

The Replication of Murine Coronaviruses in Enucleated Cells

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Accepted December 18, 1980

Coronaviruses JHMV and A59V have been shown to replicate, produce viral-specific antigens and cytopathic effects (CPE) in enucleated 17CL-1 cells.

The replication strategy of coronaviruses is not currently understood. The RNA genome of some members of the group has been demonstrated to be large, single-stranded, unsegmented, polyadenylated, and infectious (1-4). Virus multiplication is thought to occur in the cytoplasm without a requirement for the cell nucleus. Most studies of coronavirus-infected cells using coronavirus-specific immunofluorescence have shown viral-specific antigens only in the cytoplasm (5, 6). Four hours post-infection (hpi) is the earliest that cytoplasmic viral-specific antigens have been observed (5). Pensaert (7) has suggested that immunofluorescence begins in the perinuclear region. The results obtained by immunofluorescence are consistent with the results obtained by electron microscopy which show that coronaviruses assemble in the cytoplasmic factories associated with cisternal membranes of the endoplasmic reticulum and the Golgi apparatus (5, 6). Robb and Bond (8), however, reported an intranuclear viral-specific antigen(s) 2 hpi in 17CL-1 cells infected with coronaviruses MHV-JHM (JHMV) or MHV-A59 (A59V).

The sensitivity of viruses to actinomycin D during replication has been used qualitatively to determine if viral replication is dependent on DNA-dependent RNA syn-

thesis. Some coronavirus group members, HCV 229E (9) and JHMV (Leibowitz *et al.*, unpublished observations) have been shown to be sensitive to actinomycin D early in infection. In contrast, mouse hepatitis virus-3 (MHV-3) has been shown to be insensitive to actinomycin D during replication (10), as has A59V (Leibowitz *et al.*, unpublished). Analysis of the effects of actinomycin D on the replication of 229E and JHMV indicate the observed reduction of virus yield to be a secondary effect rather than a direct effect on virus-specific RNA synthesis.

The observation of nuclear viral-specific antigens as well as the sensitivity of JHMV replication to actinomycin D has led us to definitively test the role of the nucleus during coronavirus replication by infecting enucleated cells with the murine coronaviruses A59V and JHMV. Cells were enucleated by exposure to high gravitational fields in the presence of cytochalasin B.

17CL-1 cells were enucleated by a modification of the method of Prescott *et al.* (11). Because JHMV and A59V normally do not grow to high titers, it was desirable to enucleate cells as efficiently as possible. Depending on the cell system, cultures have been obtained with 95-98% enucleated cells by the method of Prescott *et al.* (11). Detjen *et al.* (12) have utilized precentrifugation at 11,000 *g* at 37° before the addition of cytochalasin B to achieve >99% enucleation. In what may be an analogous modification, we allowed exponentially growing 17CL-1 cells to attach for short periods of time (45-90 min) to glass cover-

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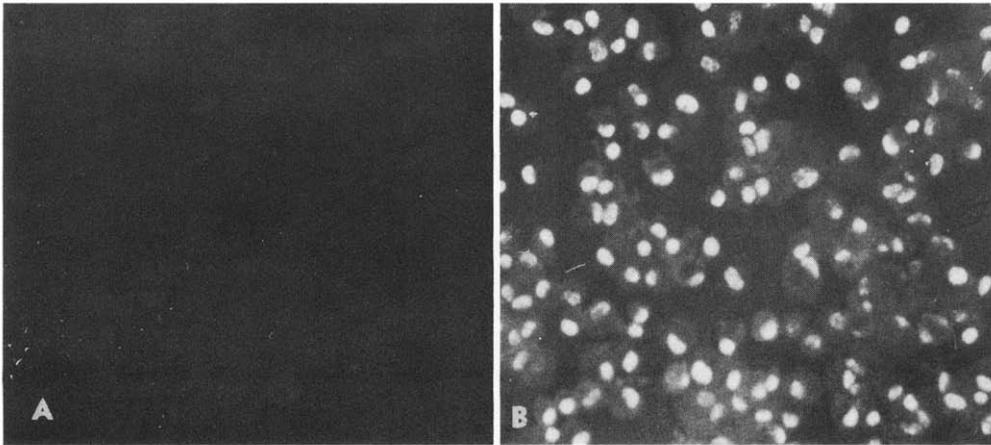


FIG. 1. Efficient enucleation of 17CL-1 cells. Enucleated 17CL-1 cells were prepared by a modification of the method of Prescott *et al.* 17CL-1 cells (16) were grown and maintained as previously described (17). Cells were trypsinized, resuspended in DEB 10 (Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum) and incubated for 45 min to attach to 1.2-mm thick, 2.2-cm-diameter glass coverslips cut from microscopic slides. Coverslips were placed inverted into Spinco SW27 polyallomer tubes with 8 ml DEB 0 (serum-free medium) containing 10 $\mu\text{g}/\text{ml}$ of cytochalasin B and 1% DMSO. Cells were enucleated by centrifugation for 60 min at 8250 rpm at 37° in a Sorvall SS-34 rotor. Seven milliliters of water was added to each tube holder to help maintain tube integrity and thermal equilibrium. The SS-34 rotor was prewarmed in a Sorvall RC2 centrifuge by operation at 15,000 rpm for 1 hr. Enucleated cells were incubated for 1 hr in 35-mm dishes with 2 ml DEB 10 and then fixed with methanol, acetic acid (3:1) and stained with the fluorescent nuclear stain bisbenzamid H 33258 (American Hoechst Corp.). Comparison of cell counts before and after enucleation demonstrated >90% recovery of cells. Panel A is a low-power (100 \times) field of enucleated cells. Panel B is a control coverslip of nucleated cells.

