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In vivo and in vitro models of demyelinating disease X. A Schwannoma-L-2 somatic cell hybrid persistently yielding high titres of mouse hepatitis virus strain JHM

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Summary

Following infection of RN2 rat Schwannoma cells with unfiltered JHMV inocula, a cell line with an altered phenotype evolved, which was shown to be a somatic cell hybrid of RN2 and mouse L-2 cells. This cell line, EJ, persistently yields JHMV at titres greater than 10^6 pfu/ml and does not show the suppression of virus production at 39.5 °C that is characteristic of a persistently infected RN2 line. Intracellular viral nucleocapsids are demonstrated. Cloning of EJ hybrids yields cell lines that show a variety of responses to infection by JHMV or MHV₃.

persistent infection, somatic hybrid, coronavirus

Introduction

The coronaviruses have been extensively studied recently, especially in light of their ability to establish persistent infections both in vitro (Holmes and Behnke, 1981; Stohلمان and Weiner, 1978; Lucas et al., 1977, 1978) and in vivo (Knobler et al., 1981; Hirano et al., 1980; Nagashima et al., 1979; Sorensen et al., 1980, 1982, 1984). Such viruses are suitable candidates to consider as factors in the etiology of chronic demyelinating diseases.

A number of cells of neural origin have been examined with respect to their

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responses to infection by murine coronaviruses JHMV and MHV₃ (Lucas et al., 1978). The neurotropic strain has been shown to establish readily a persistent infection characterized by limited cytopathology in rat Schwannoma cells, RN2 (Lucas et al., 1977). The RN2 cell line yields virus in a cyclic fashion at 32.5°C (Lucas et al., 1978) but virus production is suppressed and the infection becomes latent at 39.5°C. We have recently identified a rat–mouse hybrid cell line persistently infected with JHMV, which at 32.5°C consistently produces virus at titres greater than 10⁶ pfu/ml but which does not manifest the temperature sensitivity of virus production evident in RN2 cells. This article contains data characterizing this cell line and presents information about the nature of the persistent state.

Materials and Methods

Culture of cells and virus

The sources and routine culture of rat Schwannoma cells RN2 and mouse fibroblasts, L-2, as well as the mouse hepatitis strains, JHMV and MHV₃ have already been described (Lucas et al., 1977).

L-2 cells were used for routine propagation and plaque assays of both viruses, as described in Lucas et al. (1977). Uninfected and infected cell lines were at all times maintained completely separately. Prior to infection all cell lines maintained normal morphology, growth rates, and virus susceptibilities.

Initiation of persistent RN2 infection

Virus inocula were grown on L-2 cell monolayers. Cell debris was removed as indicated below either by centrifugation or by filtration through a 0.22 µm Millex-GV filter unit (Millipore). Monolayers of RN2 cells were inoculated at a multiplicity of infection (m.o.i.) of 0.1–0.5. Following adsorption for 1 h at room temperature or 32.5°C, unadsorbed virus was removed, and monolayers were washed with PBS, overlaid with nutrient medium and incubated at 32.5°C. Cultures were maintained with medium changes on alternate days and subculturing every 6–7 days. Routine monitoring by light microscopy was done to observe cytopathic effects (cpe).

Karyotyping

Chromosome spreads were prepared according to the procedure of Worton and Duff (1979). Centromeric staining was carried out using the formamide procedure described by Marshall (1975) and modified by Flintoff (1984). Photographs were taken on Leitz Dialux 20 microscope.

Lactate dehydrogenase isozyme analysis

The preparation of cell extracts and assay for lactate dehydrogenase (LDH) was carried out according to the procedure described by Weiss and Ephrussi (1966) as modified by Flintoff (1984).

Antibodies

Polyclonal antibodies were produced by immunizing previously tolerized Balb/c

mice with concentrated JHMV. The serum obtained could neutralize 10^6 pfu/ml of JHMV at a 1 : 512 dilution. Hybridoma (HB) antibodies specific for E2 glycoprotein and the nucleocapsid (N-C) antigen, described by Collins et al. (1982), were kindly provided by Dr. M. Buchmeier, Scripps Clinic and Research Foundation, La Jolla, Calif., U.S.A.

