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Coronavirus Species Specificity: Murine Coronavirus Binds to a Mouse-Specific Epitope on Its Carcinoembryonic Antigen-Related Receptor Glycoprotein

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Like most coronaviruses, the coronavirus mouse hepatitis virus (MHV) exhibits strong species specificity, causing natural infection only in mice. MHV-A59 virions use as a receptor a 110- to 120-kDa glycoprotein (MHVR) in the carcinoembryonic antigen (CEA) family of glycoproteins (G. S. Dveksler, M. N. Pensiero, C. B. Cardellichio, R. K. Williams, G. S. Jiang, K. V. Holmes, and C. W. Dieffenbach, J. Virol. 65:6881–6891, 1991; and R. K. Williams, G. S. Jiang, and K. V. Holmes, Proc. Natl. Acad. Sci. USA 88:5533–5536, 1991). The role of virus-receptor interactions in determining the species specificity of MHV-A59 was examined by comparing the binding of virus and antireceptor antibodies to cell lines and intestinal brush border membranes (BBM) from many species. Polyclonal antireceptor antiserum (anti-MHVR) raised by immunization of SJL/J mice with BALB/c BBM recognized MHVR specifically in immunoblots of BALB/c BBM but not in BBM from adult SJL/J mice that are resistant to infection with MHV-A59, indicating a major difference in epitopes between MHVR and its SJL/J homolog which does not bind MHV (7). Anti-MHVR bound to plasma membranes of MHV-susceptible murine cell lines but not to membranes of human, cat, dog, monkey, or hamster cell lines. Cell lines from these species were resistant to MHV-A59 infection, and only the murine cell lines tested were susceptible. Pretreatment of murine fibroblasts with anti-MHVR prevented binding of radiolabeled virions to murine cells and prevented virus infection. Solid-phase virus-binding assays and virus overlay protein blot assays showed that MHV-A59 virions bound to MHVR on intestinal BBM from MHV-susceptible mouse strains but not to proteins on intestinal BBM from humans, cats, dogs, pigs, cows, rabbits, rats, cotton rats, or chickens. In immunoblots of BBM from these species, both polyclonal and monoclonal antireceptor antibodies that block MHV-A59 infection of murine cells recognized only the murine CEA-related glycoprotein and not homologous CEA-related glycoproteins of other species. These results suggest that MHV-A59 binds to a mouse-specific epitope of MHVR, and they support the hypothesis that the species specificity of MHV-A59 infection may be due to the specificity of the virus-receptor interaction.

To determine the mechanism of the strong species specificity for infection exhibited by most coronaviruses (49), we have studied the species specificity of binding of the coronavirus mouse hepatitis virus (MHV) and anti-MHVR antibodies to cell lines and membranes from tissues of many different species. MHV causes frequent and widespread infections of feral and laboratory mice (3). Depending in part on the virus strain, MHV infection causes a variety of syndromes, including inapparent enteric and respiratory infection, neonatal enteritis, hepatitis, and acute and chronic demyelinating diseases (9, 49). Susceptibility to MHV differs markedly among inbred strains of mice (1, 17, 40). BALB/c mice are susceptible to MHV, whereas adult SJL/J mice are highly resistant to MHV infection (1, 17, 36). Using virus overlay protein blots, a 110-kDa glycoprotein receptor (MHVR) for MHV-A59 was identified on intestinal brush border membranes (BBM) and hepatocyte plasma membranes from adult BALB/c, but homologous glycoproteins from BBM of adult SJL/J mice failed to bind virus (7, 53). We suggested that the failure of MHV to bind effectively to receptors on intestinal and liver membranes from SJL/J mice may account for the marked resistance of SJL/J mice to MHV infection (7).

N-terminal amino acid sequencing of immunoaffinity-purified MHV receptor glycoprotein (MHVR) from Swiss-Webster mouse liver suggested that the receptor was a member of the carcinoembryonic antigen (CEA) family of glycoproteins in the immunoglobulin superfamily (52, 53) and antibodies to human CEA cross-reacted with affinity-purified MHVR (52). Cloning and sequencing of the MHVR cDNA from BALB/c mouse liver (11) showed that the open reading frame of MHVR was almost identical to that of mmCGM$_1$, a partial cDNA clone of a mouse CEA-related glycoprotein (4), which is a homolog of human biliary glycoprotein (14) and rat ecto-ATPase (19). Although glycoproteins homologous to MHVR were recognized in SJL/J mouse liver and intestine by antibody directed against the amino-terminal peptide of MHVR, these SJL/J glycoproteins did not bind MHV or monoclonal antireceptor antibody (53).

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Thus, the resistance of SJL/J mice to MHV infection appears to be due, at least in part, to absence of a virus-binding epitope on the SJL/J homolog of MHVR (53).

Natural MHV infection appears to occur only in mice. Although antibodies which cross-react with MHV are found in rats and humans, these may be elicited by natural infection of rats with the rat coronavirus RCV or sialodacryoadenitis virus (5, 25) and humans with the human coronavirus HCV-OC43, which are antigenically related to MHV (21). Although rats are much less susceptible to MHV than mice, intracerebral inoculation of rats with MHV-JHM, MHV-3, or MHV-A59 can cause neurological infection. Susceptibility of rats to MHV is age dependent; only young rats are susceptible, and adult rats are highly resistant to MHV infection (37, 50). Thus, like many other coronaviruses, MHV is highly species specific, causing natural infection only in susceptible strains of mice and experimentally infecting only young animals of a closely related species.

