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Replication of Enterotropic and Polytropic Murine Coronaviruses in Cultured Cell Lines of Mouse Origin

Shigeru KYUWA[1)], Kazutaka OHSAWA[2)], Hiroshi SATO[2)], and Toru URANO[1)]

[1)]Division of Microbiology and Genetics, Center for Animal Resources and Development, Kumamoto University, Honjo 2–2–1, Kumamoto 960-0811, [2)]Laboratory Animal Center for Biomedical Research, Nagasaki University School of Medicine, Sakamoto 1–12–4, Nagasaki 852-8523, Japan

Abstract: *To understand the virus-cell interactions that occur during murine coronavirus infection, six murine cell lines (A3-1M, B16, CMT-93, DBT, IC-21 and J774A.1) were inoculated with eight murine coronaviruses, including prototype strains of both polytropic and enterotropic biotypes, and new isolates. All virus strains produced a cytopathic effect (CPE) with cell-to-cell fusion in B16, DBT, IC-21 and J774A.1 cells. The CPE was induced most rapidly in IC-21 cells and was visible microscopically in all cell lines tested. In contrast, the coronaviruses produced little CPE in A3-1M and CMT-93 cells. Although most virus-infected cells, except KQ3E-infected A3-1M, CMT-93 and J774A.1 cells, produced progeny viruses in the supernatants when assayed by plaque formation on DBT cells, the kinetics of viral replication were dependent on both the cell line and virus strain; replication of prototype strains was higher than that of new isolates. There was no significant difference in replication of enterotropic and polytropic strains. B16 cells supported the highest level of viral replication. To determine the sensitivity of the cell lines to murine coronaviruses, the 50% tissue culture infectious dose of the coronaviruses was determined with B16, DBT, IC-21 and J774A.1 cells, and compared to that with DBT cells. The results indicate that IC-21 cells were the most sensitive to murine coronaviruses. These data suggest that B16 and IC-21 cells are suitable for large-scale preparation and isolation of murine coronaviruses, respectively.*

Key words: *B16 cells, IC-21 cells, murine coronavirus, replication, $TCID_{50}$*

Introduction

Murine coronaviruses, which are usually called mouse hepatitis viruses (MHV), induce not only hepatitis, but also a wide spectrum of diseases ranging from subclinical to acute fatal infection in mice. Although the detailed mechanism of disease diversity is not fully understood, factors such as the virus strain, dose and route of infection, as well as age, genetic background and immune status of the host, have been shown to be involved in pathogenesis [8, 12, 15]. It has been proposed that MHV can be divided into two biotypes, termed polytropic and enterotropic strains, based on their initial site of replication [2], but the molecular mechanisms

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Address corresponding: S. Kyuwa, Department of Biomedical Science, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyoy-ku, Tokyo 113-8657, Japan

that determine these biotypes remain obscure.

Since the discovery of MHV-permissive cell lines, (e.g., delayed brain tumor (DBT) cells) [11], great advances have been made in the molecular biology and pathogenesis of MHV. On the other hand, little work has been carried out with enterotropic strains, considered to be the most prevalent in natural outbreaks in contemporary mouse colonies [13], since it has been difficult to prepare enterotropic coronaviruses due to the lack of an optimal *in vitro* replication system using cultured cell lines. In a recent study [9], eight murine cell lines were examined for the replication of enterotropic coronaviruses MHV-Y and MHV-RI, and the macrophage-like tumor cell line J774A.1 was deemed suitable for *in vitro* propagation and quantification of these viruses, but these authors did not examine DBT cells, which are commonly used for MHV replication by many workers. In addition, we have recently discovered some other coronavirus-permissive murine cell lines, which have not been tested with enterotropic strains [14, 19]. In the present study, the replication of eight strains of MHV, including prototype strains of both polytropic and enterotropic biotypes and new isolates from a natural infection, was examined in six murine cell lines *in vitro*.

