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Enterotropic Strains of Mouse Coronavirus Differ in Their Use of Murine Carcinoembryonic Antigen-Related Glycoprotein Receptors

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Enterotropic mouse hepatitis virus strains (MHV-RI and MHV-Y) replicate in the intestine and rarely disseminate to other tissues, unlike respiratory MHV strains. Murine carcinoembryonic antigen-related glycoproteins (MHVR and mmCGM$_2$) are expressed in many murine tissues and serve as receptors for respiratory MHV strains. To assess the role of receptors in the limited tissue tropism of enterotropic MHV strains, the permissiveness of MHVR- and mmCGM$_2$-expressing cell lines and peritoneal exudate cells from BALB/c and SJL mice for MHV-RI and MHV-Y replication was determined. MHVR-transfected BHK cells were susceptible to infection with both MHV-RI and MHV-Y. Additionally, the anti-MHVR monoclonal antibody CC1 blocked MHV-RI and MHV-Y infection. mmCGM$_2$-transfected BHK cells were susceptible to infection with MHV-Y, but not MHV-RI. Peritoneal exudate cells from BALB/c mice were susceptible to infection with MHV-Y and MHV-RI, whereas peritoneal exudate cells from SJL mice were susceptible to infection with MHV-Y but not MHV-RI. These results indicate that MHV-RI probably uses a different receptor than other MHV strains to infect SJL mice and that receptors are probably not the primary determinant of the limited tissue tropism of enterotropic MHV strains.

Mouse hepatitis virus (MHV), a singular name for several murine coronaviruses, causes a wide spectrum of clinical outcomes ranging from subclinical infection to enteritis, hepatitis, encephalitis, and death. MHV strains can be segregated, based on their site of initial replication, into two biotypes: respiratory (polytropic) and enterotropic. Following oronasal inoculation, respiratory MHV strains, including MHV-1, -3, -A59, and -JHM, initiate infection in the nasal mucosa. Respiratory MHV strains then disseminate to multiple organs via the blood, lymphatics, or olfactory nerves if the mouse is sufficiently susceptible due to age, genotype, or immune status (*2, 12*). In contrast to respiratory MHV strains which rarely replicate in the intestinal mucosa, enterotropic MHV strains such as MHV-Y and MHV-RI replicate preferentially in the intestinal mucosa (*3, 6*). MHV biotypes elicit disease in their preferred target organs regardless of route of inoculation or immune status of the host. Neonatal mice inoculated oronasally, intracerebrally, or intraperitoneally with enterotropic MHV-Y rapidly develop enteritis, with minimal involvement of other organs (*4*; Barthold and Smith, personal communication). Athymic nude mice naturally infected with enterotropic MHV-RI developed lesions largely restricted to the intestine, unlike the disseminated pattern of infection that occurs in athymic nude mice infected with respiratory strains of MHV (*9*). All ages and genotypes of mice are susceptible to infection with enterotropic MHV-Y but only young mice develop disease in the form of enteritis (*4*).

Two murine carcinoembryonic antigen-related (mmCGM) alleles have been identified which encode glycoproteins that function as cell surface receptors for MHV (*11, 14, 15, 25, 26, 28, 29*). CGMs are members of the biliary glycoprotein subgroup of the carcinoembryonic antigen-related glycoprotein family in the immunoglobulin superfamily (*10, 20, 24*). Both alleles of the MHV receptor encode proteins that have a similar structure with a leader peptide, four immunoglobulin-like domains, a transmembrane domain, and a cytoplasmic domain. Differential splicing of these two isoforms generates proteins containing either two or four of the immunoglobulin-like domains (*14, 15, 28, 29*). The MHVR four domain and two domain isoforms [MHVR(4d) and MHVR(2d)] are present in many tissues, including the liver, intestine, and brain of BALB/c, C57BL/6, and C3H mice, whereas the mmCGM$_2$(4d) and mmCGM$_2$(2d) isoforms are present in tissues of SJL mice (*14, 28, 29*). Outbred CD-1 mice express the MHVR(4d), MHVR(2d), mmCGM$_2$(4d), and mmCGM$_2$(2d) isoforms (*10, 19, 20, 24*). The MHVR and mmCGM$_2$ isoforms have extensive differences in domain 1, the domain to which MHV-A59 binds (*16*). The presence or absence of specific mmCGM isoforms in particular tissues and inbred mouse strains has been linked to the susceptibility or resistance of these tissues and mouse strains to infection with some respiratory MHV strains (*11, 14, 25, 26, 28, 29*). The present study was undertaken to determine if a correlation exists between the capacity of two mmCGM receptor isoforms to serve as receptors for enterotropic MHV strains in cell culture with the susceptibility of mice expressing these isoforms to enterotropic MHV infection.