To determine whether the species specificity of MHV is determined by the specificity of virus interactions with CEA-related glycoproteins homologous to MHVR, we studied the interactions of MHV-A59 or antireceptor antibodies with membranes isolated from the intestinal BBM of a variety of vertebrate species and with cell cultures from many of these species. Cell lines and BBM from susceptible strains of mice expressed virus-binding and antireceptor antibody-binding activities, whereas cultured cells and BBM from MHV-resistant SJL/J mice or from nonmurine species failed to bind virus or antireceptor antibodies. Thus, susceptibility of cells or animals to MHV appears to depend on expression of a mouse-specific virus-binding epitope on MHVR, a CEA-related glycoprotein that serves as a receptor for MHV.

MATERIALS AND METHODS

Virus and cell propagation. The 17 Cl 1 line of spontaneously transformed BALB/c 3T3 mouse fibroblasts was used for propagation of the A59 strain of MHV (MHV-A59) as previously described (42). For studies on binding of radiolabeled virus, [^{3}H]uridine (20 μCi/ml) was present in the medium from 8 to 24 h after virus inoculation, and virions released into the supernatant medium were purified and concentrated by sucrose density ultracentrifugation as previously described (41) and stored at −70°C in medium containing 10% fetal bovine serum.

Sac$^-$ cells were obtained from W. Spaan (University of Utrecht, Utrecht, The Netherlands); L2 cells were obtained from L. Sturman (New York State Department of Health, Albany, N.Y.); RD cells were obtained from Ortwin Schmitt (University of Oklahoma, Stillwater, Okla.); fwcf-4 cells were obtained from N. Pedersen (University of California, Davis, Calif.); IMR-90 cells were obtained from R. Silverman (Uniformed Services University of the Health Sciences, Bethesda, Md.); A72 cells were obtained from L. Binn (Walter Reed Army Institute for Research, Washington, D.C.); BHK-21, CV-1, and HFF cells were obtained from J. Hay (Uniformed Services University of the Health Sciences, Bethesda, Md.); and J774A-1 and HL-60 cells were obtained from the American Type Culture Collection, Rockville, Md. Peritoneal macrophages were harvested from adult BALB/c and SJL/J mice 5 days after intraperitoneal injection of 3.0 ml of sterile 3% thioglycolate solution. Macrophages were maintained in RPMI medium (GIBCO Laboratories, Grand Island, N.Y.) as previously described (48).

Animals. BALB/c and SJL/J mice were obtained from the National Cancer Institute or Jackson Laboratories (Bar Harbor, Maine). Wistar Furth rats were obtained from Charles River Breeding Laboratories (Wilmington, Mass.). Intestinal tissues from cats, chickens, cotton rats, dogs, pigs, rabbits, and Sprague-Dawley rats were obtained from experimental animals sacrificed by other investigators for other purposes. Small samples of normal human small intestine were obtained from surgical specimens from T. Scott (Uniformed Services University of the Health Sciences, Bethesda, Md.).

Antisera. Goat antibody directed against the S glycoprotein of MHV-A59 was raised by immunization of a goat with MHV-A59 peplomers isolated from detergent-disrupted virions by sucrose density gradient ultracentrifugation (41).

Polyclonal antibody directed against the MHV receptor (anti-MHVR) was obtained by immunization of receptor-negative SJL/J mice with deoxycholate extracts from purified intestinal BBM from receptor-positive BALB/c mice. Mice which produced antireceptor antibodies, as shown in Fig. 1, were given Sarcoma 180 cells by intraperitoneal inoculation to induce the ascites fluid called polyclonal anti-MHVR. Control ascites fluid was obtained in a similar manner from nonimmunized mice.

Monoclonal anti-MHV receptor antibodies were generated by fusion of Sp2/0 cells with splenocytes from SJL/J mice immunized with deoxycholate-extracted BALB/c BBM to produce receptor-specific hybridomas by established methods (33). Monoclonal antibodies (MAbs) were screened by using an enzyme-linked immunoassay on wells coated with MHVR eluted from a 110-kDa band of a preparative polyacrylamide slab gel of BALB/c intestinal BBM. In immunoblots of BALB/c BBM, MAb CC1 (immunoglobulin G1 [IgG1] isotype) bound to the 110- to 120-kDa receptor glycoprotein and to a related 58-kDa glycoprotein (52, 53). Pretreatment of L2 and 17 Cl 1 cells with MAb CC1 protected the cells from MHV-A59 infection (53). Treatment of infant mice with MAb CC1 markedly reduced the yield of virus from the liver, nose, and brain (35). A MAb of the IgG1 isotype directed against an irrelevant antibody (cholera toxin) was used as a control for MAb CC1.

Blocking of virus attachment. L2 cells from spinner cultures in Eagles minimum essential medium without Ca^{2+} or Mg^{2+} and with 5% dialyzed fetal bovine serum were incubated for 2 h at 37°C in polypropylene tubes (5×10^6 cells in 1 ml) on a roller wheel with control medium or serial dilutions of anti-MHVR or control ascites fluid and then were incubated for 90 min at 37°C with 10^4 cpm of [^{3}H]uridine-labeled, gradient-purified MHV-A59 virions (specific activity 4.3×10^4 PFU/cpm). The cells were harvested by filtration onto 0.45-μm-pore-size Millipore filters and washed three times with medium. Radioactivity bound to the cells was determined by counting dried filters in Liquifluor scintillation fluid in a Beckman (Palo Alto, Calif.) liquid scintillation counter. Values represent the averages of triplicate samples. Percent inhibition of virus binding was calculated as $100 \times [1 - (\text{EXPT} - \text{BKGD})/(\text{MAX} - \text{BKGD})]$, where BKGD was the counts per minute on filters with virus alone (typically 500 to 1,000 cpm), MAX was the counts per minute for cells plus virus (typically 3,500 cpm), and EXPT was the counts per minute of samples containing virus and cells with antibody dilutions.

