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# Several Rat Cell Lines Share a Common Defect in Their Inability to Internalize Murine Coronaviruses Efficiently

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SUMMARY

Infection of rat cells, Schwannoma RN2, hepatoma HTC or myoblast L6, with the murine coronavirus JHM strain results in a persistent infection characterized by the release of virus over an extended period of time with a limited cytopathology. Several stages of the viral replication cycle have been examined in these cells in comparison to those in mouse L2 cells, which are totally permissive to JHM infection. Although the rat cells bound as much virus as the mouse cells their ability to internalize it was 40-fold less efficient than the mouse cells. This lower internalization efficiency was not enhanced by pH shock of infected cells, but was by treatment with polyethylene glycol. In all cell types there appeared to be no major differences in the ability of the internalized virus to replicate the viral RNA as determined by slot-blot analysis with a radiolabelled viral cDNA. A similar genetic mechanism appears to be operative in the lines because somatic cell hybrids formed between these lines in various combinations were also deficient in the ability to internalize bound virus. Taken together these results imply that rat cell lines in general share a common deficiency in their inability to internalize murine coronaviruses efficiently. This low efficiency in viral internalization may explain in part the ability of these lines to sustain persistent infections.

## INTRODUCTION

In recent years members of the murine coronaviruses have been studied extensively because of their ability to establish persistent infections both *in vitro* (Holmes & Behnke, 1981; Stohlman & Weiner, 1978; Lucas *et al.*, 1977, 1978; Leibowitz *et al.*, 1984; Lamontagne & Dupuy, 1984) and *in vivo* (Knobler *et al.*, 1981; Hirano *et al.*, 1980; Nagashima *et al.*, 1979; Sorensen *et al.*, 1980, 1982, 1984). Because of this property, these agents have been considered as useful models for several chronic disease states including demyelinating diseases.

Studies with the *in vitro* cell systems, which are simpler than the intact animal, have indicated that these agents can readily establish persistent infections in both neural and non-neural cell lines (Lucas *et al.*, 1977, 1978; Stohlman & Weiner, 1978; Leibowitz *et al.*, 1984) without requiring viral modifications or environmental manipulations such as the presence of viral antibody or interferon. These types of infections are generally characterized by the production of viral progeny at various levels over extended periods of time with little or no c.p.e. on the host cell. The mechanisms that allow these coronaviruses to be maintained in a persistent manner are not clear at present. It is apparent that mechanisms involving such factors as interferon, temperature-sensitive viral mutants and defective interfering viral particles, which are common for other viral agents that persist, are not operating for a majority of persistent coronavirus infections (Lucas *et al.*, 1977, 1978; Stohlman & Weiner, 1978; Leibowitz *et al.*, 1984; Coulter-Mackie *et al.*, 1985), although in some cases mutant viruses have been identified (Stohlman *et al.*, 1979; Holmes & Behnke, 1981). Thus it appears that the type of host cell is a major determinant in the persistent replication of these agents. In one system it has been suggested that the ability of the infected cell to resist viral c.p.e. may be an important parameter (Mizzen *et al.*,

1983), as is the efficiency of viral uncoating (Kooi *et al.*, 1988). It is conceivable that in different systems various stages in the viral replicative cycle may be altered in relation to the corresponding stages in permissive cells.

Previously, we have described a series of rat cell lines that could be persistently infected with the $MHV_3$ or JHM strains of murine coronaviruses and one line, the rat glial C6 cell, that was refractory to infection (Lucas *et al.*, 1977, 1978). Recently, we have demonstrated that the C6 cell is unable to internalize bound coronavirus (Van Dinter & Flintoff, 1987). In this report we demonstrate that the rat cell lines that can support persistent coronavirus infections are able to internalize coronavirus. This process however is relatively inefficient in comparison with mouse L2 cells which replicate coronaviruses lytically. Furthermore we demonstrate with somatic cell hybrids that in all rat cell lines a similar genetic mechanism is operative in regulating coronavirus infection. The inefficiency in internalizing the virus may account in part for the persistent state of infection.

## METHODS

*Cells.* The sources and routine propagation of the murine L2, rat hepatoma HTC, rat myoblasts L6, rat Schwannoma RN2 and rat glial C6 cells used in this study were as previously described (Lucas *et al.*, 1977, 1978; Flintoff, 1984) except that alpha medium was supplemented with 2% foetal calf serum and 8% Nuserum (Collaborative Research). For monitoring the early events of virus infection, the cells were grown on glass coverslips coated with 50 μg/ml poly-L-lysine.

*Selection of genetically marked cells.* The rat HTC and L6 cells were selected for resistance to thioguanine in the following manner. Both cell lines were treated for 18 h at 37 °C with 250 μg/ml ethyl methanesulphonate, washed and resuspended in fresh medium. Survival rates in both cases were about 40%. After 6 days of growth to allow for the expression of putative mutations, various numbers of cells were exposed to various concentrations of 6-thioguanine (TG) (Sigma). Medium and drug were replaced every 3 days and colonies surviving at a frequency of $10^{-5}$ at a drug concentration of 0·5 μg/ml were picked, cloned by limit dilution and tested for resistance. One clone from each line (HTC $TG^R4$ and L6 $TG^R6$) that was resistant to 10 μg/ml TG was used for further studies. These lines showed less than 1% of the wild-type hypoxanthine-guanine phosphoribosyltransferase activity as determined by the assay described by Chasin & Urlaub (1976).