Materials and Methods

Cell culture: A 129/SvJ-derived embryonic stem cell line, A3-1M, which had been passaged more than 40 times, was cultured as described previously, except that it was supplemented with 10% fetal calf serum (FCS) instead of 20% FCS [14, 18]. A C57BL/6-derived melanoma cell line, B16 [20], and a C57BL/icrf-derived rectal tumor cell line, CMT-93 [10], were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS. DBT, IC-21 and J774A.1 cells were cultured as described previously [16, 17, 24]. DBT is an astrocytoma cell line derived from CDF1 mice, and IC-21 and J774A.1 cells are macrophage-like tumor cell lines derived from C57BL/6 and BALB/c mice, respectively.

Viruses: MHV-RI [4] and MHV-Y [3] were obtained from Dr. Kunita (Novartis Pharmaceuticals, Tsukuba, Japan) and passaged in J774A.1 cells at least twice. MHV-A59 [18] and MHV-JHM [15] were propagated in DBT cells as described previously [11]. MHV-KU was isolated from a sentinel nude mouse in 1996 by using CMT-93 cells, and then passaged in DBT cells twice. KQ1E, KQ3E and KQ6E were isolated from three infant mice bred in separate cages by using DBT cells in November 1998, and then passaged twice.

Virus infection of cultured cell lines in vitro: Subconfluent cultures of each cell line were prepared in 12-well plates, and inoculated with murine coronavirus at a multiplicity of infection of 0.01. After adsorption for 60 min, the cultures were washed and then incubated at 37°C in 5% CO_2. Twelve, 24 and 48 hr later, a virus-induced cytopathic effect (CPE) was observed under a microscope. The supernatant was collected and stored at –80°C after removal of cell debris by centrifugation. Infected cells were fixed with methanol and then stained with Giemsa's solution. The severity of the CPE is expressed as the percentage of cells involved in MHV-induced CPE.

Plaque assay on DBT cells: The viral titer in the culture supernatants was determined by a conventional plaque assay on DBT cells [11] with minor modifications. Briefly, confluent cultures of DBT cells in 60-mm tissue culture dishes were inoculated with 0.2 m*l* of a 10-fold serial dilution of samples, and then adsorbed for 60 min. The cultures were overlaid with Eagle's minimum essential medium supplemented with 2% FCS, 1% tryptose phosphate broth and 0.5% agarose (Iwai, Tokyo, Japan), and then incubated at 37°C in 5% CO_2. Forty hours later, the cultures were overlaid with Eagle's minimum essential medium supplemented with 1% tryptose phosphate broth, 0.5% agarose and neutral red. The number of plaques was counted and expressed as plaque-forming units (PFU)/m*l*.

Relative sensitivity to MHV: The 50% tissue culture infectious dose ($TCID_{50}$) was determined as follows: subconfluent cultures of B16, DBT, IC-21 and J774A.1 cells were prepared in 96-well flat-bottom plates, and inoculated with 0.2 m*l* of a five-fold serial dilution of murine coronavirus samples. Forty-eight hours later, the cells were inspected under a microscope to observe the CPE. The relative sensitivity of each cell line to MHV was compared with that of DBT cells by means of the following formula: relative sensitivity to

MHV=log ($TCID_{50}$ titer determined in cell lines of interest/$TCID_{50}$ titer determined in DBT cells).

Results

Infection of cultured cell lines by prototype murine coronaviruses: Viral growth and virus-induced CPE were observed in six cell lines infected with polytropic (A59 and JHM) and enterotropic strains (RI and Y) of coronavirus until 48 hr after infection (Figs. 1 and 2). The virus-induced CPE was recognized first in IC-21 cells. The characteristic CPE was a formation of multinuclear giant cells as a result of cell-to-cell fusion (Fig. 1F). Subsequently, some infected cells began to detach, and almost all IC-21 cells had become detached at 48 hr post-infection. Although virus-infected B16, DBT, and J774A.1 cells underwent similar morphological changes with time, little virus-induced CPE was recognized in A3-1M or CMT-93 cells during the experiment (Figs. 1 and 2) and in the extended time period (data not shown). The kinetics and severity of the CPE were therefore dependent on the cell line. All of the prototype MHV grew in these cell lines. Enterotropic strains replicated well, not only in CMT-93 cells, a colon carcinoma cell line, but also in other MHV-permissive cells, but viral titers in the supernatants varied. Although replication of MHV-Y, in particular in A3-1M and CMT-93 cells, appeared poor, MHV-RI, which is also classified as an enterotropic biotype, showed a similar growth pattern to MHV-A59, a polytropic strain. Comparing viral growth among the cell lines, B16 and DBT cells supported good viral replication. In contrast, viral replication in CMT-93 cells was poor.