Immunofluorescence. Monolayer cultures of cells grown on glass coverslips were incubated for 1 h at 4°C with anti-MHVR or control antibody, washed three times in phosphate-buffered saline (PBS), then fixed in acetone or 2% paraformaldehyde in calcium- and magnesium-free PBS, and

washed three times in PBS with 2% normal goat serum. For some experiments, cells were fixed for 1 h with 2% paraformaldehyde and washed three times with PBS before incubation with anti-MHVR. Bound antibody was detected with affinity-purified, fluorescein- or rhodamine-labeled rabbit or goat anti-mouse IgG (Organon-Teknika Co., West Chester, Pa.). Cells were mounted in 40% glycerol and examined with a Zeiss photomicroscope III.

Intestinal BBM preparation. Mouse, rat, cotton rat, and rabbit intestines were flushed with ice-cold PBS, snap frozen in liquid nitrogen, and stored at −70°C. Mucosa were scraped from cat, dog, pig, cow, chicken, and human intestines prior to snap freezing. Intestinal BBM were prepared from frozen intestines or intestinal mucosa by the method of Kessler et al. (15). Briefly, intestinal tissue was thawed, homogenized with a Tissumizer (Tekmar Co., Cincinnati, Ohio), and clarified by precipitation with 10 mM $CaCl_2$; and BBM were prepared by differential centrifugation and stored in TE buffer (10 mM Tris HCl [pH 7.4], 1 mM EDTA). Protein concentrations of the BBM preparations were determined by the Bradford method, using bovine serum albumin as a standard (8). To determine the comparability of BBM preparations from different species, we measured the activity of sucrase, an enzyme associated with BBM, by the method of Messer and Dahlqvist (23). All BBM preparations were comparable, releasing 3 to 20 μmol of glucose/ml/min/mg of protein, except cow and neonatal rat BBM which are known to lack sucrase activity (31, 45).

Chorioallantoic membrane preparation. Chorioallantoic membranes were removed from 14-day-old chicken embryos, washed in PBS, swollen in reticulocyte standard buffer (10 mM Tris HCl [pH 7.4], 10 mM NaCl, 5 mM $MgCl_2$) with 1 μg of phenylmethylsulfonyl fluoride per ml, and homogenized. The homogenate was centrifuged at 1,000 × *g* for 5 min to remove large debris, and the resulting supernatant was centrifuged for 2 h at 125,000 × *g*. Membranes in the pellet were resuspended in TE buffer and stored at −70°C.

Solid-phase virus-binding assay. The solid-phase MHVR assay was performed as previously described (7), except that BBM were treated with 5% β-mercaptoethanol to reduce any bound immunoglobulins before being applied to nitrocellulose sheets in a 96-well minifold apparatus (Schleicher & Schuell, Inc., Keene, N.H.). This step was needed because without it, intestinal BBM of several nonmurine species bound staphylococcal protein A (SPA) in the absence of added virus or antibody. The nitrocellulose was blocked with 2% bovine serum albumin, incubated with MHV-A59 in minimum essential medium with 10% fetal bovine serum–10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Sigma Chemical Co., St. Louis, Mo.), washed, incubated with goat antibody directed against the S glycoprotein of MHV-A59 (41), and washed again. Bound antibody was detected by incubation with radioiodinated SPA (^{125}I-SPA; 2 to 10 mCi/mg; New England Nuclear Co., Boston, Mass.) and then by autoradiography. Controls for the specificity of virus binding used conditioned medium from uninfected cells in place of virus or normal goat serum or buffer in place of anti-S antibody.

VOPBA and immunoblotting. The virus overlay protein blot assay (VOPBA) was performed as previously described (7). Briefly, 100 μg of protein from BBM was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (18) and electroblotted onto nitrocellulose sheets which were then blocked with 2% bovine serum albumin. Subsequent steps of the VOPBA were performed as described above for the solid-phase virus-binding assay. For immunoblots, BBM proteins were separated by SDS-PAGE, electroblotted onto nitrocellulose sheets, and incubated with a 1:100 dilution of polyclonal anti-MHVR or approximately 20 ng of MAb CCl per ml, followed by affinity-purified rabbit anti-mouse IgG and then ^{125}I-SPA (46).

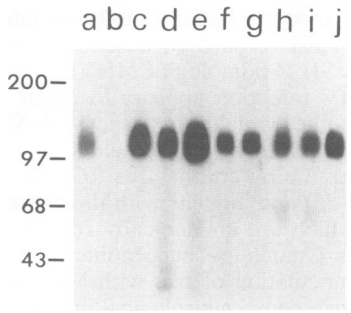

FIG. 1. Immunoreactivity with BALB/c mouse intestinal BBM of polyclonal SJL/J mouse antibody to BALB/c BBM. Strips from a preparative SDS-PAGE gel blotted to nitrocellulose were reacted with virus or antireceptor antibodies. Lane a shows a virus overlay protein blot of BALB/c BBM in which the 100- to 120-kDa CEA-related MHVR glycoprotein was detected by binding of MHV-A59 virions followed by goat anti-MHV and ^{125}I-SPA. Strips in lanes b through i were incubated with a 1:20 dilution of mouse serum; lane b was incubated with preimmunization serum of an SJL/J mouse, and lanes c through i were incubated with sera from different SJL/J mice immunized with a deoxycholate extract of adult BALB/c intestinal BBM. Lane j is an immunoblot with a 1:50 dilution of ascites fluid raised in a mouse immunized with BALB/c BBM as described in the legend to lanes c through i. This was the source of the polyclonal mouse anti-MHVR used in this report.

