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HUMAN BILIARY GLYCOPROTEINS FUNCTION AS RECEPTORS FOR INTERSPECIES TRANSFER OF MOUSE HEPATITIS VIRUS

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1. ABSTRACT

A variant Mouse Hepatitis virus (MHV), designated MHV-H2, was isolated by serial passage in mixed cultures of permissive DBT cells and nonpermissive Syrian Hamster Kidney (BHK) cells. MHV-H2 replicated efficiently in hamster, mouse, primate kidney (Vero, Cos 1, Cos 7), and human adenocarcinoma (HRT) cell lines but failed to replicate in porcine testicular (ST), feline kidney (CRFK), and canine kidney (MDCK) cells. To understand the molecular basis for coronavirus cross-species transfer into human cell lines, the replication of MHV-H2 was studied in hepatocellular carcinoma (HepG2) cells which expressed high levels of the human homologue of the normal murine receptor, biliary glycoprotein (Bgp). MHV-H2 replicated efficiently in human HepG2 cells, at low levels in breast carcinoma (MCF7) cells, and poorly, if at all, in human colon adenocarcinoma (LS 174T) cell lines which expressed high levels of carcinoembryonic antigen (CEA). These data suggested that MHV-H2 may utilize the human Bgp homologue as a receptor for entry into HepG2 cells. To further study MHV-H2 receptor utilization in human cell lines, blockade experiments were performed with a panel of different monoclonal or polyclonal antiserum directed against the human CEA genes. Pretreatment of HepG2 cells with a polyclonal antiserum directed against all CEA family members, or with a monoclonal antibody, Kat4c (cd66abde), directed against Bgp1, CGM6, CGM1a, NCA and CEA, significantly reduced virus replication and the capacity of MHV-H2 to infect HepG2 cells. Using another panel of monoclonals with more restricted cross reactivities among the human CEA's, Col-4 and Col-14, but not B6.2, B1.13, Col-1, Col-6 and Col-12 blocked MHV-H2 infection in HepG2 cells. These antibodies did not block sindbis virus (SB) replication in HepG2 cells, or block SB, MHV-A59 or MHV-H2 replication in DBT cells. Monoclonal antibodies Col-4, Col-14, and Kat4c (cd66abde) all reacted

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strongly with human Bgp and CEA, but displayed variable binding patterns with other CEA genes. Following expression of human Bgp in normally nonpermissive porcine testicular (ST) and feline kidney (CRFK) cells, the cells became susceptible to MHV-H2 infection. These data suggested that phylogenetic homologues of virus receptors represent natural conduits for virus xenotropism and cross-species transfer.

2. INTRODUCTION

The exact mechanisms of virus cross-species transmission remain largely unexplored and few model systems exist to study the sites of virus host interactions which regulate the host range of animal viruses. Given the high degree of species specificity and tissue tropism, coronaviruses provide an excellent biochemical and genetic resource to study the mechanisms of virus xenotropism and cross-species transmission. MHV was passaged in progressively decreasing concentrations of permissive murine DBT cells and increasing concentrations of nonpermissive BHK cells, a setting which may be reflective of *in vivo* conditions present in heavily immunosuppressed humans following xenotransplantation (Baric et al., 1997). The variant viruses isolated from this procedure replicated in murine and hamster cells and surprisingly in primate and human cell lines (Baric et al., 1997). Since the MHV genomic RNA is infectious in nonpermissive hosts and expression of the MHV receptor for entry (MHVR), a biliary glycoprotein, converts nonpermissive cells into MHV susceptible hosts it is likely that MHV host species specificity is mediated at receptor-binding, docking and entry (Dveskler et al., 1991; Schochetman et al., 1977). In this paper we explore the molecular mechanisms mediating the interspecies transfer of a murine virus (MHV) into human cells lines in vitro.