The HTC $TG^R4$ cells were re-mutagenized as above and exposed to 3 mM-ouabain (Oua) to select for Oua-resistant cells (Baker *et al.*, 1974). Colonies surviving at a frequency of $10^{-5}$ were isolated, cloned and shown to be resistant to at least 3 mM-Oua. One doubly marked clone, HTC $TG^ROua^R4$-4, was used in the hybridization experiments.

The L6 $T6^R6$ cells were re-mutagenized as above and exposed to 2 μg/ml α-amanitin (Ama) to select for Ama-resistant cells (Somers *et al.*, 1975). Colonies surviving at a frequency of $5 \times 10^{-6}$ were isolated, cloned and shown to be resistant to 2 μg/ml Ama. One doubly marked clone, L6 $TG^RAma^R6$-1, was used in the hybridization experiments.

Resistance to either TG, Oua, Ama, a combination of TG and Oua or of TG and Ama did not affect the ability of the cells to replicate JHM in a persistent manner.

*Cell–cell hybridization.* Somatic cell hybrids were formed between various combinations of the cell lines by exposure to polyethylene glycol (PEG) 8000 (BDH) as previously described (Flintoff, 1984) except that $Ca^{2+}$-free medium was used in the fusion and washing stages of the procedure.

For hybridizations involving the TG- and Oua-resistant markers, the selection medium was HAT with Oua ($7 \times 10^{-5}$ M-hypoxanthine, $10^{-6}$ M-methotrexate, $4 \times 10^{-5}$ M-thymidine and $2 \times 10^{-3}$ M-Oua). For hybridizations involving the TG- and Ama-resistant markers, the selection medium was HAT with Ama ($7 \times 10^{-5}$ M-hypoxanthine, $10^{-6}$ M-methotrexate, $4 \times 10^{-5}$ M-thymidine and 2 μg/ml Ama). Under these selective conditions parental cells had surviving frequencies of $< 1 \times 10^{-6}$. Putative hybrids formed between the parental lines had surviving frequencies between $5 \times 10^{-4}$ and $5 \times 10^{-3}$ depending upon the cross.

After 8 to 10 days growth in the selective medium, surviving colonies were picked, expanded and tested for susceptibility to JHM infection as soon as possible after their isolation. Hybrids were characterized by the composition and morphological distinguishing features of their chromosomes (Flintoff, 1984). In all hybrids, the chromosome composition was in the expected range for simple combinations of the two parental cell types. Although there was variation in the chromosome composition among the mouse–rat cell hybrids, there was no apparent consistent pattern in the nature and type of chromosomes that were present or absent.

*Virus.* The source and routine propagation of the murine hepatitis virus strain JHM was as previously described (Lucas *et al.*, 1977, 1978). Virus production was monitored by a plaque assay on L2 cell monolayers as previously described (Lucas *et al.*, 1977) and yields are expressed as p.f.u./ml. To determine the fraction of cells able to release virus an infectious centre assay was performed (Lucas *et al.*, 1978). Large scale preparations of virus were prepared

in the following manner. After 50% of the fused L2 cells had lifted off from the tissue culture flask, the remainder of the monolayer was scraped using a rubber policeman, pooled with the supernatant virus and passed twice through a 30-gauge needle. Cellular debris was removed by centrifugation at 2000 *g* for 10 min at 4 °C. The resulting supernatant was filtered through a 0·22 μm Millex-GV filter unit (Millipore) and used as the source of infecting inoculum.

*Virus adsorption.* Virus bound to monolayer cultures was determined as previously described (Van Dinter & Flintoff, 1987). Briefly, cell monolayers were treated with virus at 4 °C for various periods of time. Unbound virus was removed by washing the infected cells with phosphate-buffered saline (PBS) containing 0·5% bovine serum albumin (BSA) and 0·05% Tween 20. The cells were removed from the substrate by scraping into 1 ml of cold medium and disrupted by passage through a 30-gauge needle. The amount of virus present was titrated as described above.

*Virus internalization.* Virus internalized by monolayers of the various cell types was determined as previously described (Van Dinter & Flintoff, 1987). Cell monolayers were adsorbed for 60 min with virus at 4 °C, washed as above to remove unbound virus and shifted to 37 °C for various time periods. Subsequently, cultures were washed with cold PBS and treated with 0·5 mg/ml of proteinase K in PBS for 45 min at 4 °C to remove external virus. The cell suspension was diluted with an equal volume of 2 mM-PMSF, 6% BSA and centrifuged for 2 min at 650 *g* at 4 °C. The cell pellets were washed twice in 2% BSA and assayed for internalized virus by the infectious centre assay. To assay for the infectivity of internalized virus the infected cells were disrupted by passage twice through a 30-gauge needle and the amount of virus was quantified as described above.

