Genetic Resistance to Mouse Hepatitis Virus Correlates with Absence of Virus-Binding Activity on Target Tissues

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The molecular mechanism of genetic resistance of inbred mouse strains to mouse hepatitis virus, a murine coronavirus, was studied by comparing virus binding to plasma membranes of intestinal epithelium or liver from susceptible BALB/c and resistant SJL/J mice with a new solid-phase assay for virus-binding activity. Virus bound to isolated membranes from susceptible mice, but not to membranes from resistant mice. F₁ progeny of SJL/J × BALB/c mice had an intermediate level of virus-binding activity on their enterocyte and hepatocyte membranes. This correlated well with previous studies showing that susceptibility to mouse hepatitis virus strain A59 is controlled by a single autosomal dominant gene (M. S. Smith, R. E. Click, and P. G. W. Plagemann, *J. Immunol.* 133:428-432). Because virus binding was not prevented by treating membranes with sodium dodecyl sulfate, the virus-binding molecule could be identified by a virus overlay protein blot assay. Virus bound to a single broad band of *M_r* 100,000 to 110,000 in membranes from hepatocytes or enterocytes of susceptible BALB/c and semisusceptible C3H mice, but no virus-binding band was detected in comparable preparations of resistant SJL/J mouse membranes. Therefore, SJL/J mice may be resistant to mouse hepatitis virus A59 infection because they lack a specific virus receptor which is present on the plasma membranes of target cells from genetically susceptible BALB/c and semisusceptible C3H mice.

The resistance of certain strains of inbred mice to mouse hepatitis virus (MHV) is a classic example of the importance of the host genotype in determining susceptibility to viral infection (2). Susceptible strains, such as BALB/c, are readily killed by virulent strains of MHV, which replicate in the intestinal and respiratory epithelium, the liver, and, for some MHV strains, the brain and spinal cord (3, 18). In semisusceptible mouse strains, such as C3H, limited virus replication occurs in these tissues, but animals recover after mild or inapparent infection (19). The SJL/J strain of mice is profoundly resistant to replication of MHV *in vivo* and shows no signs of infection following MHV inoculation at doses which readily infect other mouse strains (9, 13). *In vivo* susceptibility to MHV is correlated with the ability of the virus to replicate *in vitro* in cultured peritoneal macrophages, neuronal cells, and hepatocytes from susceptible mouse strains (1, 2, 9, 13). A single genetic locus, *Mhv-1*, on mouse chromosome 7 determines susceptibility to the A59 strain of MHV (MHV-A59) (13).

Several hypotheses have been proposed to explain the mechanism of host genetic resistance to MHV, but no single mechanism has yet been identified. Resistance could be due to lack of a receptor for the virus on target cells, defective replication of viral RNA or virion assembly in cells from resistant animals, altered host immune response to MHV, or abnormal interferon induction by MHV. It is not surprising that the molecular mechanism of MHV resistance is not yet known, in view of the complex biology of MHV (18). There are great differences in the virulence and tissue tropism of various MHV strains (3, 16), various degrees of susceptibility of different mouse strains (9, 13), and complex effects of age, route, and dose of virus on the outcome of virus inoculation *in vivo* (6).

Because susceptibility to MHV is a dominant trait (9, 13), several investigators have tested the hypothesis that resistance to MHV is due to lack of a virus receptor on target cells of resistant mouse strains (1, 2, 9, 10). These studies did not detect differences in virus binding to cultured cells from susceptible and resistant mouse strains. There were, however, some limitations in the methods of these virus-binding studies. Virus binding to cultured macrophages, hepatocytes, or neuronal cells was studied by measurement of infectious virus remaining after adsorption to cells, electron microscopy of virus adsorption, or binding of radiolabeled virions to cells from susceptible and resistant animals. Since macrophages are phagocytic, uptake of virus by these cells may not reflect virus adsorption to other cell types. Also, most of these studies used strains of mice which were not totally resistant to the virus strain studied.

We have devised a more direct approach to test the hypothesis that MHV resistance is due to lack of a virus receptor. We have attempted to identify the MHV receptor on cells from susceptible animals and to determine whether it is present on cells from fully resistant animals. This study focuses on the A59 strain of MHV and its binding to plasma membranes of the two most prominent target tissues for MHV-A59, enterocytes and hepatocytes. In this paper, we report the development of new methods to measure binding of MHV-A59 to membranes of its target cells in susceptible strains of mice and to identify the cell surface molecule to which the virus binds. This virus-binding molecule was present on membranes of target cells from susceptible BALB/c mice and semiresistant C3H mice, but absent on membranes from fully resistant SJL/J mice. Thus, for the SJL/J mouse, the lack of a cell surface molecule required for virus attachment may be the reason for genetically determined resistance to MHV-A59.

