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Maintenance of Pluripotency in Mouse Embryonic Stem Cells Persistently Infected with Murine Coronavirus

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A persistently coronavirus-infected embryonic stem (ES) cell line A3/MHV was established by infecting an ES cell line, A3-1, with mouse hepatitis virus type-2. Although almost all A3/MHV cells were found infected, both A3/MHV and A3-1 cells expressed comparable levels of cell surface differentiation markers. In addition, A3/MHV cells retained the ability to form embryoid bodies. These results suggest that persistent coronavirus infection does not affect the differentiation of ES cells.

Coronaviruses are enveloped, positive-stranded RNA viruses associated with various diseases of both animals and humans (19, 35, 39). Mouse hepatitis virus (MHV), a member of the coronavirus family, has a genome RNA of approximately 31 kb and consists of three or four structural proteins. The spike (S) protein mediates virus binding to virus receptors, induces cell-to-cell fusion, except in some nonfusogenic strains represented by MHV type-2 (MHV-2), and elicits neutralizing antibodies and cell-mediated immunity (6). Other structural proteins include the integral membrane (M) protein, the hemagglutinin-esterase (HE) protein, and the nucleocapsid (N) protein (35). MHV induces cytopathic effects and results in cytocidal infections in most continuous cell lines, perhaps because of viral inhibition of the host macromolecular synthesis. However, in some cell lines, MHV can establish persistent infections with minimum cytopathic effect (4, 20, 21, 22, 24). Most reports state that persistent MHV infections are maintained by a small percentage of cytopathically infected cells in culture (the carrier culture) and that the type of host cell may be a major determinant in persistence (11, 15). Moreover, a recent study demonstrated that epigenetic expression of cellular receptors for MHV, members of the carcinoembryonic antigen family (8, 27, 40), is a factor involved in the establishment of persistent MHV infection (32).

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell masses of mouse blastocysts and can be maintained indefinitely in culture (9, 23). If injected into mouse blastocysts, these cells contribute to the formation of all tissues including the germ cells of developing animals (5). These characteristics of ES cells make them a very useful tool in mammalian genetic engineering. ES cells are maintained in an undifferentiated state by coculture with feeder cells or by the addition of leukemia inhibitory factor (29). They are induced to differentiate into various cell lineages and form embryoid bodies by suspension in culture in the absence of the leukemia inhibitory factor, indicating that ES cells are suitable for studies of cell differentiation. Some reports have addressed the interaction between MHV and host cells (14, 18, 37). However, cells used in those studies were terminally differentiated cells or tumor cells, which are aberrantly differentiated. We therefore attempted to examine the interaction between MHV and ES cells, which have the potential to differentiate. In this study, we succeeded in establishing ES cells persistently infected with MHV-2 and found that the cell line exhibited steady-state infection rather than that of a carrier culture. We also made some interesting discoveries by examining the relationship between MHV-2 infection and cellular differentiation.

The A3-1 ES cell line, established from a 129/SvJ mouse, was used (3, 17). Cells were maintained as monolayers in Dulbecco's modified Eagle's medium and nutrient mixture F-12 supplemented with 1,000 U of leukemia inhibitory factor (AMRAD, Boronia, Australia) per ml, 10^{-4} M 2-mercaptoethanol, and 20% fetal calf serum (complete ES medium) in 60-mm-diameter culture dishes pretreated with 0.1% gelatin at 37°C under 5% CO_2 and passed every 2 days. MHV-2 was propagated and plaque assayed with DBT cells as previously described (36). A3-1 cells were infected with MHV-2 at a multiplicity of infection of 1 and cultured as described above. Although MHV-2 is known to induce rounding and lysis of most cell lines tested, A3-1 cells survived and grew without obvious cytopathic effects after infection. After subculture, they formed characteristic insular colonies similar to those of parental A3-1 cells (Fig. 1A and B). This culture, designated A3/MHV, proliferated well, similar to A3-1 cells, and was maintained for over 60 days in complete ES medium. Virus titer in the supernatant increased until 12 h postinfection and was then sustained at 10^6 to 10^9 PFU/ml during the experimental periods (Fig. 1C), suggesting that A3/MHV cells continuously produced progeny virus. To determine the percentage of infected cells in A3/MHV cells, expression of MHV-2 S protein was examined by flow cytometry. For flow cytometric analysis, cells were detached with phosphate-buffered saline containing 0.05% EDTA and incubated with an MHV S protein-specific monoclonal antibody (MAb), number 2, a kind gift from F. Taguchi (16), and then with fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin G antibody. Samples were analyzed by a FACScan flow cytometer (Becton Dickinson). Surprisingly, almost all A3/MHV cells expressed viral S proteins, suggesting that almost all A3/MHV