*PEG-induced cell fusion.* To enhance virus internalization cells with bound JHM were fused by exposure to PEG 8000 (Flintoff, 1984; Van Dinter & Flintoff, 1987). The cultures were incubated at 37 °C and at various times monitored for the number of infected cells by the infectious centre assay.

*Virus-specific RNA synthesis.* Total RNA was isolated from $5 \times 10^5$ infected cells by the guanidine–HCl extraction procedure described by Cheley & Anderson (1984). The RNA samples in 10 × SSC (1 × SSC is 0·15 M-NaCl, 0·015 M-trisodium citrate) and 7·5% formaldehyde were heated to 50 °C for 15 min, cooled on ice, slot-blotted at various dilutions onto Biodyne paper (Pall) and treated with a germicidal u.v. light for 3 min to crosslink the RNA to the paper (Khandjian, 1987). The blots were prehybridized at 43 °C with annealing buffer consisting of 50% formamide, 5 × SSC, 5 × Denhardt's buffer (Denhardt, 1966), 20 mM-sodium phosphate pH 7·0, 20 μg/ml denatured salmon sperm DNA, 250 μg/ml yeast RNA and 0·1% SDS. After pre-annealing, $5 \times 10^6$ c.p.m. of heat-denatured $^{32}$P-labelled plasmid g344 DNA (Lavi *et al.*, 1984, kindly supplied by Dr S. Weiss) radiolabelled by random priming (Feinberg & Vogelstein, 1983) to a specific activity of $5 \times 10^8$ c.p.m./μg DNA, was added to the blots and incubation continued at 43 °C for 48 h. This plasmid clone contains a 1800 kb insert containing the E1 gene for murine coronavirus strain A59, as well as part of the nucleocapsid gene and regions of gene 5. Because of the homology among the various murine coronavirus strains and the nested set of the coronavirus mRNAs (Sturman & Holmes, 1983), this clone is capable of detecting JHM virus-specific RNA sequences in infected cells.

After hybridization with the labelled probe, the blots were washed at 37 °C in 2 × SSC–0·1% SDS and exposed to Kodak XAR X-ray films for 1 day with Cronex intensifying screens. Densitometric scanning of the slot-blot autoradiographic images was performed to quantify the level of virus-specific RNA.

To correct for varying amounts of RNA on the filters, the blots were incubated in hybridization solution at 60 °C for 5 h to remove bound probe, pre-hybridized overnight in fresh hybridization solution at 43 °C and rehybridized with $5 \times 10^6$ c.p.m. of cloned mouse rDNA (Arnheim, 1979) labelled with $^{32}$P as above to a specific activity of $4 \times 10^8$ c.p.m./μg. The cloned rDNA was kindly supplied by Dr N. Arnheim. After 48 h incubation at 43 °C, the blot was washed, exposed to X-ray film and scanned as described above.

## RESULTS

Previous work has indicated that rat glial C6 cells are unable to internalize the murine coronaviruses (Van Dinter & Flintoff, 1987). It was of interest to determine whether other rat cell lines manifested this same defect or whether other stages in the coronavirus replicative cycle were affected. Comparisons were made with corresponding stages in the permissive mouse L2 line.

### *Adsorption*

To examine the initial phase of JHM infection which involves an interaction of the viral E2 glycoprotein with cell surface receptors (Sturman & Holmes, 1983), JHM virus at an m.o.i. of 2 to 3 p.f.u./cell was added to monolayer cultures of either L2, HTC, L6 or RN2 cells and allowed to interact at 4 °C. At intervals the infected cells were washed with Tween 20 to remove non-specifically bound virus and the bound virus was determined as described in Methods. As shown in Fig. 1, the virus bound to all cell types with similar kinetics and saturation levels. In all cell

