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Difference in Bgp-independent fusion activity among mouse hepatitis viruses

Brief Report

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Summary. Mouse hepatitis virus (MHV) utilizes a mouse biliary glycoprotein (Bgp) as a receptor. Co-cultivation of MHV-nonpermissive hamster BHK cells devoid of mouse Bgp with mouse DBT cells infected with MHV-A59 or JHMV induces syncytia formation on BHK cells (Bgp-independent fusion). This study shows the difference in Bgp-independent fusion activity among various MHV strains. Under a phase contrast microscopy, JHMV (cl-2, sp-4) induced the Bgp-independent syncytia on BHK cells similar to those observed on DBT cells, while such syncytia were not seen with the infection of other MHV strains (MHV-1, MHV-3, MHV-A59, MHV-S, srr7, srr11 and srr18). Tiny syncytia detectable only by immunofluorescence were produced with the latter MHV strains except for srr7 which failed to produce syncytia. MHVs except for srr7 grew in BHK cells after Bgp-independent infection. The Bgp-independent fusion by JHMV was inhibited either by anti-S1 or anti-S2 antibodies. These results showed that the JHMV spike protein had a remarkably high Bgp-independent fusion activity.

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Mouse hepatitis virus (MHV) belongs to coronaviruses that are enveloped, positive stranded RNA viruses [24]. A variety of strains are included in MHV and they show different organ tropism and type of disease [20]. MHV has a genome with about 31 kilobases which encodes 4 to 5 structural proteins and several non-structural proteins [24].

The spike protruding from MHV virion is composed of two or three molecules of the spike (S) protein, each of which is a heterodimer consisting of S1 and S2 subunits. The S1 and S2 are derived from the N-terminal and C-terminal halves of the S protein after cleavage of the precursor S protein [25]. The S protein is responsible for the receptor-binding and fusion activities. The receptor-binding site is located in the N-terminal region of the S1 composed of 330 amino acids

(S1N330) in which a conformational structure plays an important role [12, 26], while the S2 is not involved in this activity [27]. Several different regions and amino acid residues in the S2 have been reported to be important for fusion activity [3, 7, 9, 13], although it is unclear which of those are actually involved in the fusion event.

Biliary glycoprotein (Bgp) 1, a member of carcinoembryonic antigen (CEA) gene family, serves as a highly functional receptor for MHV [5]. Several different Bgp1 isoforms are generated by an alternative splicing [6] and expressed in the liver, brain and other organs. The Bgp1 has two allelic forms. $Bgp1^a$ and $Bgp1^b$, expressed in MHV-susceptible BALB/c and resistant SJL mice, respectively [6, 31]. Both of these proteins retain the MHV receptor function, although the $Bgp1^a$ has much higher virus-binding activity than does the $Bgp1^b$ [19]. Such difference could result in the difference of MHV susceptibility in the whole animal level [18]. Two other species of protein, Bgp2 [17] and brain specific CEA [4], both of which are CEA members, have been reported to work as MHV receptor in mice.

MHV-4 (JHMV) and MHV-A59 are known to infect cells lacking MHV-specific receptors, i.e., Bgp-independent infection [8, 16]. These viruses fail to directly infect Bgp-negative BHK 21 (BHK) cells, but infect them after co-cultivation with the Bgp-positive DBT cells infected with these viruses [8, 16]. The S protein is involved in the Bgp-independent infection and fusion, since the antibodies against the S protein prevented the infection [16] and the expression of the S protein alone induced fusion on cells devoid of Bgp [8, 29]. In this paper, we compared the Bgp-independent fusion activity of various MHV strains.