MATERIALS AND METHODS

Cell cultures and virus propagation and purification. The 17C11 line of spontaneously transformed BALB/c 3T3 cells

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was grown in monolayer culture in Dulbecco modified Eagle minimal essential medium (MEM) with high glucose, 10% fetal bovine serum (FBS), and 2% of an antibiotic-antimycotic agent mixture (GIBCO Laboratories, Grand Island, N.Y.) as described previously (15). MHV-A59, obtained from L. Sturman, New York State Department of Health, Albany, N.Y., was inoculated on 17C11 cells at an input multiplicity of 0.1 to 0.5 PFU/cell and incubated at 37°C for 17 to 24 h. Released virus was harvested and purified by discontinuous followed by continuous sucrose density gradients as previously described, omitting the polyethylene glycol precipitation step (14). Purified virus was pelleted by ultracentrifugation for 2 h at 24,000 rpm in a Beckman SW27.1 rotor, gently suspended in MEM with 10% FBS, divided into portions, and quickly frozen. For some virus-binding studies, sham-inoculated or MHV-A59-infected 17C11 culture supernatants harvested 18 to 24 h postinoculation were clarified at $10,000 \times g$ for 20 min at 4°C, divided into portions, quickly frozen, and stored at -70°C.

Antiserum directed against virus proteins. Goat antibody directed against the E2 spike glycoprotein of MHV-A59 was raised by immunization of a goat with E2 purified from Nonidet P-40-disrupted virions by sucrose density gradient ultracentrifugation (14). An initial inoculation of E2 with complete Freund adjuvant was followed by three boosters with E2 in incomplete Freund adjuvant at 14-day intervals. The anti-E2 serum blotted M_r -180,000(180K) and 90K E2 in Western blots of virion proteins and MHV-A59-infected cell extracts, neutralized MHV-A59, and inhibited MHV-A59-induced fusion of the L2 line of mouse fibroblasts. With this serum, Western blots against enterocyte and hepatocyte membrane proteins and uninfected 17C11 cell proteins were negative.

Isolation and purification of plasma membranes. C3H mice were obtained from Frederick Bang, Johns Hopkins University, Baltimore, Md., and maintained by brother-sister matings in filter-topped cages in laminar flow incubators at this institution. BALB/c and SJL/J mice were obtained from the National Cancer Institute. Animals were 6 to 12 weeks old at the time organs were harvested. Livers and small intestines were held briefly on ice, rinsed or flushed with Dulbecco phosphate-buffered saline at 4°C, sealed into airtight plastic bags, snap frozen in liquid nitrogen, and stored at -70°C. Enterocyte brush border vesicles were prepared from the frozen small intestine by the method of Kessler et al. (8). Briefly, small intestine was homogenized, extraneous material was precipitated with 10 mM CaCl_2 , and brush border vesicles were pelleted by differential centrifugation. Plasma membranes from hepatocytes were prepared by the established procedure of Neville et al. (12), with homogenization, filtration, and three stages of differential and isopycnic centrifugation. Brush border membranes and hepatocyte plasma membranes were stored in TE buffer (10m M Tris hydrochloride, pH 7.4, 1 mM EDTA) and as membrane pellets, respectively, at -70°C until used. The amount of total membrane protein was determined by the Bradford method (4).

Solid-phase assay for virus binding. Equal amounts by weight of hepatocyte and enterocyte membranes in TE buffer were bound to nitrocellulose sheets in a 96-well minifold apparatus at 25°C. All subsequent steps were done at 25°C in shallow plastic boxes or screw-capped tubes with gentle rocking. The sheets were blocked for 30 min with 2% bovine serum albumin (BSA) in dilution buffer (50 mM Tris hydrochloride, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.05%