RESULTS

Antibody directed against the MHV receptor blocks binding of virus to mouse fibroblasts. In order to develop antibody to a putative MHV receptor from BALB/c BBM, we immunized SJL/J mice with extracts of these membranes. The resulting polyclonal antibody, anti-MHVR, recognized the 110- to 120-kDa CEA-related MHVR glycoprotein in immunoprecipitates (data not shown) and immunoblots of BALB/c BBM but not those of SJL/J BBM (Fig. 1). Immunofluorescence experiments showed that anti-MHVR also reacted with paraformaldehyde-fixed BHK-21 cells stably transfected with MHVR1 cDNA and expressing the MHVR glycoprotein (11) but not with control BHK-21 cells (data not shown). Thus, although SJL/J and BALB/c mice are not closely related and differ in major histocompatibility complex antigens, when SJL/J mice were immunized with extracts of BALB/c BBM, an immunodominant antigen was the MHVR glycoprotein. This suggests that the epitope(s) on MHVR recognized by anti-MHVR may not be shared by the homologous SJL/J glycoproteins.

The mouse polyclonal anti-MHVR was directed at least in part against the virus-binding determinant of MHVR, because mouse L2 cells pretreated with serial dilutions up to 1:200 of anti-MHVR were resistant to challenge with infectious MHV-A59. To determine whether this inhibition of virus infection by polyclonal anti-MHVR was due to blocking of virus attachment to the receptor, we pretreated cells with serial dilutions of polyclonal anti-MHVR, normal mouse ascites, or medium and then incubated the cells with

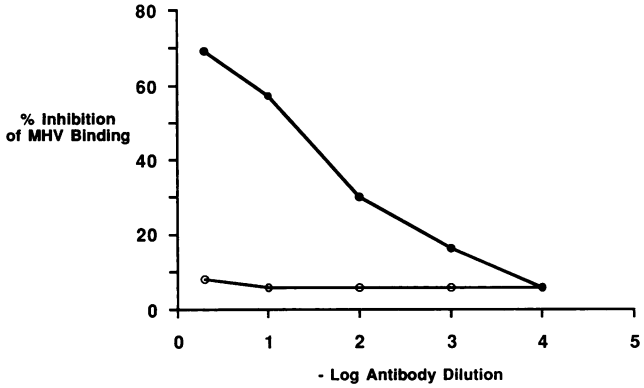

FIG. 2. Inhibition of binding of radiolabeled MHV-A59 virions to mouse fibroblasts by polyclonal antireceptor antibody (anti-MHVR). Spinner cultures of L2 cells were incubated with serial dilutions of mouse polyclonal antireceptor antibody (solid circles) or of ascites from nonimmunized mice (open circles) and then were incubated with [^{3}H]uridine-labeled, gradient-purified MHV-A59 virions. Radioactivity binding to the cells was decreased in a concentration-dependent manner by anti-MHVR.

gradient-purified, radiolabeled MHV-A59 virions. Figure 2 shows that mouse polyclonal anti-MHVR prevented binding of MHV-A59 virions to mouse fibroblasts. Thus, anti-MHVR blocks infection of mouse fibroblasts by blocking the epitope of the MHV receptor glycoprotein which is recognized by the viral S glycoprotein.

The epitope(s) of MHVR recognized by polyclonal anti-MHVR is not expressed on nonmurine cell lines. Because anti-MHVR recognized one or more epitopes on MHVR, including one at or near enough to the virus-binding site to block virus attachment, we used this antibody to test cell lines from different species for expression of homologs of MHVR that might have MHV-binding activity. Indirect immunofluorescence showed that only cell lines of murine origin bound mouse polyclonal anti-MHVR (Fig. 3 and Table 1). Like the BHK line of baby hamster kidney cells (Fig. 3B), all other nonmurine cell lines tested (Table 1) showed no specific immunofluorescence with the mouse polyclonal anti-MHVR. These data indicate that the polyclonal anti-MHVR raised in SJL/J mice was specific for antigens on mouse cells and did not recognize proteins homologous to MHVR on cell lines from other species.

Cells of nonmurine species are resistant to infection with MHV-A59. Mouse, hamster, cat, dog, monkey, and human cell lines were inoculated with MHV-A59 at a multiplicity of inoculation of 1 to 10 PFU per cell and incubated for 1 to 3 days at 37°C. Cultures which showed cytopathic effects such as cell fusion, rounding, and/or cell death were scored as susceptible to MHV (Table 1). All of the murine cell cultures tested except SJL/J macrophages developed cytopathic effects from MHV infection. Cultures which did not exhibit virus-induced cytopathic effects were fixed and examined for development of MHV-specific antigens by indirect immuno-