Immunolabelling

Virus antigens were detected on acetone-fixed cultures grown on glass coverslips. The procedure followed that described in detail by Dales and Oldstone (1982), involving sequential labelling with specific polyclonal or hybridoma (HB) antibodies from mouse (mu), diluted 1 : 50 and then with rhodamine-labelled goat anti-mu conjugates (Cappell) diluted 1 : 15.

Electron microscopy

Monolayer cultures, grown in plastic flasks or culture dishes, were washed with PBS, then treated with 0.025% trypsin to release the cells and suspended in medium containing 10% foetal calf serum to inhibit further tryptic activity. The cell suspensions were centrifuged into pellets at $800 \times g$ for 10 min. The pellets were fixed, dehydrated and embedded without further disturbance according to Dales (1963). Thinly sectioned, stained cells were examined in a Philips EM 300.

Results

Biological properties of the hybrid cell line, EJ

A persistently infected culture, designated EJ, initiated by infection of RN2 cells with centrifuged but unfiltered JHMV inoculum, was established. After several weeks in culture it was observed that the cells no longer resembled the RN2 line morphologically or in their rapid growth rate. The extracellular virus titre frequently exceeded 10^6 pfu/ml (Fig. 1A) whereas that described by Lucas et al. (1978) was usually less than 10^5 pfu/ml. It was also observed that the cells did not display the temperature sensitivity of virus production (Fig. 1A) associated with the RN2 line, as described by Lucas et al. (1978). The data in Fig. 1 commence at 300 days post infection (PI) for EJ although the high titre was noticed as early as 20 days PI and the lack of temperature sensitivity before 100 days. By contrast, initiation of persistent infection of RN2 cells with JHMV inocula that had been Millipore filtered invariably resulted in cultures behaving like bona fide RN2 cells in terms of temperature sensitivity of virus production (Fig. 1B) and cpe.

Chromosome spreads were prepared and stained to visualize the centromeres (Fig. 2). Chromosome counts showed a modal average of 74 chromosomes in EJ cells, with counts ranging from 72 to 80, as compared to an average of 38 in RN2 and 41 in mouse L-2 cells. Based on morphological features, such as size and positions of centromeres, the karyotyping indicated that EJ cells contain a complement of combined mouse and rat chromosomes (Fig. 2). The number of chromosome spreads studied was insufficient to permit a correlation between the characteristics of

the EJ cell line and the presence or absence of particular chromosomes.

To confirm that EJ is a hybrid cell line in biochemical terms, LDH isozyme analysis was performed on cultures 190 days PI. The results (Fig. 3) revealed that whereas the rat and mouse cell extracts each showed one distinct isozyme band, the EJ cell extract showed bands at intermediate positions on the electropherogram. Such bands were absent from artificial mixtures of RN2 and L-2 cell extracts.

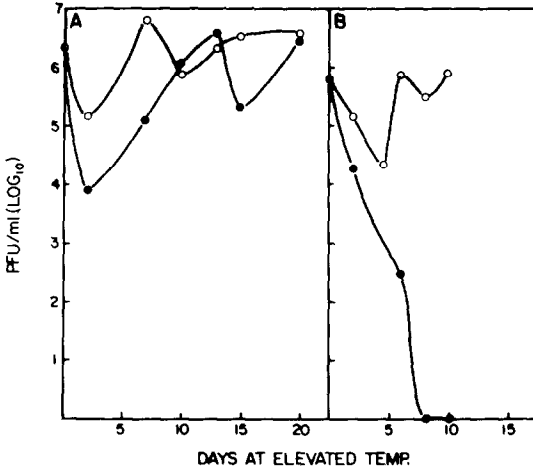


Fig. 1. Comparison of JHMV replication at 32.5°C (○) and 39.5°C (●) in EJ hybrid cells (A) and RN2 (B). Cell monolayers were infected with JHMV inoculum that had been centrifuged (A) or filtered (B) as described in Materials and Methods. After the infections were well-established (A, 305 days PI; B, 32 days PI) cells were subcultured and incubated at 32.5°C and 39.5°C.