Tween 20, and 0.1% BSA) and then incubated for 1 h with either Dulbecco modified MEM or gradient purified MHV-A59 virions in MEM, both containing 10 mM HEPES (N-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) FBS. The sheets were washed three times for 5 min each with dilution buffer and incubated for 1 h with preimmune goat serum or goat antibody to the E2 glycoprotein of MHV-A59, both diluted 1:100 in dilution buffer. Nitrocellulose sheets were again washed three times in dilution buffer, and bound antibody was detected by incubation for 1 h with radioiodinated staphylococcal protein A (New England Nuclear Corp.; specific activity approximately $8\mu\text{Ci}/\mu\text{g}$, 10^5 cpm/ml; 25 ml per 3- by 5-in. sheet of nitrocellulose). The sheets were washed three times in dilution buffer and autoradiographed on Kodak XAR-5 film. Virus-binding activity was routinely quantitated by cutting the autoradiograph to fit the carrier of a Titertek Multiscan MicroELISA reader and reading the OD at 620 nm. The OD values plotted represent the average of duplicate determinations in which values for samples with preimmune serum were subtracted from those with anti-E2. Duplicates varied by no more than 0.05 OD units. Alternatively, virus binding was quantitated by cutting dots from the nitrocellulose paper and counting them in a Packard gamma counter.

For some experiments, 0.032 to 20 μg of enterocyte membranes was treated with a 0.1% final concentration of various detergents in TE buffer, vortexed, incubated for 5 min at 25°C, vortexed again, and diluted to a final detergent concentration of <0.01% before binding to nitrocellulose. Higher concentrations of detergent apparently interfered with protein attachment to nitrocellulose.

VOPBA. Proteins from plasma membranes isolated from susceptible BALB/c, semisusceptible C3H, and resistant SJL/J enterocytes or hepatocytes were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (11). For each lane, 0.1 mg of membrane protein was used. Gels were stained with Coomassie blue or electroblotted onto nitrocellulose paper with a Trans-blot cell (Biorad Laboratories, Richmond, Calif.). Transfer buffer contained 25 mM Tris, 0.192 M glycine, pH 8.6, with 20% methanol, and transfer was for 16 h at 150 mA by the method of Towbin et al. (17). The nitrocellulose sheets were then blocked with 2% BSA in dilution buffer for 30 min at 25°C with gentle rocking. The subsequent steps of the virus overlay protein blot assay (VOPBA) were performed as described above for the solid-phase virus-binding assay. Molecular weights of proteins were determined with a series of prestained markers from 12.3K to 200K (Bethesda Research Laboratories, Bethesda, Md.).

RESULTS AND DISCUSSION

Solid-phase assay for virus binding. To evaluate MHV binding to plasma membranes of murine target tissues, we developed a solid-phase virus-binding assay. Figure 1 shows the binding of MHV-A59 to membranes from hepatocytes and enterocytes of susceptible BALB/c mice, along with appropriate controls for the assay. No binding of labeled staphylococcal protein A was observed when membranes or virus were omitted or preimmune serum was used. Binding of labeled staphylococcal protein A was only detected when membranes, virus, anti-E2, and protein A were all present. The assay was equally sensitive and specific when culture supernatant from 17C11 cells infected with MHV-A59 was used in place of gradient-purified virions. Quantitative comparison of the virus-binding activity of increasing amounts of

FIG. 1. Solid-phase assay for binding of MHV-A59 to plasma membranes. The binding of MHV-A59 to isolated membranes from BALB/c mice was demonstrated by spotting 10 μ g of purified enterocyte (ENT) or hepatocyte (HEP) plasma membranes per dot in TE buffer or buffer alone (TE) onto nitrocellulose sheets, incubating with gradient-purified MHV-A59 virions (MHV) or medium (MEM), and detecting bound virus with antibody directed against the E2 spike glycoprotein of the virus (aE2) or preimmune goat serum (PI). Conditions for the solid-phase assay are described in Materials and Methods.

BALB/c membranes was done by densitometric scanning of autoradiographs of the solid-phase assay in a MicroELISA plate reader (Fig. 2). Virus binding to BALB/c intestinal brush border membranes was directly proportional to the amount of membrane protein added.