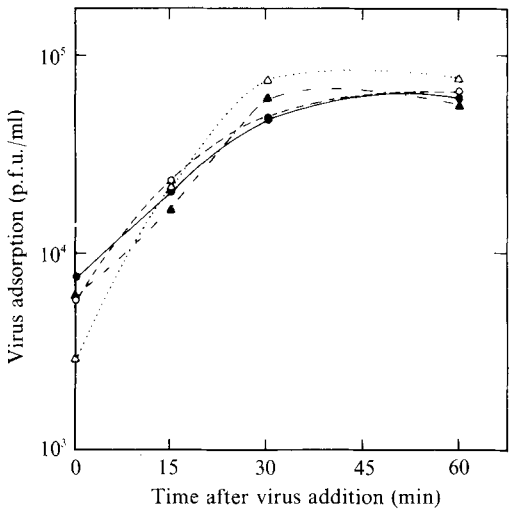


Fig. 1

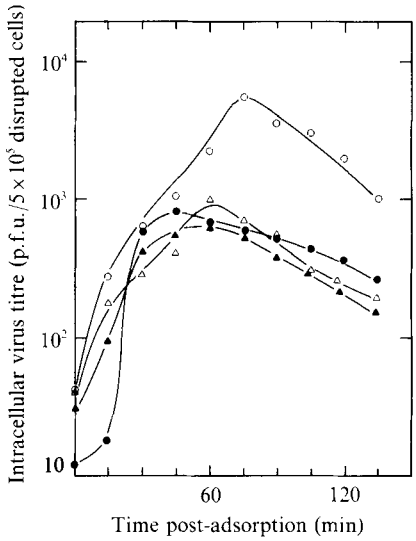


Fig. 2

Fig. 1. Adsorption of JHM to mouse L2 and rat cell lines. JHM at an m.o.i. of 2 to 3 was adsorbed at 4 °C to monolayers of L2 (○), RN2 (▲), HTC (△) or L6 (●) cells. Unbound virus was removed by washing with PBS containing BSA and 0·05% Tween 20. Bound virus was assayed at various times as described in Methods.

Fig. 2. Internalization of JHM by mouse L2 and rat cell lines. Cultures of JHM-adsorbed L2 (○) infected at an m.o.i. of 0·3, and rat cells RN2 (▲), HTC (△) and L6 (●) infected at an m.o.i. of 4 were warmed to 37 °C for set time periods, treated with proteinase K to remove external virus, disrupted by passage through a needle and assayed for infectious virus.

lines and under these binding conditions approximately 3% of the total input virus was bound to the cell. Similar results were obtained when radiolabelled virus was bound to the cells using the conditions recently described by Wilson & Dales (1988). In this case, all cell lines bound approximately 10 to 15% of the input label (data not shown). This difference in the amount of virus bound is a reflection of the nature of the assays. The former assay measures infectious particles, whereas the latter measures structural binding of all virions and so a given inoculum contains non-infectious as well as infectious virions. These data suggest that this initial phase of the replication cycle in the rat cell lines does not dramatically differ from that in mouse L2 cells.

### *Virus internalization*

Subsequent to adsorption, the murine coronaviruses are presumably internalized into the host cell by absorptive endocytosis (David-Ferreira & Manaker, 1965). To examine the internalization of JHM by the various cell types, JHM-adsorbed cells were incubated at 37 °C for various time periods and the amount of virus internalized was determined as described in the legend to Fig. 2. As shown in Fig. 2, L2 cells infected at an m.o.i. of 0·3 rapidly took up the virus, which reached a peak intracellular level in about 75 min before a decrease occurred presumably resulting from viral eclipse. The rat cells infected at an m.o.i. of 4 also took up the virus, reaching peak intracellular levels at 45 to 60 min post-adsorption prior to the eclipse phase. The temporal difference when peak intracellular levels were obtained in the mouse and rat cells was probably due to the differences in the initial m.o.i. which affects the amount of virus bound. Although the rat cells could take up virus the amount of bound virus that they internalized was dramatically different from that taken up by the mouse L2 cells. Under these assay conditions the L2 cells internalized approximately 35% of the virus bound on the cell surface. In contrast, the RN2 cells internalized 0·6% of bound virus, the HTC cells 1%, and the L6 cells 0·8%. These differences in

Table 1. *Infectious centres 60 min after temperature shift*

| Cell line | Infectious centres (%) |
|---|---|
| L2 | 5 |
| L6 | 0·01 |
| HTC | 0·4 |
| RN2 | 0·02 |

Table 2. *Percentage of infected cells after treatment with or without PEG*

| Cell line | PEG | Infectious centres (%) |
|---|---|---|
| L2 | − | 27·0 |
| L2 | + | 29·0 |
| RN2 | − | 0·035 |
| RN2 | + | 0·52 |
| HTC | − | 0·2 |
| HTC | + | 4·4 |

viral internalization were also reflected in a decreased number of infectious centres assayed 60 min after the temperature shift (Table 1).

Infecting the rat cells with an m.o.i. below 1 yielded marginally detectable levels of internalized virus. Increasing the viral m.o.i. to 40 in the rat lines allowed more virus to be internalized but this amount was still only about half of that internalized by the L2 cells infected at an m.o.i. of 2 (data not shown).

### *Attempts to enhance JHM internalization*

#### *Effect of pH shock*

The internalization of a number of envelope viruses occurs by receptor-mediated endocytosis (Marsh, 1984) and if this route is blocked by various means infection can be induced by a brief treatment of low pH conditions (Helenius *et al.*, 1980). To examine whether exposure to low pH would enhance JHM internalization in the rat cell lines, JHM was allowed to adsorb to the RN2 and L6 cells, the infected cells were exposed to a medium of pH 5·5 for 5 to 10 min and the internalization of virus was measured. The treatment did not enhance the ability of the cells to internalize the virus.

#### *PEG fusion*

Previously, we have shown that the murine coronaviruses can be introduced by fusion with PEG into the rat glial C6 cell line which is totally deficient in coronavirus internalization (Van Dinter & Flintoff, 1987). It was thus of interest to determine whether this treatment would also enhance internalization in the rat cell lines. JHM at an m.o.i. of 2 was adsorbed to monolayers of HTC and RN2 cells. After removal of non-specifically bound virus, the infected cells were treated with PEG, incubated for 60 min at 37 °C, treated with proteinase K and the amount of internalized virus was measured by an infectious centre assay. As shown in Table 2, the PEG treatment greatly enhanced the percentage of cells scoring as infectious centres for the rat lines examined but had little effect on the infectious process in mouse L2 cells.