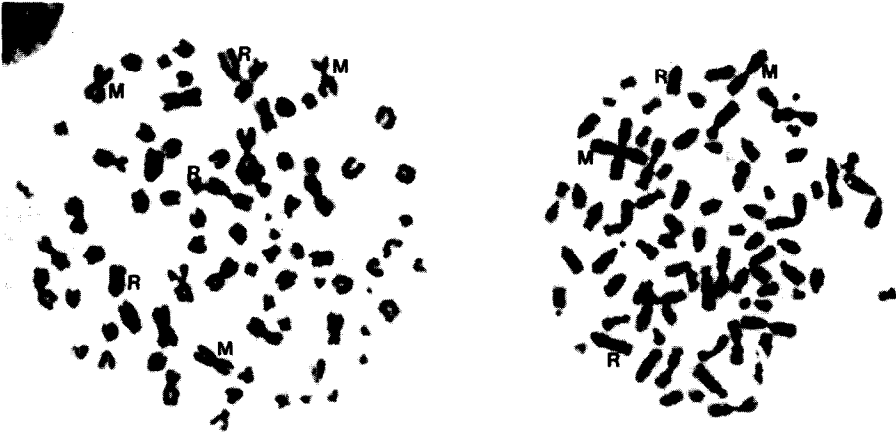


Fig. 2. Two representative spreads of chromosomes from EJ cells (230 days PI), prepared as described in Materials and Methods. Distinctive mouse and rat chromosomes are indicated by M and R, respectively. The chromosomes showed a modal average of 74.

Similar results were seen with cultures 240 days PI. Comparable isozyme patterns were observed by Flintoff (1984) in RN2 X L-2 somatic cell hybrids generated with polyethylene glycol (PEG). The occurrence of the intermediate bands indicates that an isozyme hybrid was being identified and is consistent with the presence and association of both rat and mouse isozymes of LDH within a single cell. Taken together, the cytological and isozyme observations are consistent with the assumption that EJ cells are a mouse \times rat somatic cell hybrid.

It is most likely that the hybrid line was created by addition of viable L-2 cells with the unfiltered JHMV inoculum, when initiating the infection of RN2 cells. The normal infected or uninfected RN2 cells would be expected to have been diluted out by overgrowth of the faster growing hybrid cells, whereas uninvolved L-2 cells would be destroyed during lytic infection with JHM. The phenomenon of spontaneous hybrid cell formation is a recurring one because other cell lines have been generated in a like manner and, like the EJ line, also manifested the lack of temperature sensitivity and high virus titres. As yet, however, these additional lines have not been further characterized.

Variability among cloned EJ hybrid cells

EJ cells were cloned by limit dilution in 96-well microtitre plates. All clones derived from single cells were expanded and tested for virus production. In three