Comparison of virus-binding activity of BALB/c and SJL/J membranes. We tested the hypothesis that the resistance of SJL/J mice to MHV infection is determined by the lack of a specific MHV receptor which in susceptible and semi-susceptible mice is present on target cells such as enterocytes and hepatocytes. We compared the binding of MHV-A59 to plasma membranes purified from enterocytes and hepatocytes from susceptible BALB/c and resistant SJL/J mice by the solid-phase assay for virus attachment (Fig. 3). While virus binding to BALB/c enterocyte and hepatocyte membranes increased with increasing amounts of membrane protein, little or no virus binding was detected to enterocyte or hepatocyte membranes from resistant SJL/J mice. This experiment shows that in two of the cell types which are normal targets for replication of MHV-A59 in vivo, MHV-binding activity was present on plasma mem-

FIG. 2. MHV-A59-binding activity correlates with the concentration of enterocyte membrane protein. Virus binding to various amounts of enterocyte membrane protein was quantitated by densitometrically scanning the autoradiographs from solid-phase receptor assays in a MicroELISA plate reader. Virus specificity and dependence of virus-binding activity on the amount of membrane protein was demonstrated by probing with clarified supernatant medium from mock-infected (●) or MHV-A59-infected (■) 17Cl1 cells.

FIG. 3. Absence of virus-binding activity in membranes from genetically resistant mice. The solid-phase virus-binding assay was used to compare binding of MHV-A59 to membranes purified from enterocytes (upper panel) and hepatocytes (lower panel) from genetically susceptible BALB/c (●) and resistant SJL/J (■) mice.

branes from susceptible BALB/c mice, but absent on membranes from resistant SJL/J mice. The results of this solid-phase assay are in accordance with our observation that radiolabeled MHV virions bound to the plasma membranes of splenocytes from BALB/c but not SJL/J mice (7). Thus, the ability of virus to attach to plasma membranes of target cells is determined by the genotype of the mice and correlates directly with susceptibility to MHV infection.

Using the solid-phase virus-binding assay, we evaluated the binding of MHV-A59 to membranes of F_1 mice from SJL/J $\times$ BALB/c matings (Table 1). We found that virus bound to enterocyte and hepatocyte membranes from both male and female F_1 mice at levels intermediate between those of the BALB/c and SJL/J parents. These virus-binding data correlated with published observations that such F_1 animals are susceptible to infection with MHV-A59 (13). These data are compatible with the strong genetic evidence

TABLE 1. MHV-A59-binding activity of plasma membranes purified from BALB/c, SJL/J, and F_1 hybrid mice

Membrane prep ^a	OD ₆₂₀ (% of BALB/c) ^b			
	BALB/c	SJL/J	F_1 females	F_1 males
Enterocytes	0.996	0.011 (1)	0.277 (28)	0.166 (17)
Hepatocytes	0.987	0.055 (5)	0.223 (23)	0.312 (32)

^a Membrane preparations were from pools of tissue from 5 to 10 animals.

^b Numbers in parentheses are the percentage of values for BALB/c membranes obtained in the solid-phase virus-binding assay. Data are representative of two experiments.

FIG. 4. Virus-binding activity is resistant to treatment with SDS. In the solid-phase virus-binding assay, MHV-A59 bound equally well to untreated (●) and 0.1% SDS-treated (■) BALB/c enterocyte brush border membranes.

that MHV susceptibility is controlled by a single dominant autosomal gene, which has been mapped to mouse chromosome 7 (9, 13).

Lack of effect of detergents on virus-binding activity. Plasma membrane receptors for a growing number of animal viruses are now being identified (5). These have often been found to be glycoproteins or glycolipids. To characterize the molecule(s) on enterocyte and hepatocyte membranes to which MHV-A59 bound, we examined the sensitivity of virus-binding activity to inactivation with various detergents. Enterocyte plasma membrane preparations were treated with 0.1% of either deoxycholate, Nonidet P-40, Tween 20, Lubrol-PX, or SDS in TE buffer or with TE buffer alone prior to binding to the nitrocellulose paper. Binding of MHV-A59 to enterocyte membranes of BALB/c mice was not inactivated by treatment of the membranes with SDS (Fig. 4). Similar results were observed with the other detergents (data not shown). Virus-binding activity remained in the supernatant when detergent-treated membranes were centrifuged at $200,000 \times g$ for 30 min at 4°C , indicating that the virus-binding entity had been solubilized effectively.

Thus, it appears that MHV binds to a monomeric molecule on the enterocyte membranes rather than a complex of macromolecular components, which would be disrupted by detergents. This experiment also supports the conclusion that the molecule(s) which binds MHV is on the external side of the plasma membrane, since solubilization of brush border vesicles, which are >95% rightside out and sealed (8), did not result in increased virus binding, as would be expected if the binding moiety were on the cytoplasmic side of the membrane vesicles.