Enterotropic MHV strains, including MHV-RI and MHV-

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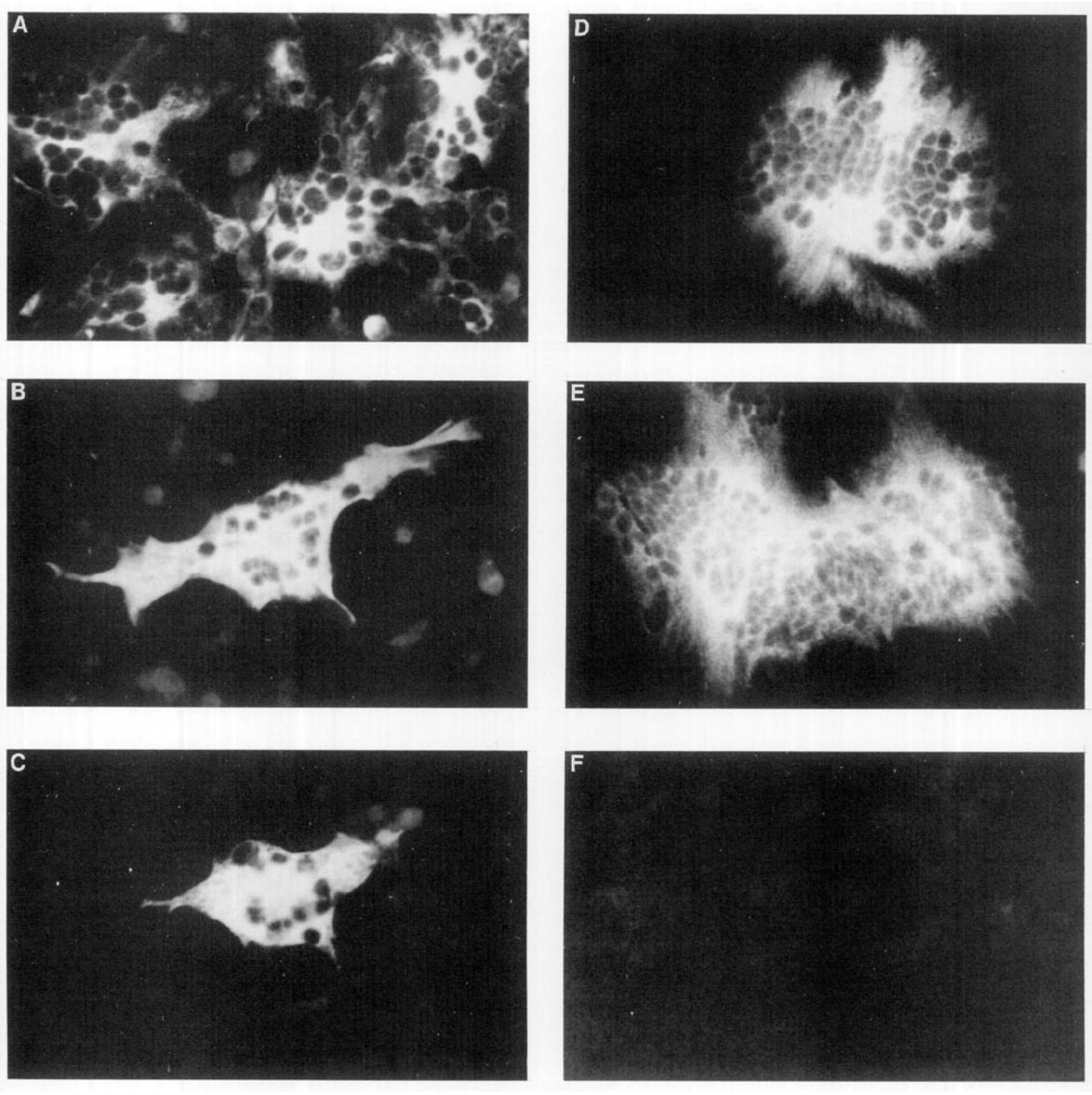