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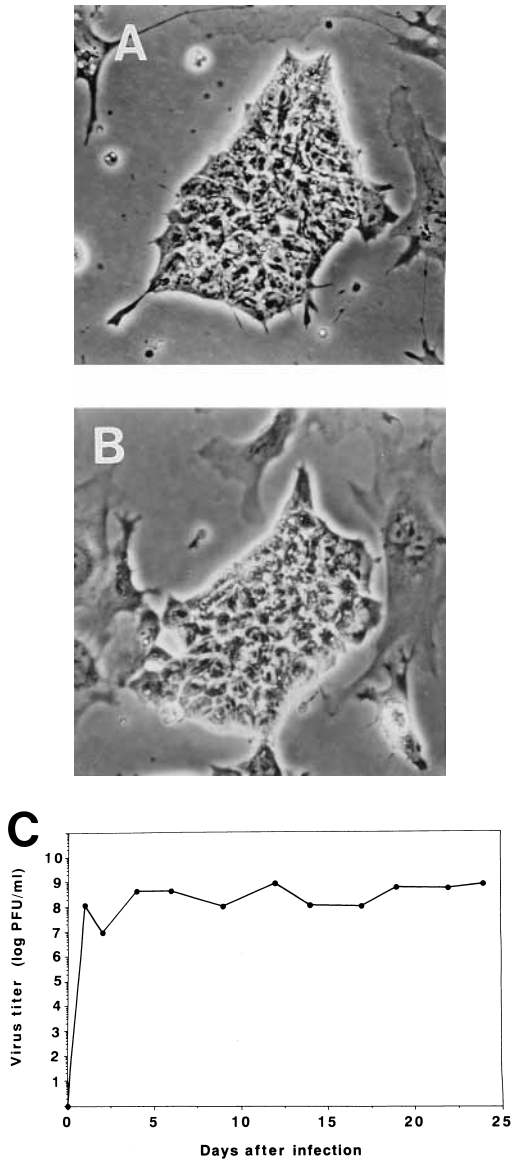

FIG. 1. Characterization of a persistently MHV-infected ES cell line (A3/MHV). Both A3-1 (A) and A3/MHV (B) cells formed insular colonies. Phase-contrast photomicrographs (magnification, ×88) are shown. (C) Virus production of A3-1 cells after MHV-2 infection. A3-1 cells were infected with MHV-2 at a multiplicity of infection of 1, and virus titers of the supernatants were determined by conventional plaque assay.

cells were infected (Fig. 2A). This was confirmed by indirect immunofluorescence of acetone-fixed A3/MHV cells with anti-S (number 2) and anti-N (J.3.3) (10) MAbs (Fig. 2B and C). The immunofluorescence was not confined to the cell membranes, indicating that the fluorescence was due to infection of cells rather than to virions bound on the cell membranes.