MHV strains MHV-1, MHV-3, MHV-S, MHV-A59 [10, 20], JHMV cl-2 [30], sp-4[28] as well as 3 different soluble receptor-resistant (srr) variants derived from cl-2 [20] were used in this study. Of these the amino acid sequences of A59 [15] and JHMV cl-2 [29] S proteins have been reported. Sp-4 S has a 141-amino acid deletion in the hypervariable region of the cl-2 S1 [23]. Srr 7 and srr18 have amino acid mutations at positions 1114(Leu→Phe) and 1163(Cys→His) in the S2, respectively. Srr 11 has a mutation at position of 65 (Leu→His) in the S1. To see the Bgp-independent fusion activity, DBT cells cultured in 12 well-plate (Corning) were infected with those viruses at an m.o.i. 1 and incubated at 37 °C for 1 h. After 3 times of washing with Dulbecco's modified minimal essential medium (DMEM, Nissui, Tokyo), infected cells were cultured in 1 ml of DMEM supplemented with 5% fetal calf serum (FCS, Gibco) for 3 to 4 h. The cells were then trypsinized and 10^4 cells were overlaid onto BHK cell monolayer cultured in 12 well-plate (1×10^6 cells). The syncytia of BHK cells were examined by a phase contrast microscopy during 48 h after overlay of MHV-infected DBT cells. Viral antigen was also examined by immunofluorescence using anti-S1 MAbs [11]. Only JHMV cl-2 and sp-4 induced the Bgp-independent syncytia on BHK cells detectable by a phase contrast microscopy. The syncytia could be observed from about 12 h after overlay. The syncytia formed by cl-2 were significantly larger than those by sp-4 (Fig. 1A and B). This size difference observed on BHK cells between cl-2 and sp-4 was in good agreement with the size difference in