FIG. 1. Immunofluorescent staining of coronaviral antigens in MHV-infected BHK_{MHVR} and BHK_{mmCGM2} cells. Cells were fixed in acetone and viral antigens were detected with anti-sialodacryoadenitis virus hyperimmune mouse ascitic fluid and FITC-conjugated goat anti-mouse IgG (Antibodies Inc., Davis, CA). (A) BHK_{MHVR} cells infected with MHV-A59 at an m.o.i. of 0.1 at 18 hr postinoculation. (B) BHK_{MHVR} cells infected with MHV-Y at an m.o.i. of 4.5 at 18 hr postinoculation. (C) BHK_{MHVR} cells infected with MHV-RI at an m.o.i. of 3 at 18 hr postinoculation. (D) BHK_{mmCGM2} cells infected with MHV-A59 at an m.o.i. of 5 at 24 hr postinoculation. (E) BHK_{mmCGM2} cells infected with MHV-Y at an m.o.i. of 5 at 16 hr postinoculation. (F) BHK_{mmCGM2} cells infected with MHV-RI at an m.o.i. of 5 at 24 hr postinoculation. Magnification, 200×.

Y, have traditionally been difficult to cultivate in cultured cells. MHV-Y was isolated in NCTC 1469 cells from the intestine of an infant mouse with acute typhlocolitis and MHV-RI was isolated in CMT-93 cells from a nude mouse intestine (*8, 9*). The murine macrophage-derived cell line J774A.1, derived from a BALB/c mouse tumor, was found to be capable of producing substantial yields of all MHV strains tested including MHV-Y, MHV-RI, and MHV-A59. J774A.1 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 medium containing 10% fetal bovine serum. J774A.1 cells have been shown to express MHVR glycoproteins on their cell surface (*13*). To determine whether MHV-RI and MHV-Y can use MHVR glycoproteins as receptors, J774A.1 cells were pretreated with a monoclonal antibody directed against MHVR (MAb-CC1) and then challenged with 100 $TCID_{50}$ of MHV-RI or MHV-Y. MAb-CC1 is specific for an epitope on the N terminal domain of MHVR

TABLE 1

GROWTH OF MHV IN BHK_{MHVR} AND BHK_{mmCGM2} CELLS

Hours postinoculation[a]	BHK_{MHVR}			BHK_{mmCGM2}		
	A59	RI	Y	A59	RI	Y
0	6.50[b]	6.25	5.25	ND[c]	ND	ND
3	3.50	3.50	≤2.50	≤2.50	2.75	2.75
24	7.50	5.50	3.50	6.75	≤2.50	3.50
48	ND	6.50	4.25	ND	≤2.50	3.25

[a] Cells were infected at an m.o.i. of 4. Two hours postinoculation, cell layers were washed and fresh medium was added. At 24 h postinoculation, supernatants were removed and fresh medium was added to the cell layers.

[b] Titers are represented as $\log_{10}$ $TCID_{50}$/ml in J774A.1 cells.