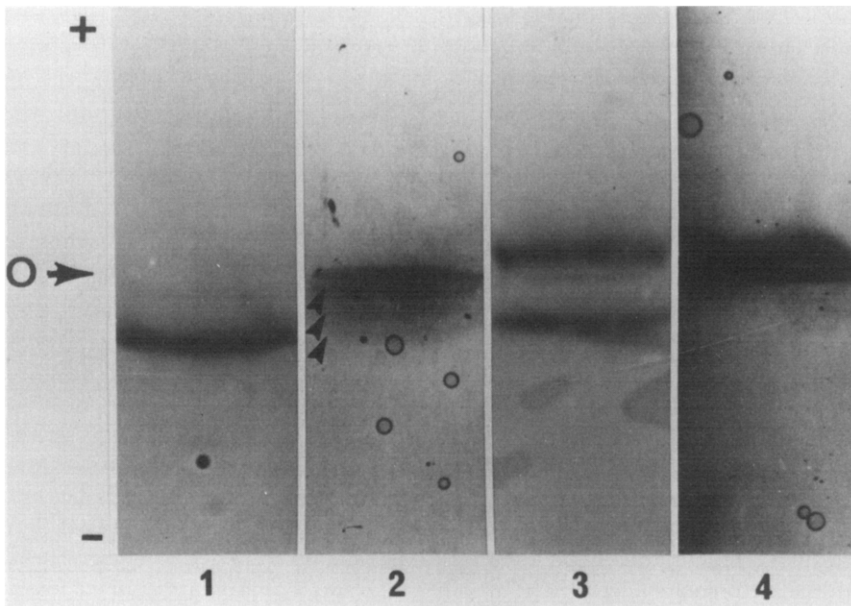


Fig. 3. Isozyme patterns of LDH prepared, as described in Materials and Methods, from hybrid cells (190 days PI) and parental cell lines. RN2 cell extract (lane 1), EJ cell extract (lane 2), mixture of RN2 rat and L-2 mouse cell extracts (lane 3), and L-2 cell extract (lane 4). The origin, anode and cathode are indicated at the left. Note the presence of intermediate bands in the EJ preparation, indicated by arrows.

separate experiments at 189, 260 and 305 days PI the proportions of virus yielding clones was 4/32, 4/19 and 0/10. Subcloning of virus yielding clones gave 100% nonyielders. Immunofluorescence studies, described below, revealed that these nonyielders were virus antigen-negative. The large proportion of nonyielding clones would not be surprising if complete virus genome information is segregated unequally in cell division leading ultimately to the generation of uninfected cells which are given a growth advantage by the conditions of limit dilution.

The nonyielding EJ clones were also tested for their infectability by MHV₃ and JHMV. It should be pointed out that with regard to the two parental cell lines, L-2 undergoes a rapid, lytic MHV₃ and JHMV infection, characterized by massive syncytiogenesis, whereas RN2 is infectable in a persistent manner, undergoing only limited cpe by JHMV but is not infectable by MHV₃ (Lucas et al., 1977). By contrast, 6/8 of the hybrid EJ clones underwent a lytic infection by MHV₃, resulting in extensive cpe and high yields of virus, much like the L-2 parental line. The 2/8 clones which underwent only minimal cpe were, nevertheless, virus producing. Following JHMV infection of the same clones, 8/8 produced titratable virus but underwent minimal cpe. Superinfection of EJ cells with the homologous virus, JHM, caused no obvious change in the culture. However, MHV₃ did induce syncytia formation. These observations revealed that a continuous somatic hybrid line not only differs from the parental cell lines but generates daughters at high frequency which are highly variable in their cpe response to different strains of coronavirus.

Detection of JHMV antigens by immunomicroscopy

Stationary cultures of EJ cells incubated at the permissive (32.5°C) and restrictive (39.5°C) temperatures, for maintenance of persistence or latency of JHMV in the RN2 Schwannoma line, were examined using virus-specific polyclonal and hybridoma (HB) antibodies. The polyclonal antibodies become bound to mononuclear, and multinucleate, large syncytia, as exemplified in Fig. 4a, c, and e. About 1–5% of the cells in persistently infected yielder-cultures kept at 32.5°C contained large, antigen-positive intracytoplasmic inclusions. In addition 10–15% of the mononuclear cells possessed antigen-positive regions at or near the cell periphery (arrow in Fig. 4a). By contrast the non-yielding subclones, EJc1-1 and EJc1-4, isolated from EJ by limit dilution, were all JHMV antigen-negative. Tagging with HB antibodies to the E₂ and nucleocapsid protein components of JHMV showed similar patterns of labelling to those described for the polyclonal antibody (Fig. 4c, e, g). However, the presence of peripheral antigen accumulations of the type illustrated in Fig. 4a was not observed with the HB reagent. Although specific tagging for the E₁ antigen was not undertaken, these data revealed that cells synthesizing JHMV probably contain all the major antigenic components of the virus, consistent with the notion that they are elaborating complete, infectious virus particles. The prominence of nucleocapsid antigen in the cytoplasm (Fig. 4e, g) implies that this component may be overproduced and accumulates in defined foci, as previously observed upon infection of L-2 cells, one of the parental lines (Massalski et al., 1982). In fact, accumulations of nucleocapsid material were evident in the cytoplasm of EJ cells examined by transmission electron microscopy, as described below.