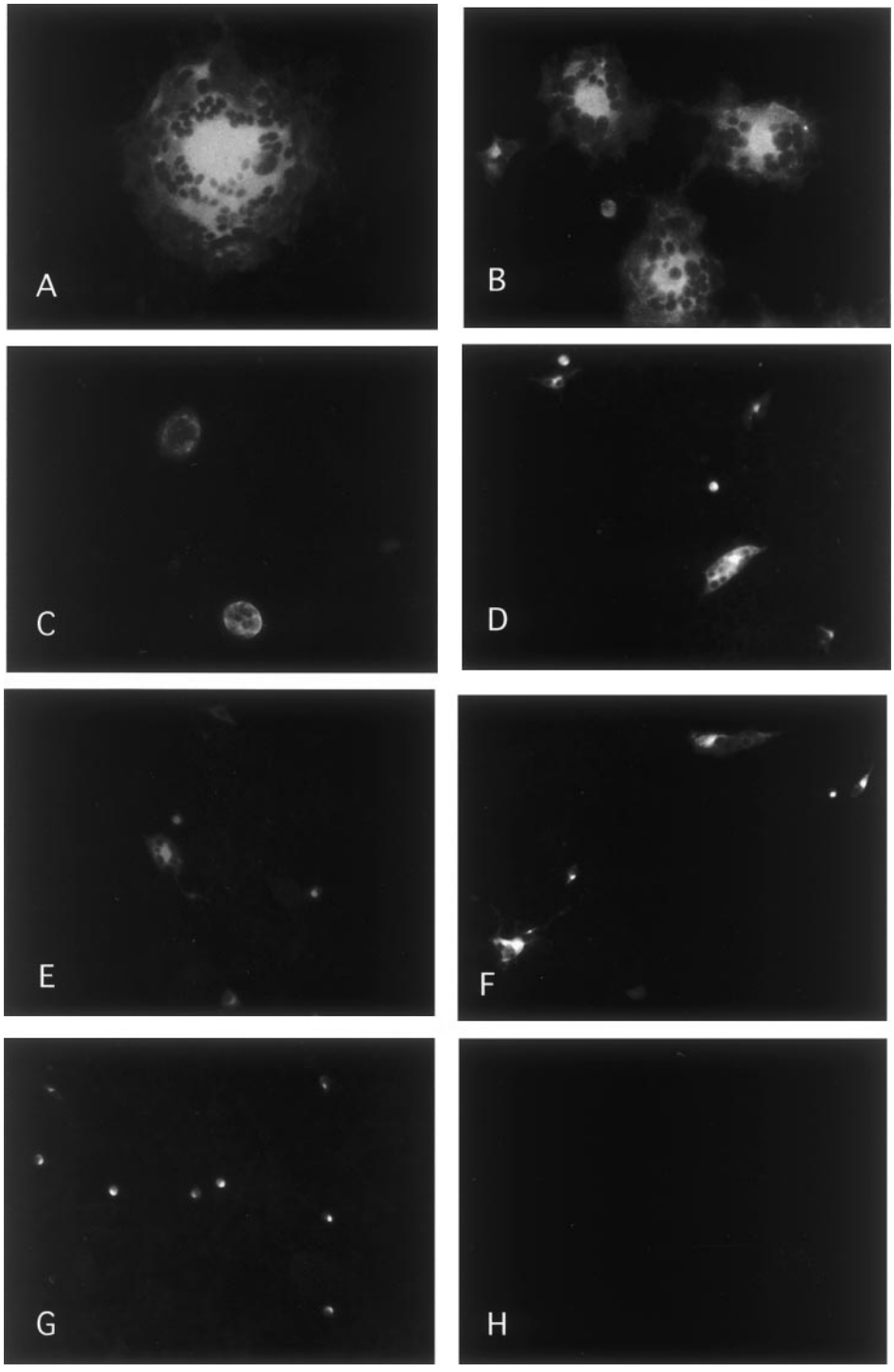

Fig. 1. Bgp-independent syncytia formation by MHV strains on BHK cells. Ten thousands of DBT cells infected with JHMV cl-2 (**A**) JHMV sp-4 (**B**), MHV-A59 (**C**), MHV-S (**D**), MHV-1 (**E**), srr11 (**F**) srr7 (**G**) or mock-infected DBT cells (**H**) were overlaid onto BHK cell monolayer and incubated for 24 h. Cells were fixed with methanol and allowed to react with anti-S1 MAbs and anti-mouse IgG labelled with FITC for immunofluorescence

plaque produced on DBT cells [30]. Interestingly, no syncytia formation was demonstrated on BHK cells with other strains or srr mutants under the phase contrast microscopy, though all of them produced syncytia on Bgp-positive DBT cells [10]. MHV-A59 induced tiny syncytia on BHK cells comprising roughly 20 to 30 cells detectable only by immunofluorescence (Fig. 1C). Other MHV strains, MHV-1, MHV-3 and MHV-S produced tinier syncytia consisting of less than 10 cells (Fig. 1D and E). Srr mutants have also failed to induce syncytia comparable to those produced by wild type cl-2. Srr11 caused syncytia including about 10 to 15 cells (Fig. 1G). Srr18 showed very tiny syncytia consisting of less than 10 cells. No obvious syncytia was observed on BHK cells overlaid with srr7-infected DBT cells. Single cells were MHV antigen-positive (Fig. 1F). These single cells presumably represented srr7-infected DBT cells overlaid on BHK cell monolayer.

We have examined whether these viruses multiply in BHK cells. DBT cells infected with JHMV-cl-2, MHV-A59 and 3 srr mutants were either overlaid onto BHK cell monolayer as described above or allowed to seed alone in the 12-well plate and virus growth in these cells was examined. As shown in Fig. 2, all viruses except srr7 grew significantly higher in BHK cells co-cultured with MHV-infected DBT cells than in DBT cells alone, indicating that all viruses examined but srr7 multiplied in BHK cells. Though cl-2 produced significantly larger syncytia on BHK cells in a Bgp-independent manner as shown in Fig. 1, it replicated to almost the same extent as other viruses. No significant difference in the growth on BHK cells between cl-2 and other MHV strains can not be accounted for at present. The cl-2 S protein could have a very strong Bgp-independent fusion activity, but its replication in BHK cells could be less efficient than the other MHV strains. No replication of srr7 in BHK may be due to its failure to spread from infected DBT cells to BHK cells.