Infection of cultured cell lines with newly isolated viruses: The same experiments were conducted with

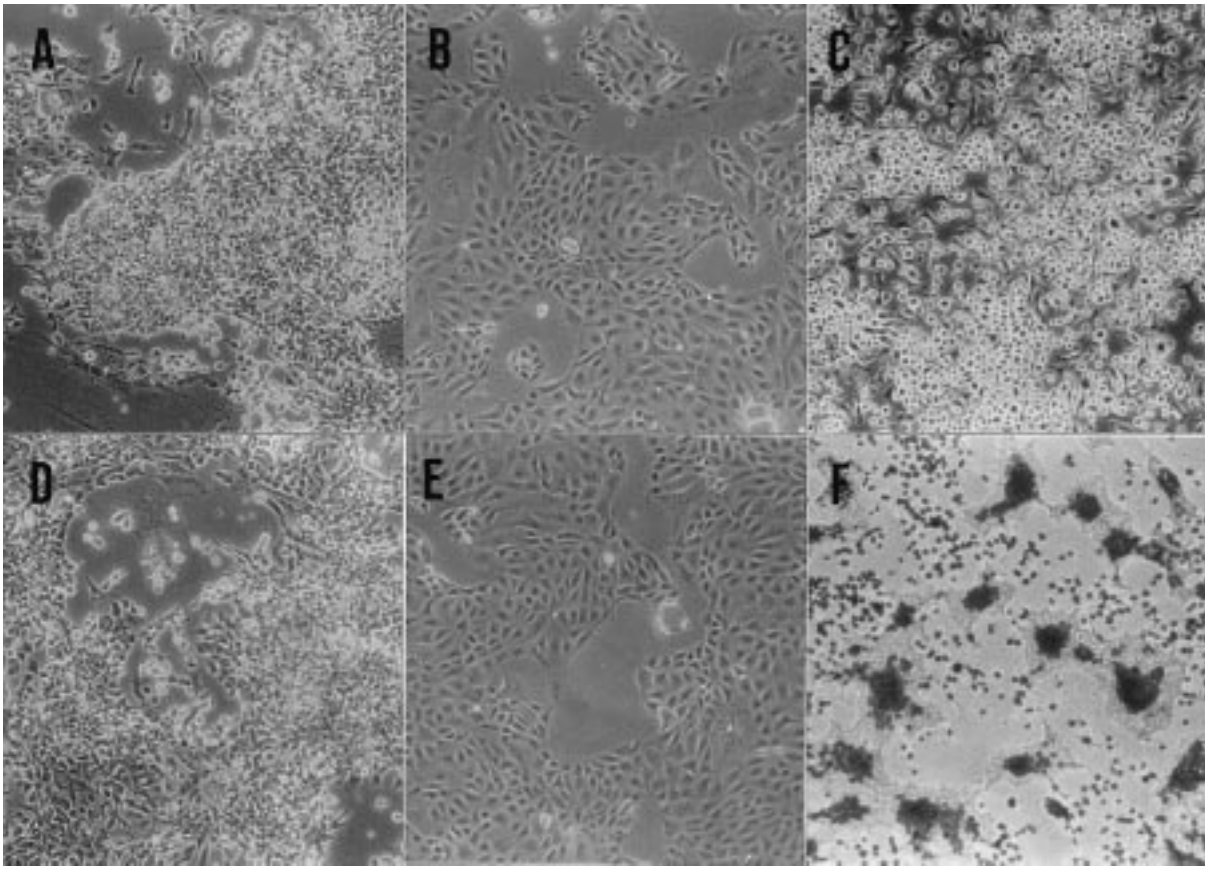

Fig. 1. Cultured cell lines of mouse origin with or without MHV infection. Uninfected A3-1M (A), CMT-93 (B), IC-21 cells (C), MHV-JHM-infected A3-1M (D) and CMT-93 cells (24 hr post-infection) (E), and MHV-RI-infected IC-21 cells (12 hr post-infection, Giemsa stain) (F).