[c] Not determined.

that is not present in $mmCGM_2$ (*16, 21, 26*). Pretreatment of J774A.1 cells with MAb-CC1 blocked infection with both MHV-RI and MHV-Y as determined by the lack of visible cytopathic effects (CPE) or viral antigens at 48 h postinoculation (data not shown). Pretreatment of J774A.1 cells with MAb-CC1 also blocked MHV-A59 infection of J774A.1 cells, though the concentration of antibody needed to block infection with MHV-A59 was substantially greater than that needed to block MHV-RI or MHV-Y infection. Since MAb-CC1 completely blocked infection of J774A.1 cells with all three MHV strains, MHVR glycoproteins are probably the receptors used by these MHV strains to initiate infection in J774A.1 cells.

To confirm that MHV-RI and MHV-Y were using the MHVR isoform and that binding of MHV to an unknown receptor was not sterically hindered by MAb-CC1 binding, BHK cells stably transfected with a plasmid encoding *MHVR (4d) (BHK_{MHVR}) were challenged with virus. Both* MHV-RI and MHV-Y infected BHK_{MHVR} cells and caused cell fusion (Fig. 1). All syncytia contained viral antigen as detected by immunofluorescence, but syncytia were much less numerous in MHV-RI- and MHV-Y-infected cultures than in MHV-A59-infected cultures even when MOIs were 50- to 100-fold higher for MHV-RI and MHV-Y than for MHV-A59. While the proportion of fused cells was similar in cultures infected with MHV-RI or MHV-Y, the infectious virus yields differed substantially (Table 1). In multiple experiments, MHV-RI produced viral yields several orders of magnitudes greater than those of MHV-Y. Attempts to increase the titers of MHV-Y produced in BHK_{MHVR} cells, by multiple passages of MHV-Y in BHK_{MHVR} cells, were unsuccessful. These cells continued to yield less than 10^5 $TCID_{50}$/ml of MHV-Y. Equivalent titers of MHV-Y were produced in BHK_{MHVR} cells using stocks which had been passed once or 10 times in tissue culture, indicating that the low titers observed were not simply the result of studying a noncell culture-adapted strain of MHV (data not shown). Equivalent titers of MHV-Y were produced in J774A.1 and L2.Percy cells at 33, 37, and 39°, indicating that the low viral titers measured in BHK_{MHVR} cells at 37° were not the result of studying a temperature-sensitive strain of MHV (data not shown). MHV-RI and MHV-Y did not infect receptor-negative BHK cells based on the lack of visible CPE and viral antigen at 24 hr postinoculation. Pretreatment of BHK_{MHVR} cells with anti-MHVR MAb-CC1 completely blocked infection with MHV-RI and MHV-Y as determined by the lack of CPE and viral antigens at 24 hr postinoculation (data not shown). Clearly, both MHV-RI and MHV-Y are capable of using the MHVR glycoprotein to initiate productive *infection.*

Infection of adult SJL mice with either enterotropic or respiratory MHV strains produces no disease. Respiratory MHV strains infect the nasal mucosa of adult SJL mice and produce low titers of virus (*3, 7*). On the other hand, enterotropic MHV strains infect the intestinal mucosa of adult SJL mice and produce high viral yields in the ascending colons (*4*; unpublished data). To determine whether MHV-RI and MHV-Y can use the $mmCGM_2$ receptor isoform to initiate productive MHV infections, BHK cells stably transfected with a plasmid encoding $mmCGM_2$(2d) [BHK_{mmCGM2}] were challenged with virus. Both MHV-Y and MHV-A59 infected BHK_{mmCGM2} cells, producing numerous large syncytia with greater than 75% of the cells forming syncytia by 24 hr postinoculation (Fig. 1). Even though the majority of cells in MHV-Y-infected cultures formed syncytia, only low yields of infectious MHV-Y were produced (Table 1). Attempts to increase the titers by multiple passages of MHV-Y in BHK_{mmCGM2} cells proved to be unsuccessful, as the cells continued to yield less than 10^5 $TCID_{50}$/ml of MHV-Y. In four separate experiments, neither visible CPE nor viral antigen was detectable in BHK_{mmCGM2} cells inoculated with MHV-RI at *m.o.i.s as high as 5 and after infection intervals as long* as 72 hr (Fig. 1). As predicted by the lack of CPE and viral antigen, MHV-RI did not produce detectable infectious virus in BHK_{mmCGM2} cells (Table 1). It is important to note that the inability of BHK_{mmCGM2} cells to support MHV-RI replication is not due to the inability of transfected BHK cells to replicate MHV-RI, since BHK_{MHVR} cells support replication of MHV-RI. Therefore the block in MHV-RI replication in BHK_{mmCGM2} cells appears to be at the receptor level, either at the binding or the internalization event.