Next we have examined whether the anti-S1 and anti-S2 antibodies inhibit the Bgp-independent fusion. We used neutralizing MAbs 3, 13 and 93 recognizing the S1N330 as well as MAbs 78 and 85 recognizing other regions in the S1 [12]. Anti-S2 antibodies with neutralizing activity were produced using rabbits. Rabbits were immunized with a synthetic peptide consisting of amino acid sequence NESPLLGCIGSTCAED which encompassed an immunodominant epitope in the S2 recognized by a neutralizing MAb 5B19.5 [14]. The rabbit sera were then affinity-purified using the synthetic peptide. BHK cell monolayer overlaid with cl-2-infected DBT cells that produced about 100 syncytia was cultured in 10-fold serial dilutions of the anti-S1 and anti-S2 antibodies. Cells were stained with Giemsa solution at 24 to 36 h after infection and the syncytium number was counted under the light microscopy. The inhibition of syncytium formation was calculated in comparison with the syncytium number formed in the absence of neutralizing antibodies. Standard neutralization test using cl-2 was also performed with these antibodies on DBT cells as described previously [30]. As shown in Fig. 3, all of these antibodies showed fusion inhibition (FI) activity, though the FI titers were variable; anti-S1 MAbs showed 10 to 10^4 higher FI and viral neutralization activities compared with anti-S2 antibodies. The antibodies with higher neutralization titer exhibited the higher FI titer. This could imply that