### *Viral RNA synthesis*

In order to evaluate whether RNA synthesis occurred with equal efficiency once the virus had penetrated into the cells, various rat cell lines (RN2 and HTC) and the mouse line L2 were infected with JHM. Because the L2 cells took up the virus more efficiently, the m.o.i. for these cells was 0·02 compared to 2 for the rat cell lines. In addition JHM was introduced into the rat cells by PEG fusion to assess viral RNA synthesis under these conditions. At various times,

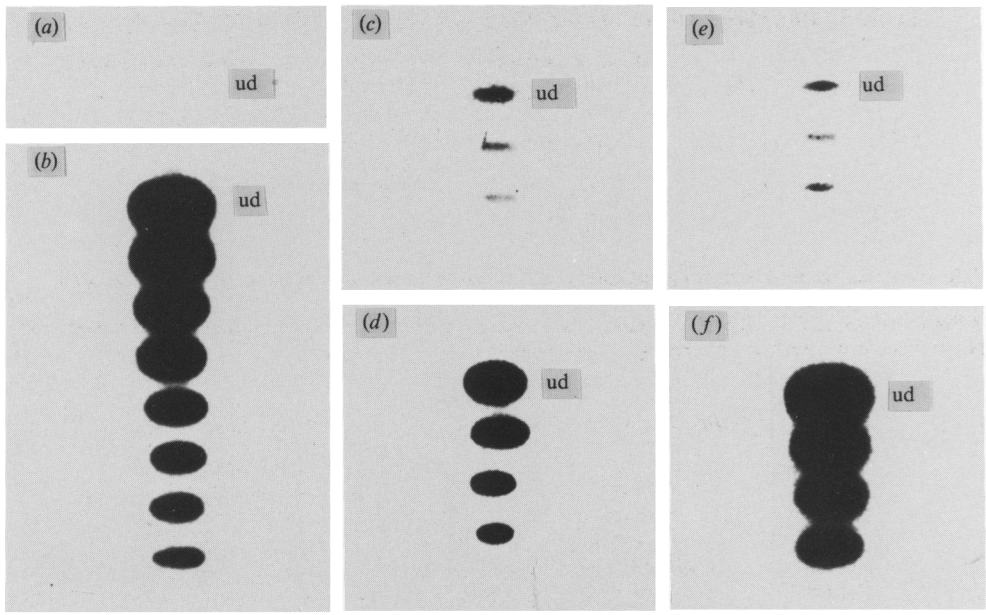


Fig. 3. Viral RNA synthesis. RNA was isolated from approximately 5 × $10^5$ JHM-infected (*b*, *c*, *d*, *e* and *f*) or uninfected (*a*) cells 24 h post-infection and slot-blotted either undiluted (ud) or in serial two-fold dilutions onto Biodyne paper. After prehybridization, the blot was hybridized with $^{32}$P-labelled A59 cDNA for 48 h, washed and exposed to an X-ray film. Exposure was for 24 h. (*a*) Uninfected L2 cells; (*b*) L2 cells infected with JHM at an m.o.i. of 0·02; JHM-infected RN2 (*c*) or HTC (*d*) at an m.o.i. of 2; JHM-infected RN2 (*e*) or HTC (*f*) at an m.o.i. of 2 and treated with PEG.

virus released into the supernatant was assayed and the number of infectious centres was scored. Total RNA was extracted from a duplicate infected culture and the amount of JHM-specific viral RNA present was determined by slot-blot analysis by using a $^{32}$P-labelled cloned cDNA representing part of the genome of the A59 strain of murine coronaviruses. As shown in Fig. 3, after 24 h incubation all cultures produced detectable virus-specific RNA sequences. It is also evident that the PEG-treated cultures produced more JHM RNA than the non-treated ones (compare Fig. 3*c* and *e*, and *d* and *f*) although the effect on RN2 cells may be marginal. Similar results were also obtained with the infected rat cell cultures after 72 h incubation although the amount of viral RNA produced was approximately one-third to one-half less than that present at 24 h (data not shown). When the amount of virus-specific RNA was quantified and correlated with the number of infectious centres, there were no major differences among the cell lines in their abilities to allow synthesis of the viral RNA (Table 3).

At 24 h post-infection the L2 cell culture had about half of the monolayer involved in syncytial formation. In contrast, there was no evidence of c.p.e. in the RN2 or HTC cultures that had not been treated with PEG. The PEG-treated cultures showed about 10% of the cells involved in syncytial formation. By 72 h post-infection, the L2 cells had been totally destroyed, both the PEG-treated and non-PEG-treated RN2 cultures showed less than 10% of the cells involved in a c.p.e., whereas both the HTC cultures had from 10 to 20% of the cells in syncytia.