Molecular mechanisms of ES cell differentiation are not fully understood. However, it is possible to examine the process of differentiation by monitoring the expression of cell surface molecules, because their expression is regulated (1, 12, 13, 25, 26, 28, 31). Flow cytometric analysis demonstrated that L-CAM, a marker of undifferentiated ES cells (1, 33), was expressed on A3/MHV cells at a level comparable to that on parental A3-1 cells (Fig. 3A and B). A3/MHV cells expressed

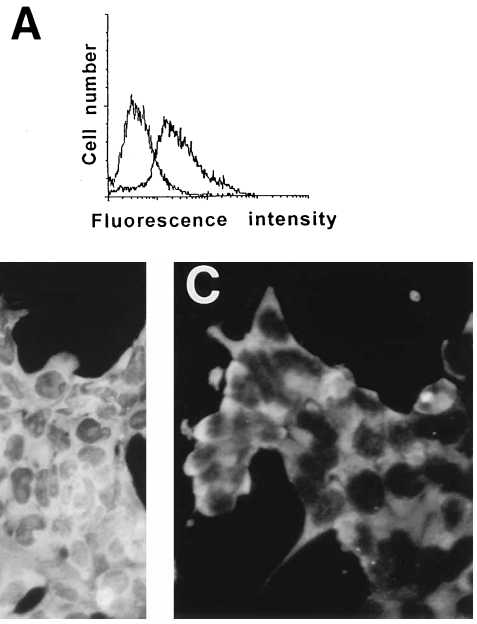

FIG. 2. Expression of MHV-2 protein in A3/MHV cells. A3/MHV cells were incubated with anti-S MAb number 2 and then with FITC-conjugated anti-mouse immunoglobulin G antibody. Samples were analyzed by flow cytometry. (A) Histogram of the negative control (second antibody only). A3/MHV cells were fixed with acetone and incubated with anti-S protein MAb number 2 (B) or anti-N protein MAb J.3.3 (C). They were further incubated with FITC-conjugated anti-mouse immunoglobulin G antibody and observed under a fluorescence microscope.

a slightly higher amount of Le^x antigen, a carbohydrate determinant of the stage-specific embryonic antigen-1 (25, 26), than A3-1 cells (Fig. 3C and D). However, the significance of the difference remains obscure. Moreover, both A3/MHV and

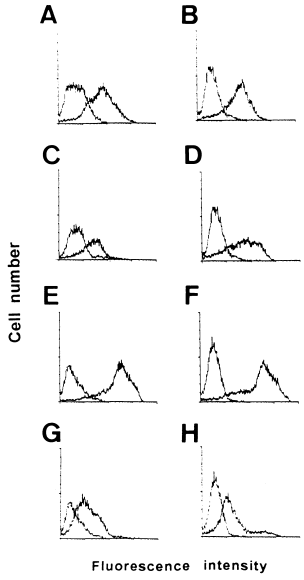

FIG. 3. Expression of cell surface differentiation markers on A3-1 and A3/MHV cells. Cell surface expression of L-CAM (A and B), Le^x (C and D), PNA-binding sites (Galβ1→αGalNac) (E and F), and DBA-binding sites (*N*-acetylgalactosamine) (G and H) on A3-1 (A, C, E, and G) and A3/MHV (B, D, F, and H) cells was examined by flow cytometry using anti-L-CAM (38) and anti-Le^x (31) MAbs and FITC-conjugated PNA and DBA lectins. See the legend to Fig. 2.