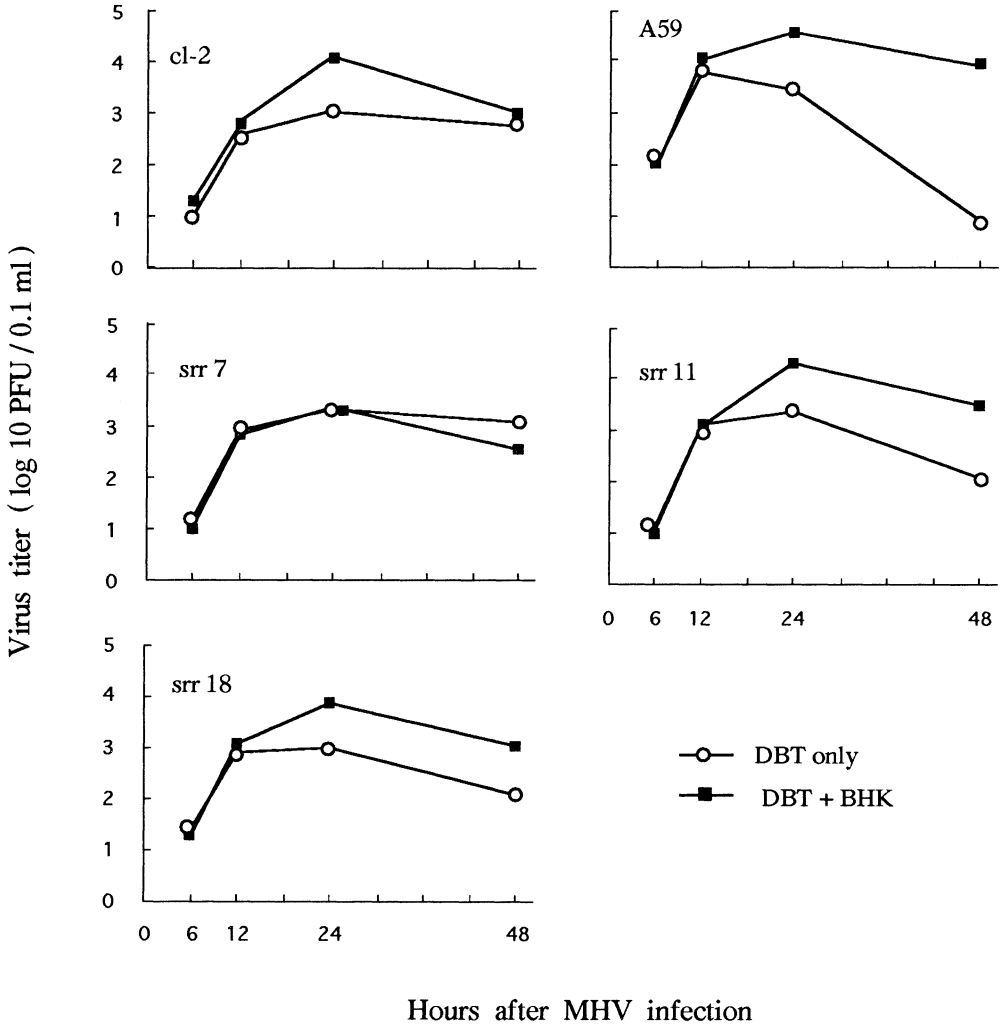

Fig. 2. Growth of MHV in BHK cells after Bgp-independent infection. Ten thousands of DBT cells infected with JHMV cl-2, MHV-A59, srr7, srr11 or srr18 were overlaied on 1×10^6BHK cells or allowed to seed alone in 12-well plate. At intervals after overlay, virus titers in the cultures were measured by plaque assay

the attachment of the S protein to an unidentified receptor on BHK cells, which is conceibably prevented by the anti-Si MAbs (3, 13 and 93) recognizing the receptor-binding domain on the S, is an inevitable step for the Bgp-independent fusion events or that the S1 is more profoundly involved in the Bgp-independent fusion activity than the S2. These results appear to suggest that both S1 and S2 are involved in the Bgp-independent fusion activity.

On DBT cells MHV-A59 and MHV-3 produce large plaques similar to those produced by cl-2, while other MHVs used in this study produce smaller plaques similar in size to those produced by sp-4. The plaque is composed of a single, large syncytium. This indicates that all these MHVs have a potential to induce fusion on