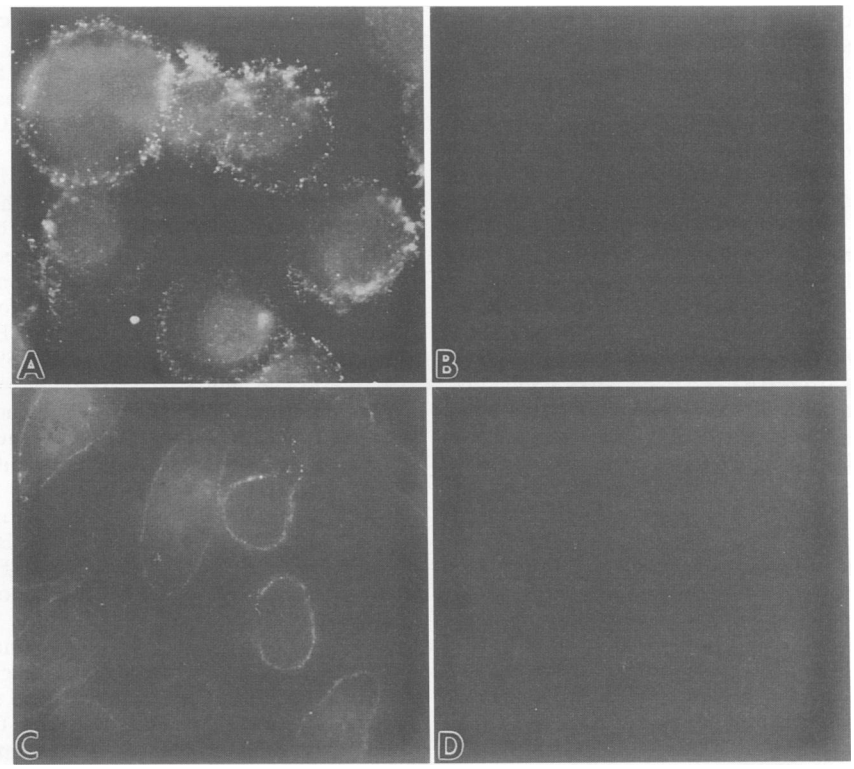

FIG. 3. Expression of the 110-kDa glycoprotein receptor for MHV on the cell surface. Polyclonal mouse anti-MHVR was incubated with an MHV-susceptible murine cell line (L2; panel A), an MHV-resistant hamster cell line (BHK-21; panel B), and primary macrophage cultures from MHV-susceptible BALB/c mice (C) or MHV-resistant SJL/J mice (D). The cell lines (A and B) were fixed with acetone after incubation with antibody, and the macrophages (C and D) were fixed with 2% paraformaldehyde prior to incubation with antibody. Anti-MHVR was visualized with rhodamine-conjugated goat anti-mouse IgG.

TABLE 1. Expression of the 110-kDa protein on cell lines from different species[a]

Species	Cell line or type	Tissue and strain	Susceptibility to MHV-A59[b]	Presence of 110-kDa protein
Mouse	L2	C3H fibroblast	+	+
	Sac$^-$	STU fibroblast	+	+
	17 Cl 1	BALB/c fibroblast	+	+
	J774A-1	BALB/c monocyte/macrophage	+	+
	JLSV9	BALB/c bone marrow fibroblast	+	+
	PM[c]	BALB/c macrophage	+	+
	PM	SJL/J macrophage	−	−
Hamster	BHK-21	Kidney fibroblast	−	−
Cat	fwcf-4	Fetal tissue	−	−
Dog	A72	Tumor tissue	−	−
Monkey	CV-1	Kidney fibroblast	−	−
Human	HFF	Foreskin fibroblast	−	−
	RD	Rhabdomyosarcoma	−	−
	IMR-90	Lung fibroblast	−	ND[d]
	HRT-18	Rectal tumor	−	−
	HL-60	Promyelocytic leukemia	−	ND

[a] Cells in monolayers were labeled by indirect immunofluorescence for surface antigen with a 1:25 dilution of antibody directed against the 110-kDa protein and rhodamine-conjugated goat anti-mouse immunoglobulin.
[b] Susceptibility to MHV-A59 was assayed by development of MHV-induced cytopathic effects, including cell fusion, rounding, and/or lysis. Negative cultures were confirmed by lack of immunofluorescence of MHV-inoculated cells, using antibody directed against MHV structural proteins.
[c] PM, primary cultures of thioglycolate-stimulated peritoneal macrophages.
[d] ND, not determined.

fluorescence with antibody to MHV structural proteins. All cell lines of hamster, cat, dog, monkey, and human origin which we tested failed to develop cytopathic effects or detectable MHV antigens after MHV inoculation, indicating that infection was blocked either at the level of virus adsorption or at another early stage in the virus replicative cycle prior to viral protein synthesis. Thus, failure of cell lines from nonmurine species to bind mouse polyclonal anti-MHVR correlated with resistance of these cell lines to MHV infection.

Susceptibility of murine cell cultures to MHV-A59 infection also correlated with binding of polyclonal anti-MHVR to the plasma membrane. The antibody detected MHVR in small patches on the membranes of L2 (Fig. 3A), Sac$^-$, 17 Cl 1, J774A-1, and JLSV9 cell lines and on primary cultures of peritoneal macrophages from BALB/c mice, all of which are susceptible to infection with MHV-A59 (Table 1). In contrast, peritoneal macrophages from SJL/J mice did not bind the polyclonal anti-MHVR (Fig. 3D) and were not susceptible to MHV-A59 infection (Table 1) (36). Thus, the SJL/J homolog of the MHVR glycoprotein, which was detected in SJL/J intestinal BBM by antibody to the amino-terminal peptide of MHVR (53), either was not expressed in SJL/J macrophages or was not recognized by mouse polyclonal anti-MHVR. Although immunofluorescence with antireceptor MAb CCl was not sensitive enough to detect the MHVR on the MHV-susceptible murine cell lines even after amplification with rabbit anti-mouse IgG, MAb CCl did recognize MHVR on mouse fibroblast cell lines such as L2 or 17 Cl 1, because pretreatment of cells with MAb CCl blocked MHV-A59 infection of these cells (53).