Detection of virus materials by electron microscopy

To ascertain whether the JHMV antigens detected by immunofluorescence microscopy were recognizable as virions and/or subviral components, pellets of EJ cells cultured at 32.5°C were prepared for thin sectioning and electron microscopy.

As evident from the selected examples, in Figs. 5–10, extensive accumulations of virions possessing the typical morphology of coronaviruses were present on the external cell surface. Such virions were evident on polykaryocytes but were also associated with smaller, perhaps mononucleate, cell profiles. In the cytoplasm of both large syncytia and smaller cells, formative stages characteristic of coronavirus assembly at surfaces of vacuoles or smooth endoplasmic reticulum, could be observed (Figs. 9, 10, arrows). Masses of a dense helical component, previously shown to be related to JHMV nucleocapsids (Massalski et al., 1982), were also present in multinucleate and smaller cells (Figs. 5, 8). This finding corroborates the intense staining of cytoplasmic foci by HB specific for the nucleocapsid, illustrated in Fig.

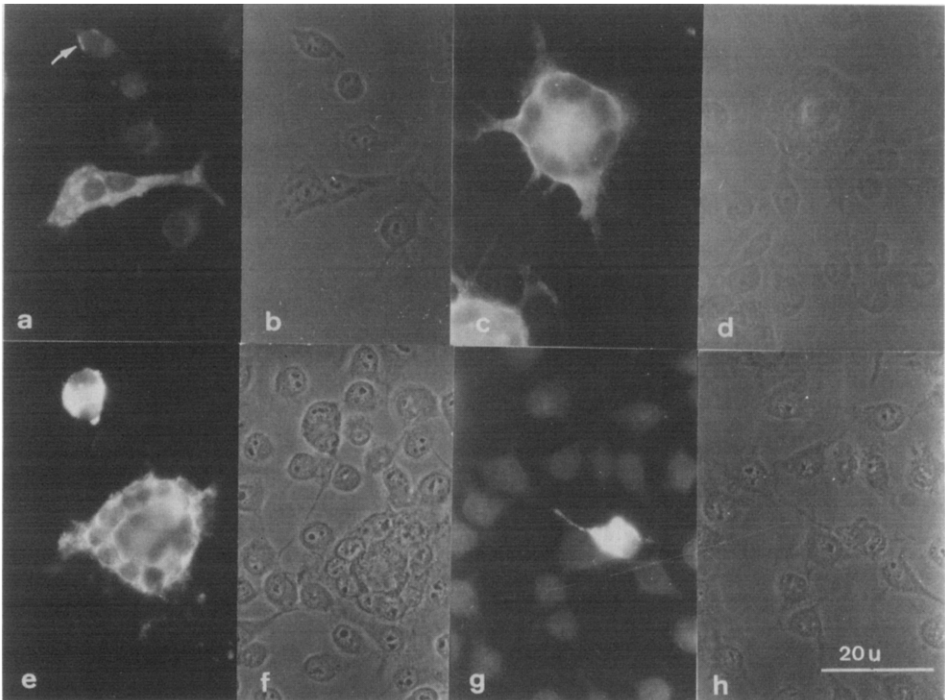
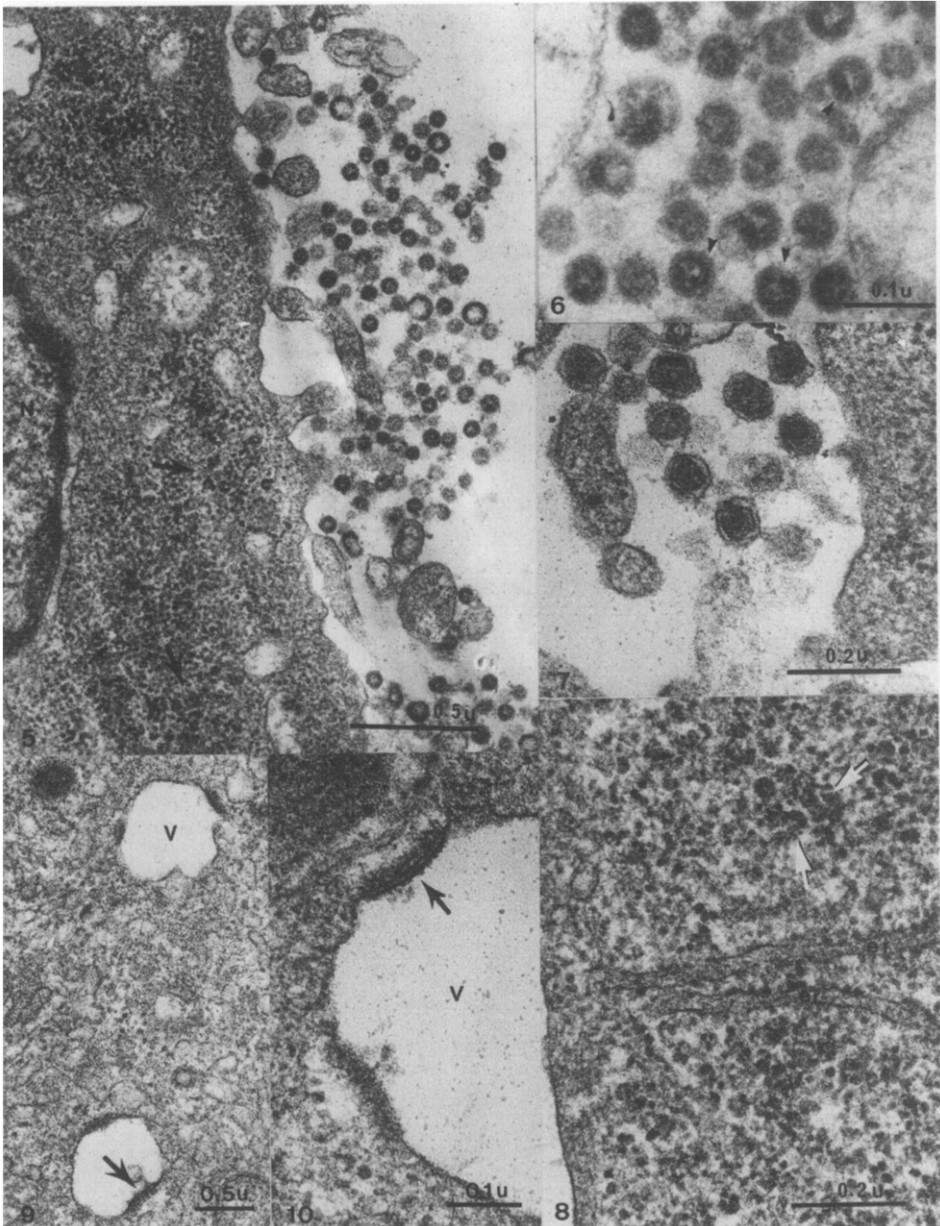


Fig. 4. Immunolabelling of JHMV antigens in cultures of persistently infected EJ cells. Images in a, c, e and g were photographed under UV optics, those in b, d, f, and h are the same areas as they appeared under phase-contrast. a, tagging with polyclonal mouse (μ) anti JHMV reveals a single binucleate cell filled with large antigen-positive inclusions and a mononucleate cell with zones of virus antigen at the surface (arrow). In c, large infected syncytia are marked by E_2 μ HB antibody. Note the absence of antigen from peripherally disposed nuclei. The two examples e and g illustrate labelling with anti N-C μ HB antibody. In e both a binucleate and large, multinucleate syncytium contain the viral antigen while in g a single, mononucleate cell is positive.



Figs. 5-10. Selected regions of thinly sectioned EJ cells shown to illustrate components of JHMV.

Fig. 5. Periphery of a mononucleate cell shows the presence of a portion of the nucleus (N), cytoplasmic nucleocapsids (arrows) and extensive accumulation of coronavirions near the cell surface.