### *Studies with somatic cell hybrids*

The results to date indicate that the rat cell lines RN2, HTC and L6 were inefficient in the internalization of bound JHM virus. Previous work has shown that the rat glial C6 cells are totally deficient in viral internalization (Van Dinter & Flintoff, 1987). It was of interest to determine whether this defect was reflected at the same genetic level in the various cell lines. In order to do this, however, it was necessary to establish that the persistent or restricted state of coronavirus infection was a genetically recessive trait. Previous work had shown that

Table 3. *Virus production and viral RNA synthesis in cell lines 24 h post-infection*

| Cell line | PEG | Infectious centres (%) | Virus yield (p.f.u./ml) | Viral RNA/infectious centre |
|---|---|---|---|---|
| L2* | − | 3 | ND† | 0·07 |
| L2 | − | 28‡ | $1 \times 10^5$ | 0·04 |
| RN2 | − | 0·06 | $5 \times 10^1$ | 0·05 |
| RN2 | + | 0·2 | $2 \times 10^2$ | 0·09 |
| HTC | − | 0·06 | $1 \times 10^2$ | 0·03 |
| HTC | + | 2 | $1{\cdot}7 \times 10^3$ | 0·04 |

* At the m.o.i. of 0·02 used in these experiments at 8 h there was no apparent syncytial formation.
† ND, Not determined.
‡ Because of extensive syncytial formation, the infectious centres may be underestimated and thus the ratio of RNA/infectious centre may as a result be overestimated.

Table 4. *Replication of JHM in hybrid cell lines: interspecies crosses*

| Cell line | Modal chromosome number | Range | Virus yield (p.f.u./ml) Day 1 | Day 3 |
|---|---|---|---|---|
| *Mouse L2 cells × rat HTC cells* | | | | |
| L2 Oua$^R$ 7-1 | 38 | 34–45 | $1{\cdot}5 \times 10^5$ | $1{\cdot}0 \times 10^3$ |
| HTC | 38 | 36–47 | $5{\cdot}9 \times 10^2$ | $4{\cdot}7 \times 10^2$ |
| Hybrid 1 | 70 | 63–73 | $1{\cdot}4 \times 10^5$ | $2{\cdot}6 \times 10^4$ |
| Hybrid 2 | 71 | 65–74 | $5 \times 10^5$ | $2 \times 10^3$ |
| *Mouse L2 cells × rat L6 cells* | | | | |
| L2 Oua$^R$ 7-1 | 38 | 34–45 | $1{\cdot}5 \times 10^5$ | $1{\cdot}0 \times 10^3$ |
| L6 | 46 | 42–47 | $1{\cdot}5 \times 10^1$ | $1{\cdot}2 \times 10^2$ |
| Hybrid 1 | 73 | 64–78 | $1{\cdot}0 \times 10^3$ | $1{\cdot}0 \times 10^4$ |
| Hybrid 2 | 76 | 67–88 | $1{\cdot}8 \times 10^5$ | $5 \times 10^5$ |
| *Mouse L2 cells × rat C6 cells* | | | | |
| L2 Oua$^R$ 7-1 | 38 | 34–45 | $5 \times 10^5$ | $1{\cdot}4 \times 10^5$ |
| C6 | 35 | 37–42 | $1{\cdot}0 \times 10^1$ | 0 |
| Hybrid 1 | 68 | 57–80 | $1 \times 10^5$ | $5 \times 10^5$ |
| Hybrid 2 | 71 | 60–74 | $8 \times 10^4$ | $1{\cdot}4 \times 10^5$ |

persistence in the RN2 cell was recessive to the lytic state manifested by the mouse L2 cells (Flintoff, 1984).

### *Replication of JHM in mouse–rat cell hybrids*

Confluent monolayers of mouse–rat hybrid cells derived from two independent fusion experiments for each cross were infected at an m.o.i. of 2 to 3 of JHM at 32·5 °C. After one hour to allow for viral adsorption, the inoculum was removed, the monolayers were washed twice with PBS, overlaid with fresh medium and monitored for the production of extracellular virus for a period of 3 days.

As shown in Table 4, the hybrid cells, formed between the mouse L2 and either the rat HTC or the rat C6 cells, were able to replicate the JHM virus. These hybrid cells produced virus yields similar to those of the parental mouse cells and considerably higher than those of the rat cells. Accompanying this virus production was a c.p.e. characterized by extensive syncytial formation in which total destruction of the monolayer occurred between 24 and 48 h, not unlike the c.p.e. of the parental L2 cells. JHM replication in these hybrid cells was similar to its replication in mouse L2–rat RN2 (Schwannoma) cells previously described (Flintoff, 1984). The ability of the hybrid cells to replicate JHM was also reflected in the ability of such cells to permit viral penetration (Table 5). The ability of hybrid cells to internalize virus was similar but not identical to that of the mouse parental L2 cells.