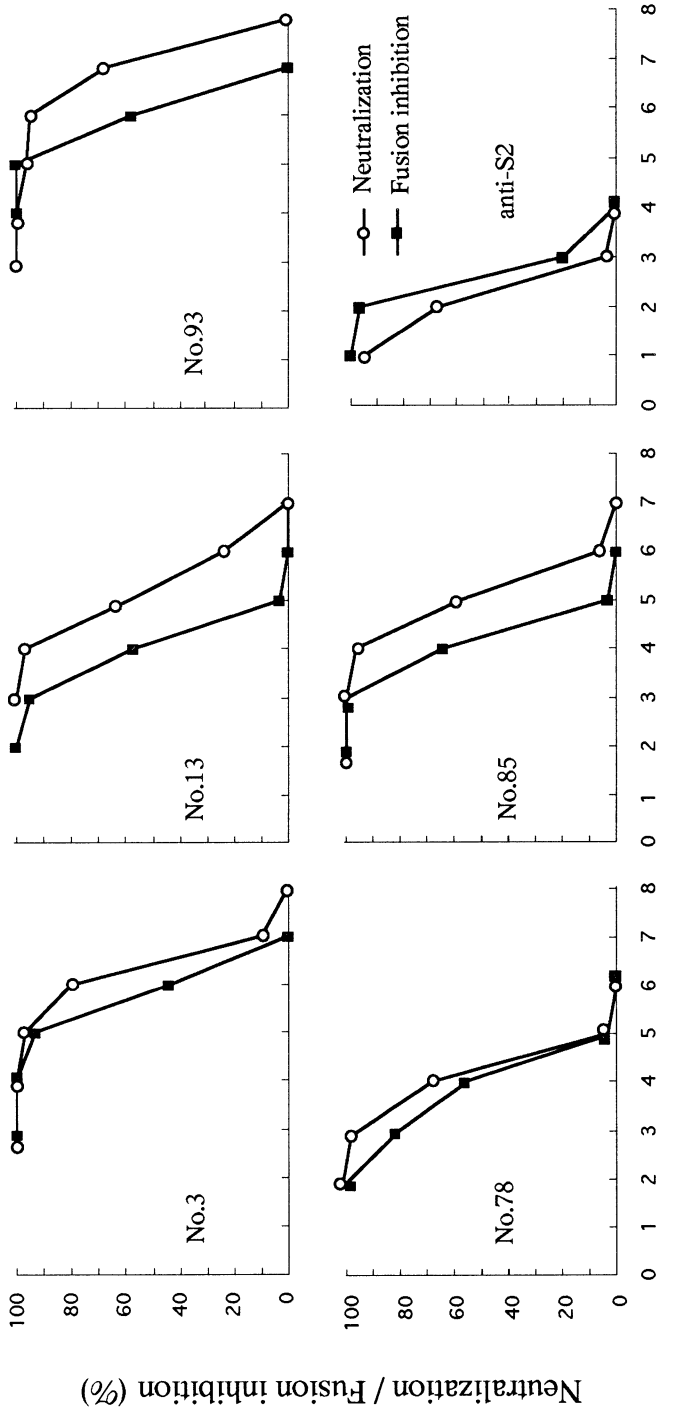

Fig. 3. Fusion inhibition and neutralization activities of anti-S1 and anti-S2 antibodies. Anti-S1 MAbs and anti-S2 rabbit serum were serially 10 fold diluted and examined for fusion inhibition and neutralization as described in the text

Bgp-positive cells. Present study showed that JHMV (cl-2 and sp-4) could induce syncytia on BHK cells detectable by microscopy, whereas the other MHVs and srr mutants failed. These findings have indicated that JHMV, particularly cl-2 with a large S protein [30], has a strong Bgp-independent fusion activity. The high fusogenicity of JHMV was also reported by Gallagher [7] who described that exogenously added JHMV viral particles had a strikingly high fusion activity on Bgp-positive cells as compared with MHV-A59.

The srr mutants showed extremely reduced Bgp-independent fusogenicity in spite of their high fusogenicity on Bgp-positive DBT cells [21]. This suggests that mutated amino acids in the srr mutants could play a critical role for the Bgp-independent fusion. Srr11 with a mutation at a position 65 in the S1 has a low receptor-binding activity [21] which also may affect on the fusion activity in Bgp-negative cells. Srr7 and srr18 have mutations in the amino acids at positions 1114 and 1163 in the S2, respectively [21]. Gallagher et al. reported that both of these residues were involved in the fusion on Bgp-positive cells [7, 9]. Their mutant virus which had mutations at a position of 1114 as well as two other positions showed an acid-pH dependent fusion activity on Bgp-positive cells [9]. This virus had a reduced fusion activity on Bgp-negative cells [16]. Gallagher also reported the importance of cystein residue at a position 1163 of JHMV S protein for syncytia formation on Bgp-positive cells using anti-cystein reagent [7]. Both amino acids at positions 1114 and 1163 are conceivably important for overall fusion activity, whether it is Bgp-dependent or Bgp-independent.

Nash and Buchmeier reported that the MAb to Bgp1 prevented MHV-4 (JHMV) infection on Bgp-positive cells, yet it failed to prevent syncytia formation in infectious center assay on mouse DBT cells [16]. This implies that the Bgp1 is not necessary for syncytia formation. In the present study, we showed all the viruses except for JHMV failed to produce microscopically-detectable syncytia on BHK cells, while they produced syncytia on DBT. These results could suggest that the Bgp1 plays an important role in the syncytia formation by MHV strains but JHMV. Expression of the S proteins of these MHVs in Bgp-positive and Bgp-negative cells will clarify the importance of Bgp in syncytia formation.

Present study suggested that both S1 and S2 were critical for the Bgp-independent fusion. The S1 could be important for the binding to the molecule on BHK cells which is an alternative receptor for MHV. The S protein-receptor interaction is an inevitable step prior to fusion events. Such receptor is conceivably not so efficient as the Bgp and therefore work as a receptor only when large amounts of S protein expressed on MHV-infected DBT cells are in contact with it [15]. It is interesting to note that the recombinant MHV between JHMV and MHV-A59 arisen in a mixed culture of DBT and BHK cells as well as mutants derived from persistent infections are able to infect BHK and other non-murine cells devoid of murine Bgp [1, 2, 22]. Some of those mutants have recently been reported to utilize human CEA and Bgp as functional receptors [1]. The receptor protein on BHK cells for these mutants may also interact with the JHMV S protein expressed on DBT cells.

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