Preliminary studies have shown that infant and adult SJL mice are susceptible to MHV-RI infection (unpublished data). In an attempt to identify a receptor for MHV-RI on SJL cells, thioglycollate-stimulated peritoneal exudate cells (PEC) from SJL mice were challenged with MHV-RI. PEC were harvested from 6-week-old mice 3 to 4 days after intraperitoneal inoculation with 1 ml of sterile thioglycollate broth (REMEL, Lexena, KS) and were maintained in RPMI medium containing 5% FBS and 50 μM 2-mercaptoethanol on glass chamber slides (Nunc Inc, Naperville, IL). SJL PEC did not yield detectable MHV-RI

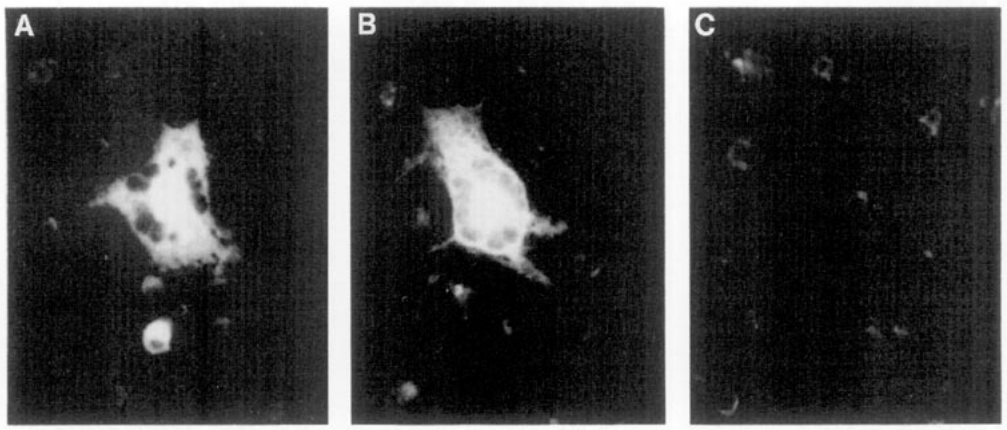

FIG. 2. Immunofluorescent staining of coronaviral antigens in MHV-infected thioglycollate-elicited peritoneal exudate cells from SJL mice. Cells were fixed in acetone and viral antigens were detected with anti-sialodacryoadenitis virus hyperimmune mouse ascitic fluid and FITC-conjugated goat anti-mouse F(ab')$_2$ fragment of IgG (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). (A) SJL peritoneal exudate cells infected with MHV-A59 at an m.o.i. of 2.5 at 48 hr postinoculation. (B) SJL peritoneal exudate cells infected with MHV-Y at an m.o.i. of 2.5 at 48 hr postinoculation. (C) SJL peritoneal exudate cells infected with MHV-RI at an m.o.i. of 2.5 at 48 hr postinoculation. Magnification, 200×.

