antigenic regions, designated A and B. Antigenic region A is clearly defined by monoclonal antibodies A.3.10, A.1.3, and A.1.9. Monoclonal antibodies A.1.3 and A.1.9 showed strong reciprocal competitions, indicating that they recognize similar determinants. A.1.3 competed with A.3.10 only at the highest concentration of unlabeled competitor (10 μg) tested. This distinguishes A.1.3 from A.1.9, which did not show competition with A.3.10, and also suggests that A.1.3 and A.3.10 recognize similar or overlapping determinants.

The inclusion of the E2 determinants recognized by A.2.1, A.1.12, and A.2.3 in region A is less clear because their competitive interactions were not reciprocal. For example, unbiotinylated A.1.12 competed only with A.3.10 and A.2.3, while biotinylated A.1.12 was unable to compete with A.2.1, A.3.10, A.1.3, A.1.9, or A.2.3. This suggests that A.1.12 recognizes determinants shared, at least in part, by A.3.10 and A.2.3. Thus, we have tentatively included A.1.12 and A.2.3 as antibodies defining antigenic region A. It also indicates that biotinylation may have altered A.1.12 binding properties, making it difficult to identify its E2 binding site.

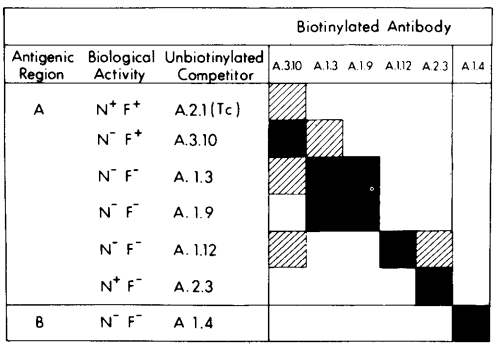

FIG. 3. Identification of antigenic regions by competitive binding. Solid squares represent greater than 50% competition over two or more $\log_{10}$ dilutions of the unlabeled competitor. Lined squares represent 50% competition only at the highest concentration (10,000 ng) of unlabeled competitor. Blank squares represent competition less than 50%. Results represent two to six experiments.

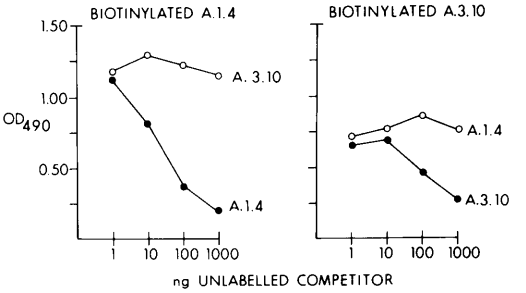

FIG. 4. Competitive binding between anti-E2 monoclonal antibodies defining antigenic regions A (A.3.10) and B (A.1.4). (Left) Competitive binding using biotinylated A.1.4 and unlabeled A.3.10 (Right) Reciprocal experiment in which biotinylated A.3.10 was used against unlabeled A.1.4.

One monoclonal antibody, A.1.4, did not compete with any of the other anti-E2 monoclonal antibodies and as such defines a second, nonoverlapping antigenic region, designated B. Figure 4 shows competitive interactions between A.1.4 and A.3.10, with A.3.10 representing the remainder of the anti-E2 monoclonal antibodies. The data illustrate that these antibodies recognize distinct antigenic sites on E2. None of the anti-E2 monoclonal antibodies defining region A or B competed with control MHV-A59 anti-N (A.1.10) or anti-E1 (A.1.8) monoclonal antibodies.

The biological activities of each of the antibodies defining the topographical relationships of the E2 binding sites are also shown in Fig. 3. The binding data indicate that the E2 determinants responsible for the induction of both neutralization (tentatively defined by A.2.1 and A.2.3) and fusion (defined by A.3.10) activities are associated with antigenic region A. Within region A, the N^-F^+ site (defined by A.3.10) appears to be distinct from

the N^+F^- site (defined by A.2.3) since A.3.10 and A.2.3 did not compete. However, the two sites may be closely related since A.1.12 competes with both A.3.10 and A.2.3. These assays could not distinguish between the N^+F^+ site (A.2.1) and the N^-F^+ (A.3.10) site since A.2.1 competed only with A.3.10. The assignment of A.2.1 to antigenic region A is tentative due to the lack of a biotinylated preparation; however, its competitive interaction with A.3.10 supports the distinction between an independent neutralization site (N^+F^-; A.2.3) and a fusion site (N^-F^+; A.3.10).