Fig. 6. A group of virions in an area similar to that in Fig. 5, shown at a higher magnification. Individual virions in which the internal nucleocapsid is distinguishable are identified by arrowheads.

Fig. 7. A group of retrovirus C-type particles occurring near the cell surface. Note the distinctive differences in the fine structure of corona- and retroviruses. The latter possess a polygonal, dense nucleoid, readily distinguishable from the 'tubular' nucleocapsids of JHMV.

Fig. 8. Nucleocapsid helical material (arrows) in the cytoplasm of a multinucleate cell, shown at a higher magnification than in Fig. 5. Elements of the rough endoplasmic reticulum (er) are also evident.

Figs. 9 and 10. Stages in JHMV assembly (arrows) at the surface of smooth er vacuoles (v) shown at low power in Fig. 9 and at higher resolution in Fig. 10.

4e and g. In addition to virions possessing the morphology of coronaviruses, EJ cells were associated with groups of particles morphologically identical to retro or C-type viruses (Fig. 7). These C-type virions occurred on cell profiles regardless of the presence of JHMV materials. Since L-2 strain cells are chronically infected with a murine retrovirus (Dales and Howatson, 1961), it is not surprising that this agent is also expressed in the L-2 × RN2 hybrid line, under investigation here.

Discussion

The development of a virus-producing hybrid cell, as described here, has obvious implications regarding the manner in which inocula are prepared. For the study of long-term infections, as in persistence, it is essential that the virus source be uncontaminated by any viable cells which might grow and become a factor in the outcome of the infection. For this reason millipore filtration was adopted in our laboratory as means of avoiding cellular contamination, since centrifugation does not appear to be adequate.

Despite its uncertain time of origin, the EJ cell line has proved to be extremely useful both as a continuous source of JHMV, yielding titres greater than 10^6 pfu/ml and as an object of study of persistence of JHMV *in vitro*. The evolution of this cell line also serves as an indication that coronaviruses may, under some circumstances, act as nonlethal fusogens capable of producing somatic hybrid cell lines.

The immunofluorescence studies suggest that less than 10% of the cells in an EJ culture are antigen-positive. This agrees with the observation that the majority of subclones isolated from such a persistently infected culture are uninfected. The accumulations of large quantities of nucleocapsids evident by immunocytological and electron microscopic procedures may provide the source material to maintain the high levels of virus production by a small fraction of EJ cells found at any time to be JHMV positive.

Flintoff (1984) reported that, with one notable exception, somatic cell L-2 and RN2 hybrids created deliberately by standard PEG-induced fusion, become lytically infected by coronaviruses, demonstrating that in such hybrids there is a dominance of the permissive state over the persistent one. However, one of the hybrid lines created by Flintoff (1984) can be infected persistently by either JHMV or MHV₃ and displays the temperature sensitivity associated with RN2 cells. In the first respect the EJ line resembles the latter hybrid line, but differs from it in that it fails to restrict virus replication at 39.5°C.

The phenomenon of restriction of virus replication in RN2 cells at 39.5°C has been observed with both JHMV and measles viruses and appears to be host-controlled (Lucas et al., 1978). The mechanism of such restriction is, as yet, unknown but in general two types of mechanisms may be considered. First, the cells may actively produce a factor which suppresses virus replication at 39.5°C; or second, RN2 cells may themselves be temperature sensitive and thus deficient at 39.5°C for some function necessary for replication of these viruses. In the EJ cell line it is possible that the L-2 cell component contributes factor(s) necessary to

overcome a deficiency in the RN2 cells, thus eliminating the temperature sensitivity of virus production.

Virus-negative subclones of EJ were altered in their response to infection by JHM or MHV₃ relative to the parental cell lines. JHMV established an apparently noncytotoxic infection in all cases whereas MHV₃ resulted in a lytic infection in most cases. Again this differs from the cell lines described by Flintoff (1984). The relationship of these characteristics to the mechanisms involved in regulation of a persistent infection is as yet unclear. The variation among the subclones of EJ compared to EJ itself and the hybrid cell lines described by Flintoff (1984) suggests that such characteristics are under multifactorial control.

Acknowledgements

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