3. MATERIALS AND METHODS

3.1. Viruses and Cells

Briefly, murine astrocytoma (DBT), and 17CL-1 cells, Syrian baby hamster kidney (BHK), Chinese hamster ovary (CHO), Syrian hamster smooth muscle (DDT-1), feline kidney (CRFK), porcine testicular (ST), primate kidney (VERO), and human colorectal (HRT) cells were maintained as previously described (Baric et al., 1997). Human hepatocellular carcinoma (HepG2) cells were maintained in Eagles minimum essential medium containing 8% fetal calf serum and 1% geneticin and kanamycin. Human colon adenocarcinoma (LS174T) cells were maintained in minimum essential medium with 8% fetal calf serum, 1% geneticin and kanamycin, and 1% non-essential amino acids. Breast carcinoma (MCF7) cells were maintained in Eagles minimum essential medium with 8% fetal calf serum, 1% geneticin and kanamycin, 1% non-essential amino acids, 1% sodium pyruvate and 10 μg/ml of insulin. Canine kidney (MDCK) cells were maintained in DMEM-H minimum essential medium containing 8% fetal calf serum and supplemented with 10% tryptose phosphate broth and 1% geneticin and kanamycin. Primate kidney cells, Cos 1 and Cos 7, were maintained in Eagle's minimum essential medium with 8% fetal calf serum and 1% geneticin and kanamycin.

MHV-A59 and MHV-JHM were plaque purified and propagated in DBT cells. The MHV-H2 variant was propagated in BHK cells as previously described. The TRSB strain of sindbis virus (kindly provided by Dr. Robert E. Johnson, University of North Carolina at Chapel Hill) was propagated in DBT cells.

3.2. Virus Growth Curves and Immunofluorescence

Different cell lines grown on LabTek chamber slides (Nunc. Inc., Naperville, Ill.) were infected with MHV-A59 or MHV-H2 at an MOI of 10 for 1 hour at room temperature. The inocula were removed and the monolayers were washed 3 times with PBS. Samples of virus were harvested at various times postinfection and stored at -70(C for plaque assay. Immediately following the last sample cells were fixed and prepared for examination by immunofluorescence as previously described (Chen et al., 1997).

3.3. Transmission Electron Microscopy

HepG2 cells were seeded on $60mm^2$ dishes and infected with MHV-H2 at an MOI of 10 for 1 hour. At 36 hours post infection these cells were then prepared for examination by transmission electron microscopy using standard techniques (Bozzola, 1992).

3.4. Blockade of MHV Infection

HepG2 or DBT cells were seeded at densities of 2×10^4 on LabTek 8 well chamber slides (Nunc. Inc., Naperville, Ill.). Cells were pretreated with 100 µl of a 1:4 dilution of either kat4c (cd66abde), a commercially available monoclonal antibody directed against the human BgpA, CGM6, CGM1a, NCA and CEA glycoproteins, or a commercially available polyclonal antibody which recognizes all human CEA gene family glycoproteins for 1 hour at room temperature. The antibodies were removed and the cells were infected with either MHV-A59, MHV-H2, or TRSB at an MOI of 10 for one hour at room temperature. Cells were washed 3 times with phosphate buffered saline (PBS) to remove residual virus, and complete media containing a 1:16 dilution of antibody were added to the individual chambers. Additionally, these experiments were repeated using a panel of monoclonal antibodies with more restrictive specificities to CEA gene family members at an original dilution of 1:3. As a control, cells were pretreated with an equivalent amount of R501, an irrelevant MAb (with the same IgG isotype as CC1) directed against the anti-E2 glycoprotein of Sindbis virus.

3.5. Transient Expression of Human Biliary Glycoprotein

The human biliary glycoprotein (hBgp1) was cloned from HepG2 cells using RT-PCR, and expressed in the $pcDNA_3$ vector system. Using electroporation, transient cotransfections were performed with 6 µg $pcDNA_3$ vector (Invitrogen) containing the human Bgp1 (hBgp1) gene and 3 µg of the pHook2 vector. Briefly the cells were incubated with pHook magnetic beads for 1–2 hours, scraped and separated with a magnet. The procedure was repeated 24 hours later. Selected cells were seeded onto LabTek slides (Nunc Inc.). Cells were infected with either MHV-H2, MHV-A59 or MHV-JHM at an MOI of 10 and examined for the presence of viral antigens at 32 or 36 hours post infection.

