REPLICATION OF MURINE CORONAVIRUSES IN SOMATIC CELL HYBRIDS FORMED BETWEEN A MOUSE FIBROBLAST CELL LINE AND EITHER A RAT SCHWANNOMA LINE OR A RAT GLIOMA LINE

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INTRODUCTION

The murine hepatitis viruses can readily establish persistent infections both in vivo and in vitro. In vivo, persistent infections, dependent upon the virus strain, and the age and genetic background of the host, can occur resulting in chronic hepatitis or chronic demyelination of the central nervous system (1). In vitro, these agents can establish persistent infections in both neural and non-neural cell lines (2,3,4) without the requirements for viral modifications or environmental manipulations such as the presence of viral antibody or interferon.

Our previous results have indicated that when infection of various cell lines with either the MHV_3 or JHM strain of the murine coronaviruses resulted in persistence, virus replication was almost invariably thermosensitive and this was due to unknown factors under host control, since the progeny virions themselves were not temperature-sensitive (2,3). In addition, one cell line, the rat RN2 Schwannoma, had the unique ability to discriminate between the MHV_3 and JHM strains (2). JHM was replicated persistently in this line, whereas, MHV_3 replication was aborted. Another rat cell line, the C6 glioma, did not support the replication of either agent (3). At present, it is unclear as to the mechanism of persistence or restriction demonstrated by these cell lines. These observations coupled with others (4,5) strongly imply that the host cell has a profound influence in regulating the replication of these agents.

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As a further approach to analyzing the host functions involved in viral persistence and restriction, somatic cell hybrids have been formed between mouse L2 cells, a cell line totally permissive for both MHV_3 and JHM replication, and either the RN2 or the C6 cells. The results described in this report, indicate that the L cell functions appear to be dominant over the RN2 and C6 ones, since both viral agents replicated lytically in the somatic cell hybrids.

MATERIALS AND METHODS

Cells and Virus. The sources and routine propagation of the L2 and RN2-2 cell lines, and the MHV_3 and JHM strains of mouse hepatitis virus were as previously described (2,3) except that alpha medium (6) was used in place of Eagle's minimal essential medium. The C6 thymidine kinase minus ($C6TK^-$) cells were obtained from Dr. B.P. Schimmer, Banting and Best Institute, Toronto, Ontario, Canada.

Virus propagation was monitored by a plaque assay on L2 cell monolayers as previously described (2). Yields are expressed as PFU/ml (plaque forming units/ml).

Selection of genetically marked L2 cells. L2 cells were treated for 3 hours in the presence of 0.2 μg/ml N-methyl-N'-nitro-N-nitrosoguanidine at 34^o, washed, and resuspended in fresh medium. Survival was usually about 50%. The cells were allowed to grow 6 days to allow for expression of putative mutations before selections were carried out. The basic procedure for mutant selections is described elsewhere (7). Cells, at 5×10^5/100 mm tissue culture dish, were exposed to 0.2 μg/ml 6-thioguanine (TG) (Sigma Chemical Co.) for 8 days at 34^o with replacement of drug and medium every 2 days. Colonies surviving at a frequency of 4×10^{-6} were picked, cloned by limit dilution, and tested for resistance. One clone, L2 TG , was resistant to at least 50 μg/ml TG, a concentration 10^3 higher than was cytotoxic for the wild-type cells, and contained 0.1% of the wild-type hypoxanthine phosphoribosyl transferase activity as determined by the assay described by Chasin and Urlaub (8).

The L2 TG^R7 cells were exposed to 3 mM ouabain (Oua) to select for Oua resistant cells (9). Colonies surviving at a frequency of 10^{-5}were isolated, cloned, and shown to be resistant to at least 3 mM Oua. Wild-type cells were unable to grow at concentrations above 0.5 mM. One doublely marked clone, L2 TG^R Oua^R7-1, and designated L2 TG^R Oua^R was used in the hybridization experiments.

Neither resistance to TG, nor Oua, nor the presence of both of these markers affected the ability of the cells to support the replication of either the MHV_3 or JHM strains of mouse hepatitis virus.