The replication of JHM in hybrids formed between mouse L2 and rat L6 cells occurred somewhat differently than the hybrids described above. Although these hybrid cells produced

Table 5. *JHM internalization by hybrid cells*

| Cell line | Internalized virus* |
|---|---|
| L2 | 5000 |
| RN2 | 140 |
| HTC | 60 |
| L6 | 100 |
| C6 | 0 |
| L2 × RN2 | 2000 |
| L2 × HTC | 3800 |
| HTC × RN2 | 80 |
| L6 × C6 | 40 |

* Virus titre is per $5 \times 10^5$ cells at 60 min post-adsorption with an initial m.o.i. of 1·0.

Table 6. *Replication of JHM in rat cell line hybrids: complementation analysis*

| Cell line | Modal chromosome number | Range | Virus yield (p.f.u./ml) Day 1 | Day 3 | Day 5 |
|---|---|---|---|---|---|
| *Rat L6 cells × rat RN2 cells* | | | | | |
| L6 $TG^RAma^R$ 6-1 | 46 | 42–47 | 115 | 30 | 50 |
| RN2 | 39 | 35–43 | 185 | 25 | 640 |
| Hybrid 1 | 75 | 65–82 | 110 | 45 | 10 |
| Hybrid 2 | 70 | 60–85 | 55 | 10 | 60 |
| *Rat L6 cells × rat HTC cells* | | | | | |
| L6 $TG^RAma^R$ 6-1 | 46 | 42–47 | 115 | 30 | 50 |
| HTC | 38 | 36–47 | 185 | 355 | 100 |
| Hybrid 1 | 80 | 68–93 | 50 | 150 | 220 |
| Hybrid 2 | 81 | 63–85 | 755 | 445 | 185 |
| *Rat HTC cells × rat RN2 cells* | | | | | |
| HTC $TG^ROua^R$ 4-4 | 38 | 36–47 | 185 | 355 | 100 |
| RN2 | 39 | 35–43 | 185 | 25 | 640 |
| Hybrid 1 | 72 | 67–86 | 105 | 30 | 50 |
| Hybrid 2 | 76 | 68–101 | 165 | 50 | 250 |
| *Rat L6 cells × rat C6 cells* | | | | | |
| L6 $TG^RAma^R$ 6-1 | 46 | 42–47 | 115 | 30 | 50 |
| C6 | 35 | 37–42 | 5 | 0 | 0 |
| Hybrid 1 | 70 | 65–82 | 75 | 10 | 50 |
| Hybrid 2 | 72 | 67–89 | 150 | 50 | 100 |

virus at levels comparable to those from the parental mouse L2 cell (Table 4), there was no readily apparent c.p.e. However, upon closer examination small syncytia containing few nuclei were apparent in these infected cultures. This restriction of syncytial formation was similar to that found in the parental L6 cells (Lucas *et al.*, 1978; Beushausen, 1986). These mouse L2–rat L6 hybrid cells have been shown to produce high titres of virus over at least a week in culture with no readily apparent destruction of the monolayer.

### *Replication of JHM in rat–rat cell hybrids*

The data described above and previous information (Flintoff, 1984) indicated that the ability of cells to replicate JHM virus to initial high titres was a genetically dominant trait and therefore that the host-controlled functions for persistent and restricted infections were recessive. Because the various rat lines internalized JHM poorly (HTC, RN2, L6) or not at all (C6), it was of interest to determine whether this defect(s) was operative at the same genetic level in all the cell lines. Somatic cell hybrids were thus formed in various combinations of these four lines as described in Methods. These hybrid cells were considered to contain the genetic information from both parental lines as their chromosome content was in the expected range for simple combinations of the two cell types (Table 6).

Monolayer cultures of hybrids from two independent fusion experiments for each of the crosses were infected with JHM at an m.o.i. of 2 to 3 at 32·5 °C and viral production was monitored for 5 days as described above. In all the cases examined there was no indication of the initial production of high yields of virus (Table 6) which would be indicative of a complementation among cell functions involved in viral replication. In fact the hybrid cells produced low levels of virus accompanied by a restricted c.p.e. not unlike the infection obtained with the parental rat cells. This was true whether crosses were made between the rat cell lines that produced persistent JHM infections, or between the rat glial C6 line (which was restrictive to infection) and the other rat lines. In these latter cases, the replication of the virus was similar to its replication in the parent that supported a persistent infection. The efficiency of virus internalization in the hybrid cells was similar to that of the parental cells (Table 5) indicating that the inability of the hybrid cells to produce high yields of virus is a reflection of the inability to internalize the virus.

## DISCUSSION

Several studies have indicated that the host cell can modulate or restrict murine coronavirus infection at various stages of the viral replicative cycle. These effects can occur at the adsorption phase due to a deficiency in receptors (Tardieu *et al.*, 1986; Boyle *et al.*, 1987), at the uncoating stage by as yet undetermined mechanisms (Beushausen *et al.*, 1987; Kooi *et al.*, 1988) which may be related to the differentiation state of the cell (Beushausen & Dales, 1985; Beushausen, 1986; Beushausen *et al.*, 1987) or at later stages during the spread of infection (Wilson & Dales, 1988) perhaps due to variations in proteolytic activity (Frana *et al.*, 1985). In this report and in a previous one (Van Dinter & Flintoff, 1987) we demonstrate that internalization of bound virus can also be a stage at which host control can occur.