4. RESULTS

4.1. Virus Growth Curves in Various Mammalian Cell Lines

Previous work has demonstrated that MHV-H2 replicated efficiently in hamster (BHK, CHO, and DDT-1), murine (DBT and 17CL-1), and human (HRT) cell lines. Feline

Table 1. Host range phenotype of MHV-H2

	MHV-A59	MHV-JHM	MHV-H2
Mouse			
DBT	+	+	+
17Cl1	+	+	+
Hamster			
BHK	–	–	+
CHO	–	–	+
Feline			
CRFK	–	–	–
Canine			
MDCK	–	–	–
Pig			
ST	–	–	–
Primate			
Vero	–	–	+
Cos1	–	–	+
Cos7	–	–	+
Human			
HRT	–	–	+
HepG2	–	–	+
LS174T	–	–	–
MCF7	–	–	+/–

+ indicate evidence of viral growth; - indicates no evidence of viral growth by plaque assay and detection of viral proteins by radiolabelling or flourescence; + / – indicates low levels of immunofluorecence (< 4.0%).

kidney (CRFK) and porcine testicular cells (ST), however, were resistant to MHV-H2 infection (Baric et al., 1997). Infection of the canine kidney, MDCK, cell line with MHV-H2 also resulted in a nonproductive infection. In contrast, MHV-H2 infection of various primate cells, (VERO, COS 1 and COS 7), resulted in a productive infection with virus titers ranging between $1 \times 10^4 - 1 \times 10^5$ PFU/ml (Table 1).

MHV-H2 failed to replicate efficiently in human MCF7 cells and in human LS174T cell lines which express high levels of carcinoembryonic antigen (CEA) (Tom et al., 1976). In contrast MHV-H2 infection of HepG2 cells, which express high levels of human biliary glycoprotein (Hauck et al., 1994), resulted in virus titers approaching 1.0×10^8 (Table 1 and Figure 1). In addition, when viewed in a scanning electron microscope, significant CPE were visible in HepG2 cells infected with MHV-H2 (data not shown). Not surprisingly, the parental strains MHV-A59 and MHV-JHM only replicated in murine cells (Table 1). The ability of MHV-H2 to replicate in select cell lines suggested that one or more specific cellular factors were necessary for a productive MHV-H2 infection in human cell lines.

4.2. Pretreatment of HepG2 Cells with CEA Antibodies

Both human and mouse species have been shown to express members of the carcinoembryonic antigen gene family, including Bgp (Barnett et al., 1993; Rudert et al., 1992). Sequence comparisons have revealed that the human Bgp1 gene is highly homologous with the murine Bgp1 gene and to a lesser extent with the Bgp2 gene (data not shown). In addition human Bgp and CEA glycoproteins may function as receptors for

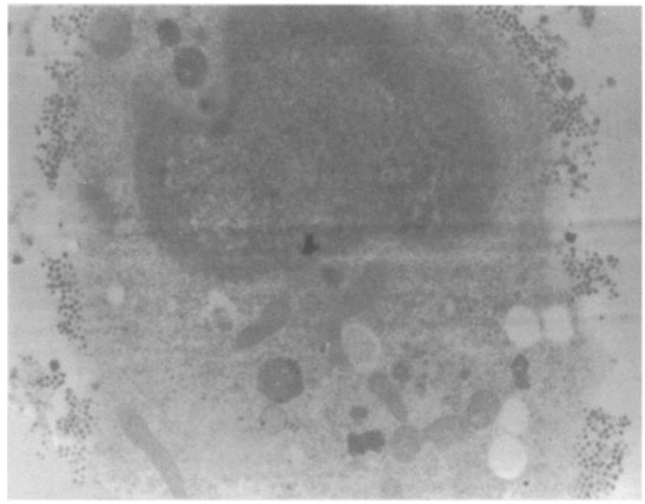

Figure 1. MHV-H2 infection in HepG2 cells. HepG2 cells were infected with MHV-H2 at an MOI of 20 for 1 hour. Cells were fixed and prepared for examination by electron microscopy 36 hours post infection. Magnification: 7,750X.