Cell-cell hybridizations. Somatic cell hybrids were formed between the L2 TG^R Oua^R and RN2-2 cells, and the L2 TG^R Oua^R and C6Tk$^-$ cells by exposure to polyethylene glycol (PEG) 6000 (British Drug House) for 1 min using the procedure described by Pontecorva (10). Cells were plated in complete medium for 1 day to allow recovery from the fusion process prior to the addition of HAT (1) + Oua selection medium (7×10^{-5}M hypoxanthine, 2×10^{-8}M methotrexate, 4×10^{-5}M thymidine, 2×10^{-3}M Oua) for the L2 TG^R Oua^R x RN2-2 cells, or HAT (2) medium (7×10^{-5}M hypoxanthine, 10^{-6}M methotrexate, 4×10^{-5}M thymidine) for the L2 TG^R Oua^R x C6TK$^-$ cells. In the latter hybridization, the Oua resistant marker was not used in the selection scheme. Hybridization frequencies are shown in Table 1. After 8 to 10 days incubation at 34°, surviving colonies were picked, expanded, maintained in normal medium, and used for study. Subclones of some of these isolates were obtained by limit dilution. There were no differences in the response of these subclones and the original isolates to virus infection.

Karyotypic analyses. Exponentially growing cells were incubated with 0.25 μg/ml colcemid (Grand Island Biological Co. for 1.5 hrs at 34°. The cells were washed with hypotonic and fixing solutions and chromosome spreads prepared (11). To distinguish between mouse and rat chromosomes the formamide-Giemsa staining procedure of Marshall (12) was used. With this technique a majority of mouse chromosomes contain centromeric regions which stain intensely, whereas, the centromeric regions of the rat chromosomes do not. From 5 to 10 chromosome spreads were examined for each cell line. In addition standard trypsin-banding techniques were also employed (11).

Lactate Dehydrogenase Assay. The preparation of cell extracts and the assay for lactate dehydrogenase was essentially as described by Weiss and Ephrussi (13) using Gelman Sephraphore III cellulose acetate strips. In vitro hybridizations were carried out in 1M NaCl and 70 mM Na phosphate pH 8.0 as described by Markert (14).

RESULTS

Properties of hybrid cells. Using the genetically marked cell lines, cell-cell hybrids were formed between either the mouse L cells and rat RN2 cells or the L cells and rat C6 cells. Although the frequency of hybrid formation was greater than the survival of either the mouse or rat parental cell lines under

Table 1 Hybridzation Frequencies

Cross	PEG	Experiment	
L2 TG^R Oua^R x L2 TG^R Oua^R a,b	+	6×10^{-7}	2.5×10^{-6}
RN2-2 x RN2-2[a]	+	1×10^{-7}	--
$C6TK^-$ x $C6TK^-$	+	--	2.5×10^{-6}
RN2-2 x L2 TG^R Oua^R a	-	6×10^{-6}	--
RN2-2 x L2 TG^R Oua^R a	+	4×10^{-5}	--
$C6TK^-$ x L2 TG^R Oua^R b	-	--	2.5×10^{-5}
$C6TK^-$ x L2 TG^R Oua^R b	+	--	1.7×10^{-3}

[a]Determined by growth in HAT (1) + Oua selection medium.
[b]Determined by growth in HAT (2) selection medium.

these selective conditions (Table 1), it was conceivable that parental cells might have survived the selection scheme. Thus, it was important to distinguish between authentic cell hybrids and parental survivors. Hybrid cells formed between these mouse and rat cells could be readily distinguished from the parental cells on the basis of their chromosome content and on the production of species-specific gene products.

As shown in Table 2, the mouse-rat hybrid cells had average chromosome numbers that were greater than those of either the rat or mouse parental cells used to form them.

To demonstrate that the chromosome content of the hybrid cells consisted of both mouse and rat chromosomes, a centromeric-staining procedure for mouse chromosomes using formamide-Giemsa was employed (12). As shown in Figure 1, the mouse chromosomes can be readily distinguished from the rat chromosomes because of the intense centromeric staining of the former. It is also evident form this Figure that in the two L2 TG^R Oua^R x RN2-2 cell hybrids shown, both mouse and rat chromosomes were present. Using this procedure and standard trypsin-Giemsa chromsome banding techniques, it was possible to identify both mouse and rat chromosomes in the hybrid cells. The chromosome compositions of

Table 2 Chromosome Content of Hybrids

Cell Line	Chromosome Number[a]	Mouse Chromosomes[b]	Rat Chromosomes[b]
Parental L2 TG^R Oua^R	42 ± 3		
RN2-2	42 ± 1		
$C6TK^-$	35 ± 5		
Hybrids (A) L2 TG^R Oua^R x RN2-2			
Hybrid 1	82 ± 5	38 ± 5	43 ± 4
2	80 ± 8	34 ± 3	46 ± 4
3	72 ±7	40 ± 3	32 ± 3
4	70 ± 6	33 ± 4	37 ± 5
5	75 ± 5	35 ± 3	38 ± 2
6	60 ± 2	22 ± 3	37 ± 3
7	69 ± 7	30 ± 4	40 ± 4
8	76 ± 4	36 ± 5	37 ± 5
(B) L2 TG^R Oua^R x $C6TK^-$			
Hybrid 21	55 ± 5	27 ± 7	28 ± 8
22	65 ± 8	27 ± 7	38 ± 5
23	71 ± 4		
24	69 ± 7		
25	70 ± 6		
26	73 ± 2	36 ± 2	38 ± 4

[a]Average chromosome number based on 5 to 10 chromosome spreads with standard deviation indicated.