MHV-binding epitope of MHVR is expressed on intestinal BBM from mice but not on those from other species. In membrane preparations of BALB/c mouse liver and small intestine, sufficient quantities of MHVR are expressed to permit detection with MAb CCl as well as with virus (52). Therefore, to explore the species specificity of expression of the virus-binding epitope of MHVR, we studied binding of MHV-A59 virions and polyclonal and monoclonal anti-MHVR to intestinal BBM from many species by using solid-phase virus-binding assays, VOPBAs, and immunoblots with anti-receptor antibodies. Figure 4 shows that although MHV-A59 bound to BBM of BALB/c mice in solid-phase binding assays in which the membrane glycoproteins were reduced but not denatured by SDS, MHV-A59 did not bind to BBM of rats, cats, dogs, pigs, cows, rabbits, cotton rats, chicken, or humans or to chicken chorioallantoic membranes. The same result was found in virus blots of SDS- and mercaptoethanol-treated BBM from many species (Fig. 5A). Thus, binding of MHV-A59 to apical membranes of enterocytes was species specific.

To determine whether proteins homologous to MHVR could be detected on BBM of nonmurine species by using mouse polyclonal anti-MHVR or anti-receptor MAb CCl, immunoblots were performed (Fig. 5B and C). Both the polyclonal and the monoclonal antireceptor antibodies detected MHVR in BALB/c mouse BBM. In addition, MAb CCl detected a 58-kDa glycoprotein (Fig. 5C) (53) which was only very weakly detected by polyclonal anti-MHVR or by MHV-A59 (Fig. 5A and B). Neither polyclonal nor monoclonal anti-MHVR reacted in immunoblots with any BBM proteins from nonmurine species (Fig. 5B and C), although immunoblotting of BBM from these species with antibody directed against human CEA showed CEA-related glycoproteins of various molecular weights in many of the species (data not shown). Thus, not only the virus-binding domain but also all epitopes recognized in immunoblots by the anti-MHVR antibodies were absent from BBM of nonmurine species.

Role of MHVR in interactions of MHV-A59 with rat intes-

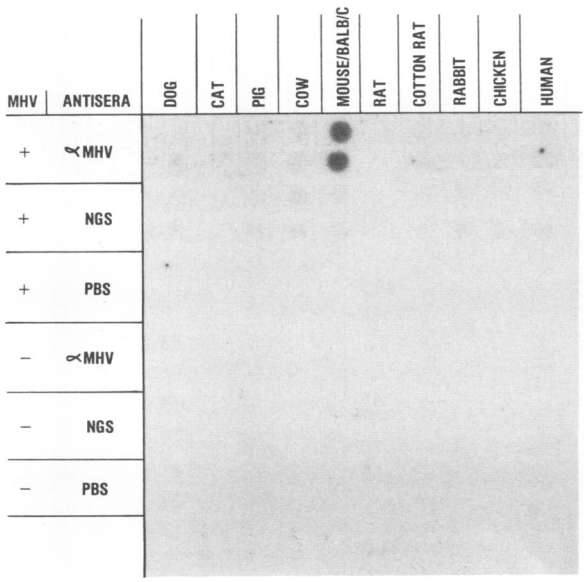

FIG. 4. Solid-phase assay of MHV-A59 binding to intestinal BBM from different species. Ten micrograms of BBM pretreated with 5% β-mercaptoethanol, from dog, cat, pig, cow, BALB/c mouse, rat, cotton rat, rabbit, chicken, or human intestines, was bound to nitrocellulose in duplicate. The immobilized membranes were incubated with MHV-A59 (+), and bound virus was detected with goat antibody directed against the spike glycoprotein S antiserum of MHV-A59 and ^{125}-SPA. Controls included samples incubated with medium (−) instead of virus or with normal goat serum (NGS) or dilution buffer (PBS) in place of anti-MHV S antiserum.

tinal BBM. The finding that MHV-A59 did not bind to rat intestines was interesting, since several MHV strains replicate in brains of neonatal or weanling rats after intracerebral inoculation (24, 37, 43, 50). We therefore studied the effect of host age and strain on the ability of rat BBM to bind MHV-A59 or anti-MHVR antibodies in order to determine whether a rat homolog of the MHVR could be detected in rat intestinal tissue. Sprague-Dawley rats were used to determine whether the expression of MHV receptors on rat BBM was age dependent, and Wistar Furth rats were used because they are the rat strain most susceptible to MHV infection, sustaining greater than 90% mortality when rats less than 10 days old were inoculated intracerebrally with MHV-JHM (37). Figure 6 shows that MHV-A59 did not bind to BBM of adults or neonates of either strain of rat.

DISCUSSION

Like several other animal viruses, the murine coronavirus MHV-A59 utilizes a glycoprotein in the immunoglobulin superfamily as a virus receptor (11, 51, 52). Both the poliovirus receptor PVR and the rhinovirus receptor ICAM1 have been cloned and expressed in cells of species resistant to the virus and were shown to confer susceptibility to virus infection (13, 22, 39). Similarly, expression of MHVR in BHK-21 hamster fibroblasts and human RD cells makes these cells susceptible to MHV-A59, overcoming a species barrier to infection which operates at the level of virus-receptor interactions (11). We previously showed that adult SJL/J mice express on their intestinal BBM and hepatocyte membranes a glycoprotein related to MHVR that lacks virus-binding and anti-MHVR antibody-binding activities (7, 53). We suggested that absence or mutation of the virus-binding domain in the SJL/J glycoprotein may be responsible for the observed resistance of adult SJL/J mice to MHV infection (53). This article explores whether nonmurine species may be resistant to infection with murine coronavirus because the CEA-related glycoproteins on their intestinal BBM lack an MHV-binding domain.