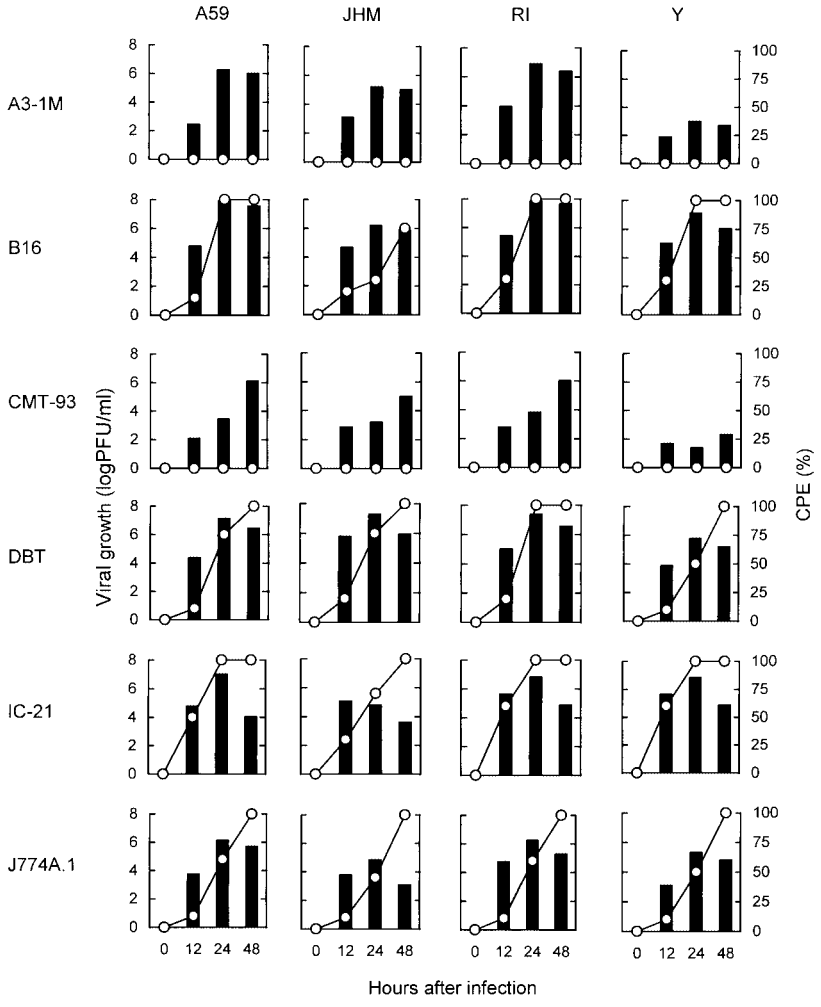

Fig. 2. Kinetics of the CPE and viral replication in murine cell lines infected with prototype MHV. A3-1M, B16, CMT-93, DBT, IC-21 and J774A.1 cells were infected with A59, JHM, RI and Y strains at a multiplicity of infection of 0.01. Viral titers and CPE are expressed by vertical bars and open circles, respectively.

new isolates of murine coronaviruses instead of prototype strains (Fig. 3). Some differences were observed between prototype viruses and new isolates. First, although the new isolates also induced a CPE in B16, DBT, IC-21 and J774A.1 cells, but not in A3-1M and CMT-93 cells, the severity of the CPE was much milder than with prototype viruses, except in IC-21 cells. In addition, growth of the new isolates was somewhat limited. The viral titer in supernatants from KQ3E-infected A3-1M, CMT-93 and J774A.1 cells was below the detectable level of the assay, even though KQ3E-infected J774A.1 cells showed a slight CPE. The pattern of viral replication of KQ1E appeared to be similar to that of KQ6E, but was clearly different from that of KQ3E, although these strains were all isolated from a natural outbreak of MHV infection. When viral growth in the various cell lines was compared, B16 cells were found to support the replication of newly isolated MHV best. During viral titration by plaque assay on DBT cells, the plaque size of new isolates was found to be smaller than that of prototype viruses (data not shown). This phenomenon was independent of the cell line infected.