[b]Determined by standard trypsin-Giemsa banding and the centrometric staining procedure of Marshall (12).

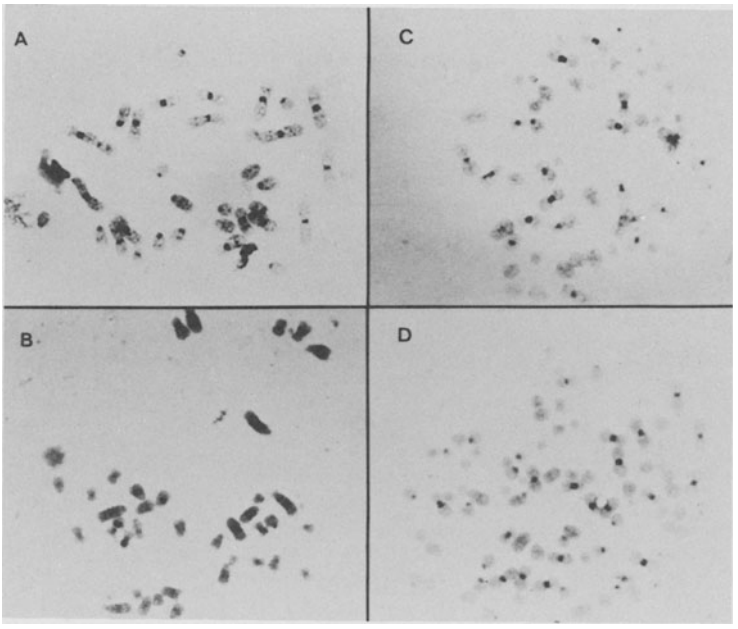

Figure 1

Figure 1. Chromosomes of selected cell lines. Chromosomes were prepared and treated with trypsin and formamide and stained with Giemsa as described in Materials and Methods. Chromosomes from (A) L2 TG^R Oua^R, (B) RN2-2, (C) HYBRID 6, and (D) HYBRID 2 cells.

the hybrids are summarized in Table 1. For the most part for both types of hybrids, a majority of the parental rat chromosomes was present with some variability in the parental murine chromosomes.

The authenticity of the hybrid cells was also demonstrated by the presence of both murine and rat lactate dehydrogenase isozymes. As shown in Figure 2 the rat RN2-2 cells produced a lactate dehydrogenase enzyme that migrated more cathodally than similar enzyme from mouse L2 TG^R Oua^R cells. When a mixture of rat and mouse lactate dehydrogenase was dissociated and reassembled in vitro, four major bands and a faint fifth band of enzymatic activity were obtained (Figure 2, lane D). Such a pattern was absent in a mixture of parental extracts (Figure 2, lane C). When extracts of hybrid cells were assayed for lactate

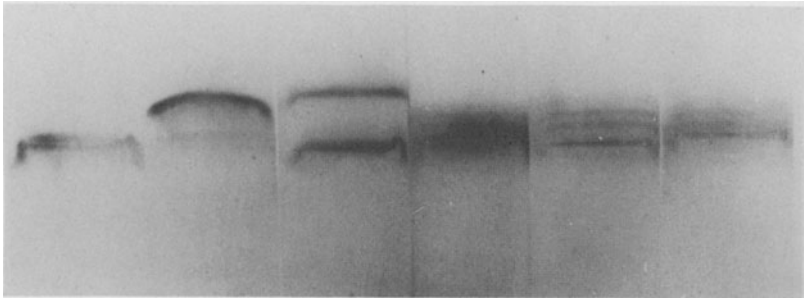

Figure 2. Lactate dehydrogenase isozymes in hybrid cells. Lactate dehydrogenase was assayed as described in Materials and Methods. Lactate dehydrogenase from (A) L2 $TG^{R}Oua^{R}$ extract. (B) RN2-2 extract; (C) a 1:1 mixture of L2 $TG^{R}Oua^{R}$ and RN2-2 extracts; (D) a 1:1 mixture of L2 $TG^{R}Oua^{R}$ and RN2-2 extracts assembled in vitro; (E) HYBRID 6 extract; and (F) HYBRID 2 extract.

dehydrogenase activity (Figure 2, lanes E,F) an isozyme pattern similar to that of the in vitro assembled isozymes was obtained. These results are consistent with the presence and association of both mouse and rat forms of lactate dehydrogenase in the hybrid cells. Similar results were obtained for the L2 TG^{R} Oua^{R} x C6 TK^{-} cells.