wildtype MHV entry into nonpermissive when expressed at high levels in non-permissive cells (Chen *et al.,* 1997). Consequently, it appeared likely that virus scanning for receptor homologues in different species may mediate cross-species transmission of MHV-H2 into human cells. A variety of polyclonal and monoclonal antibody were available that recognized the human CEA genes. Pretreatment of HepG2 cells with two commercially available antibodies, kat4c (cd66abde) and a polyclonal CEA (DAKO) significantly reduced MHV-H2 titers (Figure 2) and the number of infected HepG2 cells as observed by immunofluorescence (data not shown) Pretreatment with an irrelevant antibody, R501, failed to block MHV-H2 infection (Table 2). Consequently, pretreatment of HepG2 cells with these antibodies also failed to block Sindbis virus infection or inhibit MHV-H2 and MHV-A59 infections in VERO or DBT cells (data no shown). These data suggested that the blockade was specific and not due to antibody toxicity or other nonspecific effects.

Human CEA genes are highly homologous and the polyclonal CEA and kat4c (cd66abde) monoclonals' have broad crossreactivities and bind many CEA glycoproteins. Therefor an additional panel of monoclonal antibodies with more discrete ranges of CEA crossreactivities were obtained. Pretreatment of HepG2 cells with this panel of anti-CEA monoclonal antibodies (kindly provided by Dr. Jeffrey Schlom, NIH, Bethesda MD) produced mixed results (Table 2). Two antibodies, Col-4 and Col-14, which have been reported to react with hBgp1, successfully blocked MHV-H2 infection in HepG2 cells. Col-1, Col-6, and B6.2 which did not recognize hBgp1, all failed to block infection. A sixth antibody, Col-12 whose crossreactivity with hBgp1 has yet to be determined failed to block MHV-H2 infection in HepG2 cells. Pretreatment of HepG2 cells with these antibodies did block TRSB infection in these cells or block MHV-H2 (Table 2) or MHV-infection of murine cells (data not shown). These data indicated that human CEA genes likely functioned to initiate MHV-H2 docking and entry into human cells.