Identification of the virus attachment molecule in membrane preparations. Since MHV-binding activity was not destroyed by treatment of membranes with SDS, we performed a VOPBA to determine the molecular weight of the receptor (Fig. 5). This experiment also allowed us to compare virus-binding molecules from membranes of different tissues and mouse strains. Coomassie blue-stained membrane proteins from enterocytes and hepatocytes of BALB/c and SJL/J mice were examined after separation by SDS-PAGE (Fig. 5A). No mouse strain-specific difference between membrane proteins was detected on the stained preparation, although, as expected, numerous tissue-specific differences between enterocyte and hepatocyte membrane proteins were observed.

VOPBA for identification of the virus overlay molecules with MHV-binding activity was also done (Fig. 5B). Proteins from hepatocyte and enterocyte membranes from susceptible BALB/C, resistant SJL/J, and semiresistant C3H mice were separated by SDS-PAGE, electroblotted to nitrocellulose, and probed for virus-binding activity as in the solid-phase virus-binding assay. This experiment showed that all of the virus-binding activity of membranes from BALB/c and C3H enterocytes and hepatocytes was found in a single broad band of 100K to 110K. In contrast, none of the membrane proteins of cells from SJL/J mice showed any virus-binding activity. Comparison of the Coomassie blue and VOPBA in Fig. 5 suggests that the virus-binding molecule is a minor component of the plasma membrane. Receptors for many other viruses are also present in low copy numbers at the plasma membrane of susceptible cells (5).

To demonstrate the virus specificity of the 100K to 110K molecule, we performed VOPBAs on the same membrane preparations with bovine rotavirus and Theiler's virus, a murine picornavirus, using antibodies against each of these viruses for detection of virus binding. The 100K to 110K MHV-binding band did not bind these other viruses (data not shown). Additionally, when the VOPBA was performed with conditioned medium from uninfected cells for the virus probe step, no bands were seen in the autoradiographs (data not shown). Thus, the 100K to 110K band bound MHV-A59 specifically.

Several experiments provided some insight into what type of molecule the MHV-binding entity detected by the

FIG. 5. Comparison of proteins and virus-binding activity in plasma membranes from genetically susceptible, semiresistant, and fully resistant mice. (A) Enterocyte (ENT) and hepatocyte (HEP) plasma membranes from BALB/c (lanes B) and SJL/J (lanes S) mice were analyzed by SDS-PAGE and Coomassie blue staining (B) A VOPBA of the same amount of membrane protein from genetically susceptible BALB/c (lanes B), semi-resistant C3H (lanes C), and resistant SJL/J (lanes S) mice was performed by probing electroblotted membrane proteins with MHV-A59 as was done for the solid-phase virus-binding assay. Positions of molecular weight standards (in thousands), are shown.

VOPBA may be. The virus-binding activity of BALB/c enterocyte membranes was destroyed by treatment of the membranes with trypsin (data not shown). The protease-sensitive nature, size, and location of the virus attachment molecule suggest that it may be a protein or a glycoprotein. The reason that the MHV-binding molecule has a wider range of molecular weight in enterocyte membranes than in hepatocyte membranes is not yet known. Possibly a common protein is processed differently in the two cell types. Since the two tissues have MHV-binding molecules of comparable size and a single gene controls susceptibility to MHV-A59 (13), it is likely that the 100K to 110K virus-binding molecules of enterocyte and hepatocyte membranes are products of the same gene. Additional experiments are in progress to characterize the molecule in detail.

The data from the VOPBA in Fig. 5B confirmed the conclusion drawn from the solid-phase virus-binding assays (Fig. 2) that membranes of target cells from resistant SJL/J mice lacked the virus-binding activity which was present in membranes from susceptible BALB/c mice. A single monomeric molecule, probably a protein, on the membranes of susceptible BALB/c mice appears to be the only entity needed to bind MHV to membranes from the target cells. The same virus-binding molecule was found on the membranes of semi resistant C3H mice, suggesting that virus replication in these animals is partially restricted at a stage subsequent to virus attachment. In SJL/J mice, the absence of this virus-binding protein correlates with the genetically determined resistance of SJL/J enterocytes and hepatocytes to infection with MHV-A59. These experiments suggest that profound resistance to MHV may be due to the absence of an MHV-A59-specific receptor on target cells from resistant animals. We are now attempting to clone and sequence the *Mhv-1* gene from susceptible mice to determine whether its gene product is the 100K to 110K MHV-binding molecule. These studies should show whether the gene is functional or the gene product is inactive in profoundly resistant animals and provide further information about the nature of the coronavirus receptor.

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