Replication of JHM and MHV_3 in hybrids between mouse fibroblasts and rat Schwannoma cells. Previous results indicated that mouse L2 cells supported the replication of both the JHM and MHV_3 virus strains in a lytic fashion which involved extensive cell destruction through syncytial formation. When RN2 cells were

used as host, JHM replicated persistently with restricted cytopathology in a temperature sensitive manner. MHV_3 replication was totally restricted in the RN2 cells (2,3). The availability of somatic cell hybrids between these 2 cell lines permitted an examination of which host cell type dominantly affected the virus replication process.

Confluent monolayer cultures of several independently selected hybrid cells were infected at a multiplicity of infection (moi) of 0.05 with either JHM or MHV_3, maintained at either 32^o or 39^o, and virus production determined after 24 hours. As shown in Table 3, the hybrid cells could replicate both JHM and MHV_3 at both temperatures. The virus yields for the most part were similar to those obtained with the L2 TG^R Oua^R cell line as host and considerably higher than those obtained with the RN2 cell. Accompanying these high levels of virus production was an extensive cytopathic effect (cpe) resulting from syncytial formation. By 24 hours at 39^o, essentially all the cells in the monolayer were involved and total destruction and cell lifting had occurred. A similar cpe was observed at 32^o, however, total destruction was delayed until 30 to 36 hours post-infection. These effects were apparent with the L2 TG^R Oua^R, and hybrid lines 1, 2, 3, 4, 5, 7, and 8. Hybrid 6 showed some differences. This hybrid, which produced lower yields of virus than the other hybrid lines at 24 hours (Table 3), showed very little, if any, cpe, at both temperatures with JHM virus. If a cpe was present it was restricted to less than 10% of the cells in the population. Similar results were also obtained with MHV_3 infections, although the cpe was somewhat more extensive, perhaps involving 20 to 30% of the cells in the population. The cpe observed with either virus in this line appeared to be restricted since longer incubation periods up to 7 days did not result in a more extensive cpe even though 30-80% of the cells scored as infectious centers. The extent of the cpe was also not affected by increasing the moi to 5 even though the number of cells scoring as infectious centers was 80%. Initially hybrid 6 cells could produce either JHM or MHV_3 virus at 39^o. However, after about 1 week in culture these infected cells lost the ability to shed virus at 39^o even though they continued to produce virus in a cyclical manner at 32^o. At this time, if cells shedding virus at 32^o were shifted to 39^o there was a cessation of virus production. The properties of cyclical release of virus and restricted virus replication at the elevated temperature is reminiscent of the JHM infection of RN2 cells in which from 0.1 to 10% of the cells are infected (2,3). In the case with hybrid 6 cells, however, both JHM and MHV_3 were replicated and the number of infected cells was from 50 to 80%. Ten subclones of the hybrid 6 cells behaved similarly when challenged with virus.

Table 3 Replication of JHM and MHV_3 in Parental and Hybrid Cell Lines

	Virus Yield (pfu/ml)			
	JHM		MHV_3	
CELL LINE	32^o	39.5^o	32^o	39.5^o
L2 $TG^R Oua^R$	4×10^5	2×10^5	5×10^5	4×10^5
RN2-2	4.5×10^2	5	50	5
HYBRID 1	5×10^5	4×10^5	1×10^5	1.5×10^5
2	3×10^5	2×10^5	5×10^5	3×10^5
3	2×10^5	1.5×10^5	1.8×10^5	1.1×10^5
4	5×10^5	3×10^5	5×10^5	3×10^5
5	2×10^5	3×10^5	5×10^5	7×10^4
6	6.5×10^3	2.9×10^3	7.5×10^4	3.3×10^4
7	2×10^5	2×10^5	5×10^5	3.6×10^5
8	4×10^5	3×10^5	3×10^5	1×10^5

[a]Confluent monolayers of the various cell lines were infected at a moi of 0.05 with either JHM or MHV_3 at either 32^o or 39^o. After 1 hr to allow for virus adsorption, the infected cells were washed 2 times with phosphate buffered saline, fed fresh medium, and incubated at the appropriate temperature. Virus released into the medium was assayed at 24 hrs post-infection by the plaque assay at 32^o on L2 monolayer as described in Materials and Methods.