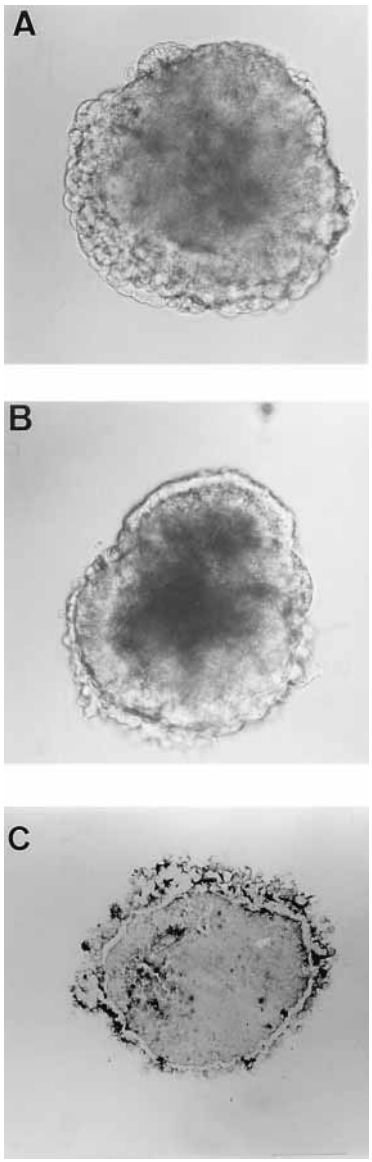

FIG. 4. Embryoid body formation by A3-1 and A3/MHV cells. The appearances (on day 7) of embryoid bodies that developed following the suspension in culture of A3-1 (A) and A3/MHV (B) cells are shown. (C) Immunohistological staining of A3/MHV-derived embryoid body. Frozen sections of embryoid bodies were stained with anti-S protein MAb. Magnification, ×80.

A3-1 cell lines expressed other carbohydrate antigens, such as peanut agglutinin (PNA)-binding (Fig. 3E and F) and *Dolichos biflorus* agglutinin (DBA)-binding (Fig. 2G and H) sites, which are also expressed on undifferentiated embryonal carcinoma cells (30, 31). These results suggest that A3/MHV cells maintain an undifferentiated state, judging from the expression of cell surface differentiation markers, even though they are persistently infected with MHV-2.

The formation of embryoid bodies in vitro is a hallmark of the pluripotency of ES cells (7, 34). To examine whether A3/MHV and A3-1 cells retain this ability, both cell lines were cultured in the ES medium lacking leukemia inhibitory factor in untreated dishes. One week later, embryoid bodies with the ectoderm-like cells interior to the endodermal cells had developed from both cell lines (Fig. 4A and B). To exclude the possibility that the embryoid bodies developed from a few uninfected ES cells in A3/MHV cells, the distribution of viral antigens in A3/MHV-derived embryoid bodies was examined by immunohistochemistry. Frozen sections of embryoid bodies were prepared, fixed with acetone, and stained with anti-MHV S MAb. As shown in Fig. 4C, MHV-2 antigens were harbored in most of the lateral endodermal cells and a few cells in the core, suggesting that the embryoid bodies truly developed from MHV-infected ES cells. These results show that A3/MHV cells have most of the characteristics of A3-1 cells, which can contribute germinal cell lineage (3, 17).

Generally, MHV produces a lytic infection and almost all MHV-infected cells die. However, a few resistant cells remain, and a carrier culture sometimes establishes itself. This lytic virus can persist in vitro under conditions in which a small fraction of the total cells is infected (4, 22, 24). In contrast, almost all A3/MHV cells were infected with MHV-2 and proliferated with the concomitant production of progeny viruses. The results suggest that A3/MHV cells exhibit steady-state infection with MHV-2 but not with a carrier culture. This determination was supported by a preliminary result, namely, that the inhibition of macromolecular synthesis was not observed in A3/MHV cells (data not shown), although such inhibition was observed in most cell lines following MHV infection (14, 18, 37). A3/MHV cells may provide a unique opportunity to investigate the molecular mechanism of steady-state infection with MHV.

It is possible that viral infection induces cellular differentiation, since viruses sometimes modulate host-cell structural and metabolic components to facilitate their replication. However, in this study, we have shown that the influence of MHV-2 infection on ES cell differentiation, if any, is minimal and that MHV-2 and ES cells can live symbiotically by establishing a steady-state infection. On the other hand, it is also possible that cellular differentiation may influence sequels of viral infection, since viral replication heavily depends on host-cell structural and metabolic components. It is of interest that MHV antigens were observed primarily in lateral endodermal cells of A3/MHV-derived embryoid bodies, not in all cells. This might be explained by tissue tropism of MHV in adult mice, if one remembers that endodermal cells develop into liver, alimentary epithelium, and respiratory epithelium cells in adult mice, where MHV replicates well. It may be important to examine the expression of the cellular receptor for MHV in embryoid bodies, since it is a candidate for the factor(s) which determines MHV-2 tropism. However, we cannot rule other possibilities out (2, 41). Although the molecular mechanisms of the phenomena described here remain largely unclarified, this study shows that MHV infection in ES cells is unique and that ES cells are a useful tool in virological studies as well as in the genetic engineering of mammals.

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