Sensitivity of cell lines to MHV: It is important to use cell lines for virus isolation that are highly sensitive to MHV. To find the most suitable cell line for this pur-

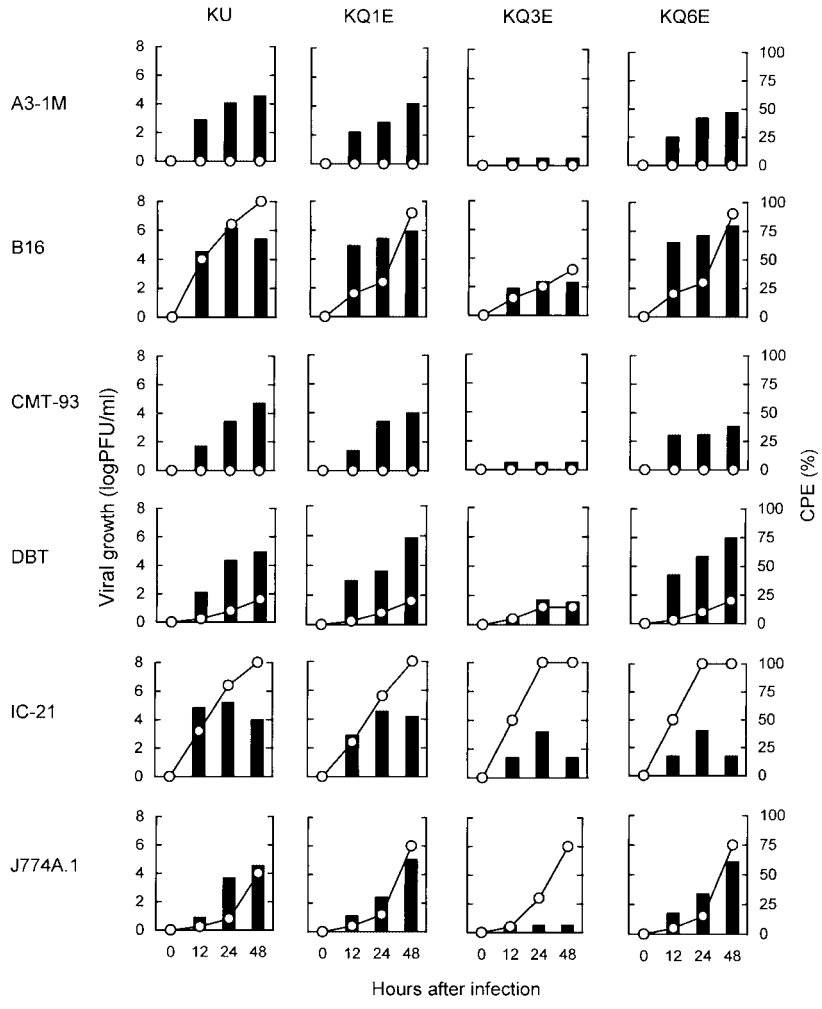

Fig. 3. Kinetics of the CPE and viral replication in murine cell lines infected with newly isolated MHV. A3-1M, B16, CMT-93, DBT, IC-21 and J774A.1 cells were infected with KU, KQ1E, KQ3E and KQ6E strains at a multiplicity of infection of 0.01. Viral titers and CPE are expressed by vertical bars and open circles, respectively.

pose, the $TCID_{50}$ titer of the polytropic and enterotropic biotypes, as well as the new isolates, was determined in B16, DBT, IC-21 and J774A.1 cells, and compared with that in DBT cells (Fig. 4). IC-21 cells were the most sensitive among the cell lines tested. Although J774A.1 cells were also more sensitive than DBT cells, B16 cells were found to be less sensitive to MHV.

Discussion

Although the microbiological environment of laboratory animals has improved considerably in the last 20 years, MHV infection is still found among mouse colonies housed in experimental animal facilities. It may be true that frequent transfers of live transgenic mice between experimental animal facilities increase the opportunity for MHV infection, but inadequate understanding, as well as insufficient control of the virus, might be responsible for the present situation. To increase our understanding of the virus-cell interactions that occur during MHV infection, the replication of several strains of MHV was compared in various cell lines of murine origin.