The gene organization of the CEA family of glycoproteins is quite complex, resulting in great diversity in the structure and number of isoforms found in various tissues in different species and in different mouse strains. The many isoforms appear to be derived by complex alternative splicing of transcripts from one or more related genes (2). More than 14 human glycoproteins and at least 4 rat and 4 mouse glycoproteins in the CEA family have been identified in intestinal epithelium, hepatocytes, macrophages, placenta, and other tissues (4, 32, 44). Until recently, few specific immunological reagents have been available for analysis of CEA-related glycoproteins of nonhuman species, and the total number of isoforms expressed on specific tissues and cell lines is not yet known. The antibodies to BALB/c BBM raised in SJL/J mice recognized the MHVR glycoprotein in immunoblots of BALB/c intestinal BBM (Fig. 1 and 5B and C) and blocked virus attachment to murine cell lines (53; data not shown). MAb CCl and MHV-A59 virus were recently found to bind to the N-terminal immunoglobulin-like domain of MHVR (12). The data presented in this report suggest that anti-MHVR and MAb CCl antibodies bound to the receptor glycoprotein either at the site recognized by MHV-A59 or close enough to that site to block virus binding. Because they block the virus-binding domain of MHVR, these antibodies, together with virus-binding assays, were used to investigate the species specificity of cellular and intestinal BBM interactions with MHV-A59.

In cell cultures, immunofluorescence with polyclonal anti-MHVR showed antigen on all but one of the murine cell lines or primary cell cultures tested and these cell lines were able to support replication of MHV-A59 (Table 1). SJL/J macrophages were not recognized by anti-MHVR (Table 1) and are resistant to infection with MHV-A59 (36). Enterocyte and hepatocyte membranes from SJL/J mice also did not express any glycoprotein recognized in immunoblots by anti-MHVR or MAb CCl or by MHV-A59 virions (Fig. 5) (7, 53). These data provide further support for the hypothesis that absence of or differences in the virus-binding epitope from the SJL/J mouse's CEA-related glycoprotein may explain the resistance of cells of adult SJL/J mice to MHV infection.

Cell lines from nonmurine species were not susceptible to infection with MHV-A59, and the MHV receptor glycoprotein was not detectable on their plasma membranes by immunofluorescence with polyclonal anti-MHVR. Although they were resistant to infection with murine coronavirus MHV-A59, the human (HRT-18, RD, and IMR-90), feline (fcwf-4), and canine (A72) cell lines are quite susceptible to the coronaviruses which naturally infect those species, namely HCV-OC43, HCV-229E, feline infectious peritonitis virus, or canine coronavirus (6, 26, 38). Thus, in these cell lines, the virus-binding epitopes on the receptors for these other coronaviruses must be different from the MHV-A59-binding epitope of MHVR and from the epitope(s) recognized by anti-MHVR.

In solid-phase virus-binding assays and in VOPBAs, MHV-A59 did not bind to intestinal BBM of rats, cats, dogs, pigs, cows, rabbits, cotton rats, humans, or chickens. In addition, in immunoblots of BBM from these nonmurine species, neither MAb CCl nor polyclonal anti-MHVR detected any glycoproteins that serologically cross-reacted

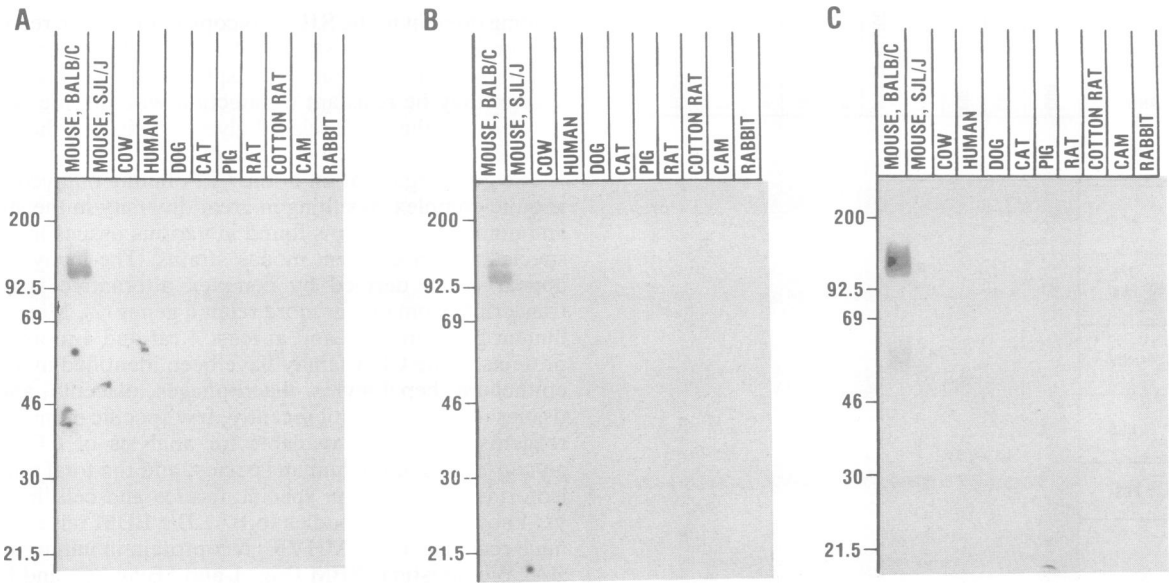

FIG. 5. Binding of MHV-A59 virus and polyclonal and monoclonal receptor antibodies to intestinal BBM from different species. One hundred micrograms of BBM from BALB/c or SJL/J mice, from cow, human, dog, cat, pig, cotton rat, or rabbit intestines, or from chicken chorioallantoic membranes (CAM) was run on SDS-PAGE gels, transferred to nitrocellulose, and probed with MHV-A59 and goat antibody directed against the S glycoprotein of MHV (A); polyclonal anti-receptor antibody (B); and monoclonal anti-receptor antibody CCl and rabbit antibody to mouse immunoglobulin (C). ^{125}I-SPA was used to detect bound antibodies. Molecular sizes of protein standards (in kilodaltons) are shown at the left of the panels.