The rat cell lines used in this study bound the virus with an efficiency similar to that of the permissive mouse L2 cells, but the amount internalized was approximately 40-fold less. The virus that was internalized appeared to be uncoated at a similar rate as in the mouse cells. A similar genetic mechanism for this inefficient viral internalization appears to be operative in these cell lines because somatic cell hybrids formed between various combinations of the cell lines failed to internalize the virus efficiently. At present, the nature of the host components that regulate the viral penetration into the cell is not clear; treatment with altered pH conditions which might have been expected to enhance the internalization had no effect. One possibility is that the virus is binding to different receptors on the mouse and rat cells. In the former case, the receptor–virus complex may be effectively internalized whereas in the latter it is not. The partial blockage of internalization was overcome to some extent by treatment with PEG. It is of interest to note that in other systems different host cells may have different receptors for the same virus. For example, the cell receptors for Sindbis virus are different between mosquito and mammalian cells (Smith & Tignor, 1980) and ecotropic murine leukaemia virus appears to bind to a class of receptor that is slowly internalized (Handelin & Kabat, 1985).

Once the JHM virus had been internalized by the rat cells either with or without PEG fusion, the ability to replicate its RNA was as efficient as that in the mouse L2 cells, indicating that there were no major restraints on overall viral RNA synthesis in these cells. It is possible however that there may be variations in the abundance of the intracellular viral RNA species among the cell lines. In this present study, no attempt was made to quantify the levels of virus-specific proteins made by the rat cells because of the low level of infected cells and progeny virus particles produced. It seems unlikely that there were major differences between the rat and mouse cell lines in the ability to translate the viral messages because the yields of extracellular virus per infected centre differ by only two-to-three-fold (see Table 3).

In a persistently infected culture it is possible to envisage how an inefficient viral internalization process could contribute to the maintenance of the infection. At any given time only a fraction of the bound virus will be internalized to allow replication to proceed. Extracellular virus released from this infection would bind to other cells in the culture, but again only a small fraction would be internalized to undergo replication. Thus in this manner an infection could be maintained over an extended period of time. For the persistent JHM

infections of the rat cells this may not be the complete explanation. Although virus is produced with apparently equal efficiency to that in the mouse L2 cells the accompanying c.p.e. was limited suggesting that viral spread by cell fusion may also be defective. Cell-to-cell fusion is a property of the viral E2 glycoprotein which requires activation by proteolytic cleavage (Sturman *et al.*, 1985). The restricted cytopathology in these infections could result from several factors: lack of cleavage of the E2 protein, lack of the E2 protein on the plasma membrane which is required for cell fusion (Sturman & Holmes, 1983) or an inherent resistance of the rat cell lines to undergo virus-induced cell fusion. This latter possibility has been suggested as a mechanism for coronavirus persistence in another cell system (Mizzen *et al.*, 1983). In this regard it is of interest to note the occurrence of virus replication in somatic cell hybrids formed between the mouse L2 and rat L6 cells. Although high yields of virus were obtained that were comparable to the parental mouse cell line the c.p.e. was restricted not unlike the infection of the parental rat L6 cells. In preliminary experiments treatment of these hybrid cells with trypsin and subsequent addition to monolayers of mouse L2 cells resulted in a rapid induction of cell-to-cell fusion in the L cell monolayers (unpublished observations). In view of the apparent requirement for proteolytic cleavage of the E2 protein for cell fusion activity it is interesting to speculate that perhaps the E2 protein in these hybrid cells is present in an uncleaved form or that it is in a membrane environment which does not permit cell fusion to occur. Thus it will be of interest to evaluate the nature and location of this viral membrane glycoprotein in these hybrid cells. Such a restriction of cell fusion was evident only in the mouse L2–rat L6 hybrid cells and not in the other mouse–rat hybrid cells examined although the parental rat cell lines all show a restricted c.p.e. after infection with JHM (Lucas *et al.*, 1978). This implies that the requirements for cell fusion may vary among the various cell lines. This is consistent with observations that these cell lines were about a thousandfold less efficient as indicators for mouse L2 infection centres (unpublished observations) and that there is cell variability in virus-induced cell fusion (Frana *et al.*, 1985).

*In vivo* susceptibility to JHM infection has been localized to mouse chromosome 7 and it has been suggested that the function involved may be a proteolytic activity involved in the processing of the viral E2 glycoprotein which is involved in cell fusion (Knobler *et al.*, 1984). If this is the case it is conceivable that in the mouse L2–rat L6 hybrids, mouse chromosome 7 may be absent. Owing to the heterogeneity in chromosome number and content of the various hybrid cell lines, it is not at present possible to make a correlation between the presence of mouse chromosome 7 and the ability to undergo cell fusion.

A combination of inefficient viral internalization and an inability to undergo virus-induced cell fusion may be the contributing factors in the ability of some rat lines to support persistent coronavirus infections. Understanding the nature of these processes may be useful in elucidating mechanisms of viral persistence at the cellular level.

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