Replication of JHM and MHV_3 in hybrids between mouse fibroblasts and rat glioma cells. Previously results indicated that the rat C6 glioma cell line was restrictive to the replication of both the JHM and MHV_3 strains (3). The availability of somatic cell hybrids between the L and C6 TK^- cells permitted an examination of which host functions were dominant.

Confluent monolayers of several independently selected hybrid cells were infected at an moi of 0.05 with either virus strain at 32°, and virus production monitored after 30 hours. The hybrids could replicate both JHM and MHV_3 (Table 4). The virus yields from the hybrid cells were similar to those from the parental mouse cells (L2 TG^R Oua^R). Accompanying this virus production in all the lines tested with both viruses was extensive syncytial formation involving about 80-100% of the cells in the culture.

DISCUSSION

The results described in this report indicate that the permissive or lytic state of coronavirus infection, characteristic of the mouse L fibroblast cell was a genetically dominant trait over the persistent or restrictive host states of the rat RN2 Schwannoma or rat C6 glioma cells. This conclusion is based on the observation that when somatic cell hybrids, formed between either the L and RN2 cells or the L and C6 cells, were infected with either JHM or MHV_3, virus yields and cytopathic effects were

TABLE 4 Virus Production From Mouse Fibroblast X Rat Glioma Cells

Cell Line	JHM	MHV_3
L2 TG^R Oua^R	5×10^5	5×10^5
$C6TK^-$	5	5
Hybrid 21	5×10^5	3×10^5
22	2×10^5	5×10^5
23	3×10^5	7×10^5
24	4×10^5	1×10^5
25	5×10^5	3×10^5
26	2×10^5	2×10^5

[a]Confluent monolayers of the various cell lines were infected at a moi of 0.05 with either JHM or MHV_3 at 32°. After 1 hr to allow for virus adsorption, the infected cells were washed 2 times with phosphate buffered saline, fed fresh medium, and incubated at 32°. Virus released into the medium was assayed at 30 hrs post-infection by the plaque assay at 32° on L2 monolayers as described in Materials and Methods.

similar to those of the L2 parental cell line. Such features are not characteristic of the infections of RN2 or C6 cells (2,3). However, one hybrid, hybrid 6, formed between RN2 and L2 TG^R Oua^R cells, and its subclones differed from the others of its type in their response to virus infection. These cells could initially replicate both virus strains at 32^o and 39^o without extensive syncytial formation. After about one week in culture such cells lost the ability to produce virus at 39^o but continued to produce virus at 32^o. The reason for the difference between this hybrid and the others is at present unclear. It may be related in some way to the chromosome content of this hybrid cell since its chromosome number is lower than that of the others (Table 2).

Since chromosome loss does occur in cell-cell hybrids (15) it is conceivable, however, that one of the rat chromosomes codes for a dominantly acting factor which is responsible for virus resistance or persistence. This chromosome may be among those that are frequently lost in the hybrids. This would thus lead to the viral susceptibility of the hybrids. Preliminary karyotyping results have indicated that the mouse-rat hybrids appear to contain a majority of the parental rat chromosomes. The individual hybrids do not appear to have consistently lost the same rat chromosomes. However, more extensive studies will be required to address this possibility. Once established these hybrids appear to be phenotypically stable since several of the lines have been kept in continuous culture for up to 8 months and periodically screened for susceptibility to lytic virus infection. Todate, all lines after prolonged culture behaved similarly in their responses to infection as they did shortly after isolation. The result that the totally permissive state (ie. the L cell) is dominant over the restrictive/persistent state (ie. the RN2 cell) or the totally restrictive state (i.e. the C6 cell) is not unlike other host-virus systems where it has been demonstrated that permissive host functions are dominant over nonpermissive ones (16,17,18). At present, it is unclear as to the nature and role that the L2 functions play in overcoming the RN2-2 controlled persistent, thermosensitive replication of JHM, and the restriction of MHV_3 and the C6 restriction of both viruses. Knowledge of these L cell functions might prove useful in an understanding of both coronavirus persistence and restriction. It is of interest to note that although MHV_3 replication is restricted in RN2 cells, if it is permitted to initiate replication as it does in hybrid 6 then after about 1 week in culture a persistent infection not unlike that of the JHM infection of RN2-2 cells can be obtained.

Since there are available several different cell lines that can become persistently infected with the JHM and MHV_3 virus strains (3) it will be of interest to determine whether these cell lines

behave in a manner similar to the RN2 cells when somatic cell hybrids are formed with the mouse L2 cells. Such studies are currently in progress.

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