antigen or infectious virus (Fig. 2 and Table 2). In contrast, inoculation of SJL PEC with MHV-Y resulted in detectable viral antigen in single cells and small syncytia (Fig. 2), although no infectious virus was detectable at 24, 48, 72, or 96 hr postinoculation (Table 2). Inoculation of SJL PEC with MHV-A59 resulted in virus antigen production in single cells and small syncytia (Fig. 2). Unlike MHV-Y, infectious MHV-A59 was produced in SJL PEC with peak yields of 10^5 $TCID_{50}$/ml at 48 hr postinoculation (Table 2). As expected, MHV-A59, MHV-RI, and MHV-Y infected PEC from BALB/c mice (Table 2). Peak virus yields in BALB/c PEC occurred within 24 hr postinoculation with both MHV-Y and MHV-A59 but were not reached until 72 hr postinoculation with MHV-RI which yielded $10^{6.25}$ $TCID_{50}$/ml. These studies indicate that MHV-RI probably uses a molecule other than $mmCGM_2$ as a receptor in SJL mice. They further suggest that this molecule is either not expressed on SJL PEC membranes or that binding does not lead to productive infection of these cells. It is clear from these studies as well as those of other investigators (*17, 18, 22*) that SJL PEC are extremely resistant to infection with MHV-A59, JHM, and Y, but a small proportion of SJL PEC can be infected producing detectable viral antigen and/or low titers of infectious virus. The ability of a respiratory MHV strain, MHV-A59, to replicate in SJL PEC harvested from 6-week-old mice is not completely unexpected as virus replicates locally in the nasal mucosa of 6-week-old SJL mice inoculated intranasally with MHV-JHM or MHV-S and resistance to MHV-JHM-induced encephalitis following intracerebral inoculation has been shown to be incomplete in 6-week-old SJL mice (*5, 7, 23*).

TABLE 2

GROWTH OF MHV IN PERITONEAL EXUDATE CELLS

	BALB/c		SJL	
Virus	Yield	Antigen	Yield	Antigen
MHV-A59	9.25[a]	>90[b]	5.00	<10
MHV-RI	3.37	20	≤1.5	0
MHV-Y	4.00	50	≤1.5	<10

[a] BALB/c cells were infected at an m.o.i. of 0.1 and SJL cells were infected at an m.o.i. of 2.5. Two hours postinoculation, cell layers were washed and fresh medium was added. Supernatants were removed from BALB/c cells 24 hr postinoculation and from SJL cells 48 hr postinoculation, and slides were acetone-fixed for immunofluorescent staining. Viral yields are represented as $\log_{10}$ $TCID_{50}$ in J774A.1 cells.

[b] Approximate percentage of viral antigen-containing cells as detected by immunofluorescence using mouse anti-sialodacryoadenitis virus hyperimmune mouse ascitic fluid and FITC-conjugated goat anti-mouse F(ab')$_2$ fragment of IgG (Kirkegaard and Perry, Gaithersburg, MD).

Both respiratory and enterotropic MHV strains use MHVR to initiate productive infection in cell culture, but care must be taken when extrapolating findings obtained in cell culture to what is occurring in the whole animal. While cell culture has allowed us to identify mmCGM isoforms that can serve as functional MHV receptors, it will be important to ascertain the levels and distribution of each receptor protein in various tissues of the animal before drawing conclusions about the importance of a single protein as a determinant in the pathogenesis of MHV infections. Initiation of infection is only the first step in the virus life cycle. While many cell types may express functional receptors, the virus may not have access to these cells during natural infection. Also, not all cell types that express functional receptors may be capable of supporting the complete virus life cycle and producing infectious virus (*1, 27*). While MHV-RI and MHV-Y use MHVR glycoproteins to initiate infection, low or undetectable concentrations of virus are produced by most cell lines used to grow respiratory MHV strains (unpublished data),

indicating that the limited tissue tropism of enterotropic MHV strains may be due to the absence of cellular factors, other than receptors, necessary to complete the viral life cycle in tissues other than the intestine. More detailed studies of the cellular factors necessary to replicate respiratory and enterotropic MHV strains are necessary to understand better the differences in the pathogenesis of these different MHV biotypes.

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