It has been proposed that MHV is divided into two

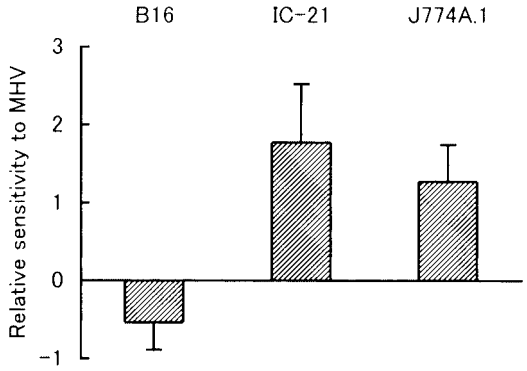

Fig. 4. Comparison of the sensitivity of four murine cell lines to MHV. Viral titers were determined from the $TCID_{50}$ with B16, DBT, IC-21 and J774A.1 cells. Relative sensitivity to MHV=log ($TCID_{50}$ titer determined with target cells/ $TCID_{50}$ titer determined with DBT cells). The results show the mean ± standard deviation of three to four experiments.

overlapping biotypes, termed polytropic and enterotropic [2], but the molecular basis for these biotypes is not yet known. In this investigation, we had expected that enterotropic strains would replicate preferentially in the colon carcinoma cell line CMT-93, because this cell line was assumed to provide an environment comparable to that *in vivo*, but no substantial differences between polytropic and enterotropic strains in viral replication were observed in any of the cell lines tested. The reason for this is unclear but the following possibility is suspected: the enterotropic strains used in this study might have lost their enterotropism by passage in J774A.1 cells [9]. CMT-93 cells are tumor cells and might be different from normal enterocytes for the replication of enterotropic MHV.

Although the tissue tropism of a virus may be complex, much attention has focused on the molecules responsible for viral attachment. To date three molecules, biliary glycoprotein 1 (Bgp1), Bgp2 and carcinoembryonic antigen-like molecule isolated from brain (bCEA) have been reported as viral receptors for MHV [5, 23]. It has been suggested that the receptor activity of these molecules is virus strain-dependent [5]. Moreover, evidence exists that enterotropic MHV-Y and -RI strains utilize different molecules, other than Bgp1, as viral receptors [6, 7]. Preliminary experiments by reverse transcription-polymerase chain reaction indicated that six of the cell lines used in this study expressed equal amounts of Bgp1 and different amounts of Bgp2 and bCEA (Kyuwa, unpublished data). Further experiments are necessary to address the critical factor(s) determining the biotype.

It is interesting that the profile of KQ6E replication in the six cell lines appeared to be similar to that of KQ1E, but was clearly different from that of KQ3E, even though these strains were isolated from three mice during a natural MHV infection. It is possible that this was due to the quasi-species nature of MHV during natural infection [1]. Moreover, the critical element(s) affecting replication of MHV could be clarified by comparing the viral nucleotide sequences of KQ6E and KQ3E.

Plaque size is a characteristic of viruses and is sometimes used as a marker in selecting variant MHV [21]. Although it has been known that most newly isolated MHV produce small plaques on DBT cells, the isolates in the present study also produced small ones. Although it can be speculated that this is related to the poor CPE induced by the isolates in DBT cells, the mechanisms determining plaque size remain unknown.

Various murine cell lines, including 17Cl1, L2 and Sac(-) cells, have been used in studies on MHV [9, 22]. DBT cells have been used widely for isolation, titration and large-scale preparation of MHV, especially in Japan. In this study, we demonstrated that IC-21 cells were the most susceptible to MHV and had a visible CPE following MHV infection. This cell line would therefore be suitable for virus isolation. MHV-infected IC-21 cells might also be useful as antigens for indirect immunofluorescence assays to detect antibodies to MHV, since the MHV-induced CPE in IC-21 cells was characteristic, but the viral titer in the supernatant of IC-21 cells was not so high partly due to the early death of the infected cells. On the other hand, the supernatants of B16 cells contained the highest titer of MHV and would therefore be suitable for large-scale preparation of MHV, including newly isolated strains.

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