with MHVR. Taken together, these data suggest that the MHV-A59-binding epitope of MHVR is not expressed on membranes of nonmurine cell lines or BBM. Absence of the MHV-A59-binding epitope may account for the resistance of cells from these nonmurine species to infection with MHV-A59. Some coronaviruses of nonmurine species might utilize as receptors CEA-related glycoproteins of their own host species that are not recognized by MHV or anti-MHVR. However, the human coronavirus HCV-229E and the porcine coronavirus transmissible gastroenteritis virus utilize as receptors aminopeptidase N, human and porcine metalloproteases unrelated to CEA, or immunoglobulins (54, 10), and protein receptors for other coronaviruses have not yet been identified.

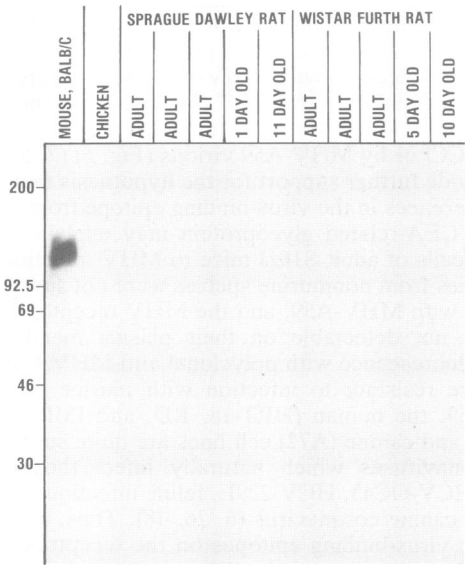

FIG. 6. Binding of MHV-A59 to proteins of intestinal BBM from adult mice, adult and suckling rats, and chickens. One hundred micrograms of intestinal tissue from BBM from BALB/c mice, chickens, three female adult rats, or pools of 5- or 10-day-old Wistar Furth rats was separated on SDS-PAGE gels, transferred to nitrocellulose, and probed with MHV-A59 virus and antibody to the S glycoprotein. ^{125}I-SPA was used to detect bound antibody. Molecular sizes of standards (in kilodaltons) are shown by the numbers at the left of the panel.

The investigation of MHV receptors in rats is of particular interest because, although rat and mouse coronaviruses are closely related, they are usually species specific for infection, exhibit markedly different tissue tropisms, and, consequently, induce different diseases (27). Natural infection of rats with the rat coronavirus RCV and sialodacryoadenitis virus does not involve the small intestine but is targeted to the salivary and lacrimal glands and respiratory tract (25, 28–30). Therefore, it is not surprising that our studies demonstrated that intestinal BBM from rats of different ages and strains did not bind MHV-A59 or anti-MHVR. Nevertheless, some kind of receptor for MHV must be expressed in the brains of young rats since they are susceptible to MHV infection. Williams et al. (52) showed that much less MHVR is expressed in BALB/c mouse brain than in mouse intestinal or liver tissue. It therefore appears unlikely that MHV binding to rat brain tissue would be detectable by the methods used in this report. Possibly, experimental MHV infection of the rat brain utilizes a different receptor than that used by rat coronaviruses in natural infections. Alternatively, the MHV that replicates in the rat brain may represent a virus subpopulation containing a mutation, such as an altered S glycoprotein, that binds to an as-yet-unidentified natural receptor in the rat brain for the S glycoprotein of RCV. Isolates of MHV-JHM from brains of infected rats have been demonstrated to differ from the inoculum virus in

several ways (24, 43), but the significance for receptor binding of these differences is not yet known.

This article focusses upon the role of the expression of the MHV-binding epitope of MHVR in the species specificity of MHV infection. The A59 strain of MHV appears to interact with cell membranes through binding of its S glycoprotein to a specific domain of MHVR. In contrast, some other strains of MHV and antigenically related coronaviruses of other species, including bovine, rat, and human coronaviruses (BCV, RCV, and HCV-OC43, respectively), express an additional spike glycoprotein, hemagglutinin-esterase (HE), not found in MHV-A59 virions (16, 20). Coronavirus HE, like the related HE glycoprotein of influenza virus C, binds specifically to a carbohydrate moiety, 9-*O*-acetylated sialic acid (47), which is found on many glycoproteins. The S glycoprotein of the bovine coronavirus BCV also recognizes 9-*O*-acetylated sialic acid (34), and this interaction can initiate virus infection. For other HE-expressing coronaviruses, it is not yet clear whether the interaction of HE with its carbohydrate receptor can lead to virus infection of cells in the absence of an interaction between S glycoprotein and its specific receptor. Possibly, the expression of HE on some coronaviruses could extend their tissue tropism and/or host range by permitting entry via many cellular glycoproteins or glycolipids which express the carbohydrate moiety recognized by HE.

In conclusion, the MHVR isoform of the murine CEA-related glycoproteins (also known as $mmCGM_1$) (4) is a functional receptor for MHV-A59, and absence of the virus-binding epitope of this protein may explain the resistance to MHV-A59 infection of adult SJL/J mice and most other species of animals.

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