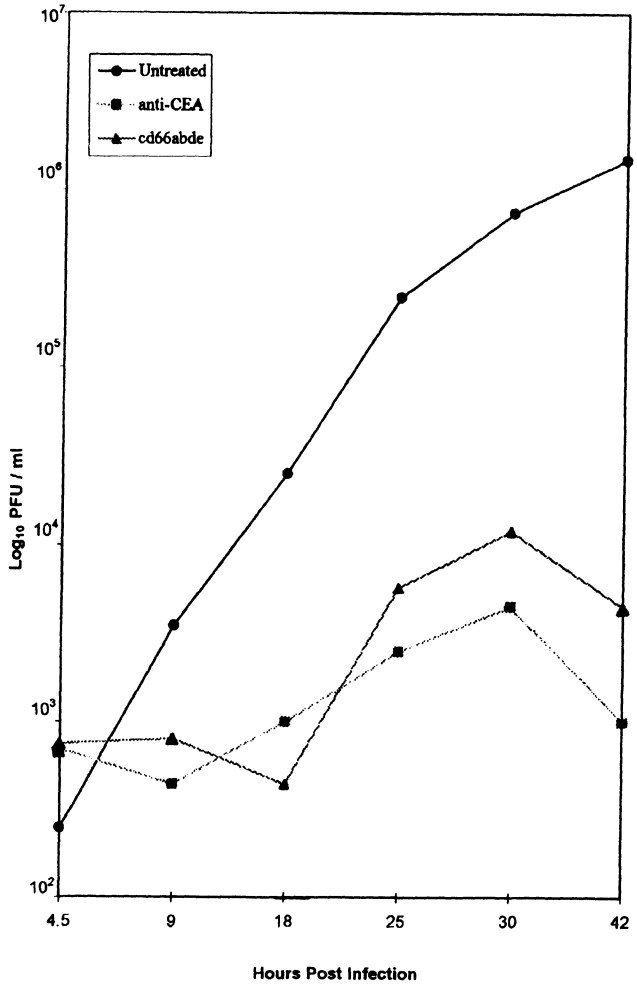

Figure 2. Anti-CEA and MAb cd66abde blockade of MHV-H2 in HepG2 cells. Cultures of HepG2 cells were pre-treated with the polyclonal anti-CEA antibody, which is directed against all human CEA members and with the MAb kat4c (cd66abde) directed against cd66abde for 1 hour. Cells were challenged with MHV-H2 at an MOI = 10 PFU/ml for one hours. Virus samples were taken at various times post infection and analyzed by plaque assay in DBT cells.

4.3. Expression of hBgp1 Promotes MHV-H2 Infection

The blockade data coupled with the observation that cell lines expressing high levels of human Bgp were susceptible to MHV-H2 infection, suggested that human Bgp1 may serve as a receptor for entry into human cells. To investigate this possibility, the human Bgp under the control of the CMV promoter was expressed in non-permissive ST cells. Following MHV-H2 infection of hBgp1-transfected cells, a significant increase in the

Table 2. Antibody blockade of MHV infections in HepG2 cells

Virus	Antibody	Virus Titer Pfu /ml
MHV-H2	Untreated	2.4×10^7
MHV-H2	TRSB	4.0×10^7
MHV-H2	Col-1	1.0×10^6
MHV-H2	Col - 4	5.5×10^3
MHV-H2	Col -14	7.0×10^3
MHV-H2	B1.13	3.0×10^6
MHV-H2	B6.2	5.0×10^5
MHV-H2	Col-6	1.5×10^6
MHV-H2	Col-12	5.0×10^5
TRSB	Untreated	1.5×10^7
TRSB	R501	6.0×10^7
TRSB	Col - 4	2.0×10^6
TRSB	Col - 14	2.5×10^6
TRSB	Polyclonal CEA	3.5×10^7
TRSB	kat 4c	5.0×10^6

number of fluorescent cells was observed by immunofluorescence. Under identical conditions 1–2% of the cells were susceptible to MHV-H2 and <0.05% of the cells to MHV-A59 infection. When transfected cells were sorted using the pHook system (Invitrogen) prior to infection, approximately 15–20% of the transfected cells were susceptible to MHV-H2 infection (see Figure 3) while only 1–2% of transfectants were permissive to MHV-A59 as observed by immunofluorescence (data not shown).

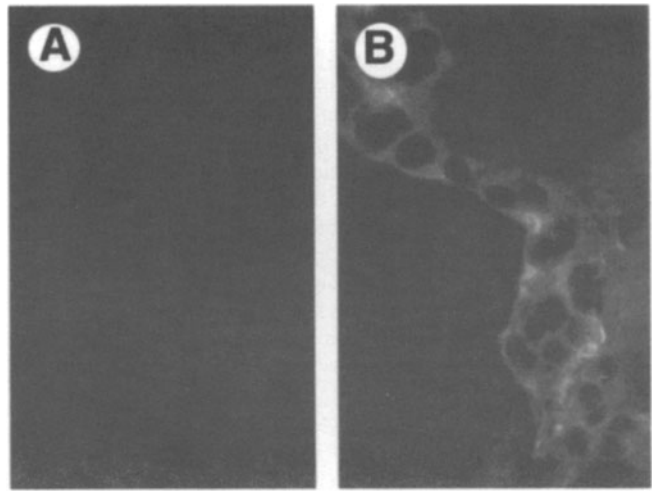

Figure 3. Virus infection in ST cells expressing hBgp1. ST cells transiently expressing hBgp1 were infected with MHV-H2 for 1 hour at an MOI of 10. Cells were fixed 36 hours later and examined by immunofluorescence. Panel A: ST cells infected with MHV-H2. Panel B: ST cells expressing hBgp1 infected with MHV-H2.