Discussion. Nineteen monoclonal antibodies isolated from mice immunized with MHV-A59 were characterized with respect to viral protein specificity, capacity to neutralize virus, and ability to prevent virus-induced cell fusion. In addition, to examine the serological relatedness of coronavirus strains, we tested the binding of the monoclonal antibodies to a group of murine and two human coronaviruses. Our laboratory has previously examined the serological relatedness of coronaviruses using anti-MHV polyclonal antisera (2) and anti-MHV-JHM monoclonal antibodies (7). In general, the current studies confirm that a high degree of serological relatedness exists among the murine coronavirus strains MHV-A59, MHV-K, MHV-D, and MHV-3. This is in agreement with additional studies of the genetic relatedness of the murine coronavirus strains, in which oligonucleotide finger printing showed that genomic RNA of MHV-A59 was very similar to that of MHV-D, MHV-K, and MHV-3 (13, 14). When evaluated on the level of the individual structural proteins, the nucleocapsid proteins of MVH-A59, MHV-K, MHV-D, MHV-2, and MHV-3 were also found to be highly conserved when both MHV-JHM (7) and MHV-A59 anti-N antibodies were tested for binding to the coronavirus panel. However, while MHV-JHM monoclonal antibodies identified a significant degree of conservation in the E1 matrix protein (7), MHV-A59 antibodies identified significant diversity. Only two out of the five MHV-A59 anti-E1 antibodies recognized MHV-JHM; all three MHV-JHM anti-E1 antibodies recognized MHV-A59 (7). In addition, the binding pattern of the MHV-JHM anti-E2 monoclonal antibodies showed that considerable diversity exists in this protein, while MHV-A59 anti-E2 antibodies showed greater conservation. Three out of seven MHV-A59 anti-E2 antibodies recognized MHV-JHM and 3 out of 10 MHV-JHM anti-E2 antibodies bound MHV-A59. The MHV-A59 anti-E2 antibodies were not able to distinguish between MHV-A59, which is weakly pathogenic in mice, and MHV-3, which causes severe bone marrow disease (12). Finally, anti-MHV-A59 monoclonal antibodies were not able to distinguish between the weakly pathogenic MHV-A59 and a virulent strain of MHV-A59, kindly supplied by Dr. K. Holmes (Uniformed Services University, Bethesda, MD) (15). These data indicate that the identifiable degree of diversity among viral strains depends on the specificities of the particular panel of monoclonal antibodies used, and that the degree of specificity may not reflect differences in viral pathogenesis.

Neutralization and fusion inhibition studies divided the MHV-A59 anti-E2 monoclonal antibodies into four functional groups: (i) N^+F^+, (ii) N^-F^+, (iii) N^+F^-, and (iv) N^-F^-. The N^-F^+ characteristic has not previously been described for anti-MHV antibodies. The existence of the N^+F^- and the N^-F^+ characteristics suggests that the E2 determinants which induce fusion and the determinants involved in virus neutralization are not identical. Our competitive binding studies suggest that the two sites are topographically distinct within a larger heterogeneous antigenic region, which we have tentatively designated A. They are indirectly related only by specificities recognized by the N^-F^- monoclonal antibody, A.1.12, which cross-reacted with both N^+ and F^+ specificities. Whether this relationship actually represents a topographical proximity of two distinct sites or that the two sites share nonoverlapping amino acid sequence homology cannot be determined on the basis of our competitive binding data since A.1.12 was only capable of competing in its unbiotinylated form. For MHV-JHM, the antigenic region designated A is similarly heterogeneous and could be separated into two subregions, based on the ability of the monoclonal antibodies to immunoprecipitate different forms of the E2 glycoprotein (30). These two subregions were also not distinguished in competitive interactions. On the basis of preliminary competition assays, it appears that the anti-

genic region we have designated A on MHV-A59 E2 is closely related to site A on MHV-JHM E2, as described by Wege (Wege, personal communication; Fleming and Gilmore, unpublished observations).

Topographically distinct neutralization sites have also been demonstrated for other viral systems, including influenza virus (8), Sindbis virus (30, 31), and VSV (29). It is reasonable to speculate that there is more than neutralization site represented on the MHV-A59 E2 molecule since A.2.1 (N^+F^+) and A.2.3 (N^+F^-) do not show competition. These data suggest that one neutralization site is associated with or overlaps the fusion site, while another is independent of the fusion site. This interpretation, suggesting two distinct sites of neutralization, is in agreement with the data presented for MHV-JHM by Talbot *et al.* (28) and Wege *et al.* (30).

Monoclonal antibodies to MHV-A59 have been previously produced and characterized according to protein specificity, immunoglobulin isotype, and neutralization titer (13). In this report, a unique antibody with the N^-F^+ biological phenotype provided preliminary insight into the topographical relationships between the two previously described epitopes exhibiting the N^+ phenotype. We are presently attempting to determine the topographical relationship of these sites on the two proteolytic products of the E2 molecule which have recently been described (25).

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