5. DISCUSSION

Host range specificity and the evolution of new viral diseases are complex phenomena involving interactions between the virus, the host and the environment (Morse, 1990). The cellular receptor is an essential component for virus entry and a major determinant of host range specificity, tissue tropism and pathogenesis (Wimmer, 1994). Cellular receptors for virus docking and entry have been demonstrated to represent a major site of virus-host interaction that regulate virus xenotropic spread. Other sites may include additional cofactors for entry, transcription factors, and host factors for virus assembly and release (Morse, 1994). Since little information is available regarding receptor utilization among phylogenetically related viruses that replicate in distinct species (Haywood, 1994; Wimmer 1994), our knowledge of virus cross-species transmission is incomplete. Focusing at the level of entry, the available literature suggests that new viral diseases may either emerge by a homologue scanning or a receptor augmentation mechanism (Baric *et al.,* 1997; Haywood, 1994; Wimmer 1994). The homologue scanning model predicts that phylogenetic homologues of the normal receptor function as natural conduits for the cross-species transmission and entry of viruses into alternative host species. For example, HIV likely evolved from SIV. Both viruses utilize CD4 and chemokine genes as receptors and co-receptors for entry into cells and target similar tissues in humans and primates (Signoret et al., 1993). Among the group I coronaviruses, transmissible gastroenteritis virus (TGEV), and human coronavirus 229E use the aminopeptidase N glycoprotein as a receptor, and produce similar infections in their respective hosts (Delmas *et al.,* 1992; Yeager et al., 1992). It appears likely that host range expansion among these virus families, in part, evolved along phylogenetically related receptor molecules. In contrast, the receptor augmentation model proposes that host range expansion is mediated by virus recognition of entirely new receptor molecules. For example, murine adapted strains of poliovirus do not recognize the murine homologue of the poliovirus receptor (PVR), rather, these variants recognize unique receptor moieties for docking and entry into murine cells (Morrison *et al.,* 1992; Morrison *et al.,* 1994).

The results presented in this paper strongly support the hypothesis that MHV-H2 utilizes human CEA glycoproteins, most likely hBgp1, for entry into permissive cell lines. MHV-H2 was able to replicate efficiently in human cell lines expressing high levels of hBgp, but was not able to replicate efficiently in LS174T cells which express high levels of CEA. In addition pretreatment of HepG2 cells with antibodies that crossreact with hBgp1 were successful in blocking infection. Finally expression of hBgp1 permitted MHV-H2 infection in non-permissive cells. These data indicate that MHV cross-species transmission evolved by virus recognition of phylogenetic homologues of the natural receptor. Expression of hBgp also appeared to permit low levels of MHV-A59 infection. This observation is consistent with previously published data, and is suggestive of an extremely low affinity or inefficient utilization of the hBgp1 homologues by MHV-A59 (Chen, D.S., et al., 1997). Since the human CEA genes are highly conserved it remains possible, if not likely, that MHV-H2 may also recognize other CEA gene family members for docking and entry. This is currently under investigation.

Transplantation of organs has become the preferred treatment for end-stage organ failure. Chronic shortages in human organs coupled with improved immunosuppressive therapies have established the feasibility of using animal organs in human transplantation as a life-saving alternative treatment (Michler, 1996). Using an in vitro model, which may be reflective of mixed species tissues that are present in human xenograph recipients, we have demonstrated that host range mutants emerge which replicate efficiently in the new

host species. While further studies are warranted, xenograph recipients may represent excellent ecosystems for the emergence and cross-species transmission of animal viruses into the human host.

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