slips after trypsinization. Optimal enucleation was achieved by allowing the cells to attach for only 45 min prior to being placed inverted in polyallomer tubes and exposed to high gravitational fields. In the presence of cytochalasin B, 10 $\mu\text{g}/\text{ml}$, 17CL-1 cells were enucleated by this method with >95% recovery with less than 0.1% contaminating nucleated cells as determined by phase microscopic examination of coverslip cultures and by examination of fixed, Giemsa- or bisbenzamid H 33258-stained cultures. Panel A in Fig. 1 shows a representative low-power field of enucleate cells. A control coverslip of nucleated cells is shown for comparison in panel B. These methods of examination gave equivalent results. In some experiments a few nucleated cells were desirable for purposes of comparison. In this case the cells were allowed to attach to the coverslip for 90 min and were then enucleated. Figure 2 demonstrates immunofluorescent staining of virus-specific

antigens in JHMV- and A59V-infected and enucleated 17CL-1 cells. These infected cells undergo cell fusion and develop typical coronavirus CPE as well as synthesize virus-specific antigens.

Enucleated 17CL-1 cells support JHMV and A59V multiplication (Table 1). Mengovirus, a member of the picornavirus group, served as a positive control (13). The yield from the following types of infected coverslip cultures was compared: enucleated 17CL-1 cells, nucleated 17CL-1 cells exposed to cytochalasin B and a moderately increased gravitational field, and untreated cells. To preclude the detection of input virus, following virus adsorption for 30 min the cultures were incubated for 60 min with neutralizing antisera to A59V, JHMV, and mengovirus, respectively. The coverslips were then washed free of antiserum, one set of coverslips was frozen and stored at -40° and later processed and titrated in the same manner as the other coverslip cul-

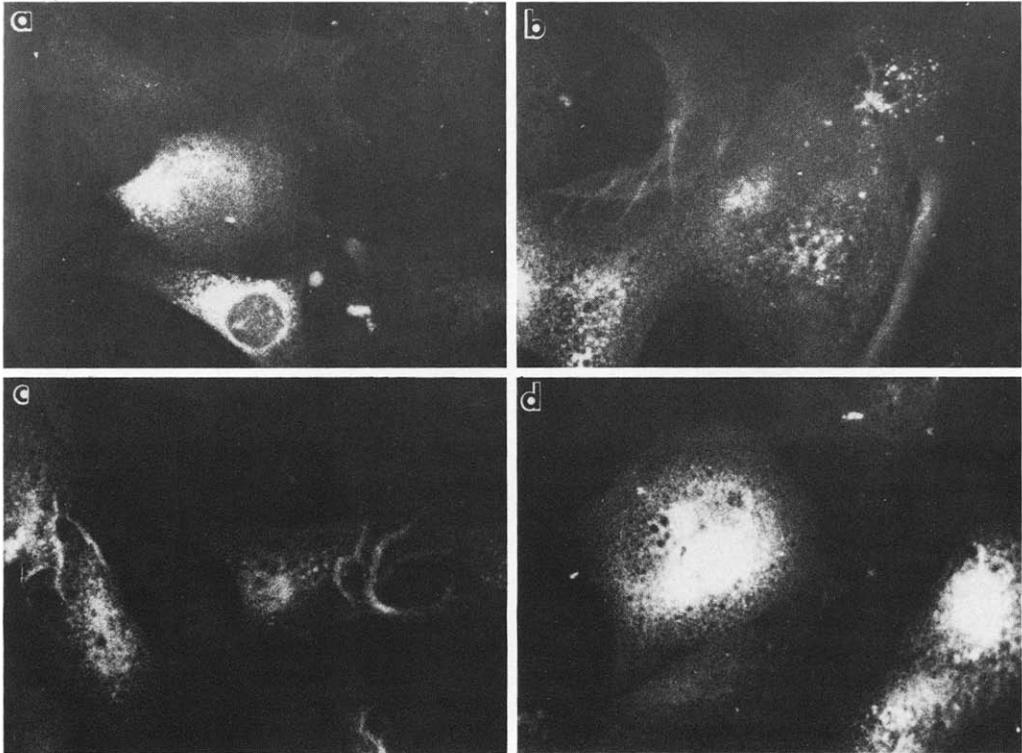


FIG. 2. Viral-specific antigen production in enucleated 17CL-1 cells. Enucleated 17CL-1 cells were prepared as described in Fig. 1 with the exception that cells were allowed to attach to the coverslips for 90 min prior to enucleation in order to provide some nucleated cells as positive controls for antigen production. Enucleated 17CL-1 cultures were infected at a multiplicity of 5 with A59V and JHMV, fixed 8 hpi, and stained as previously described (8). Panel (a) shows both enucleated and nucleated cells with and without JHM viral-specific antigen production. Panel (b) shows a typical field of JHMV-infected enucleated cells. Panels (c) and (d) show representative fields of A59V-infected enucleated cells.

tures which were incubated at 37° for 10 hr. All determinations were made on duplicate coverslips. The titer of A59V and JHMV increased from undetectable levels at 1.5 hpi (following neutralization of input virus) to 1.3×10^3 and 4.1×10^2 IU/ml, respectively. Enucleation reduces the yield of A59V by 2.1 log, JHMV by 0.84 log, and mengovirus by 1.15 log when compared to cultures treated with cytochalasin B but not enucleated. Using the percentage of nucleated cells in enucleated coverslip cultures and the amount of virus produced in control cultures, the percentage of the reported yields for enucleated cell cultures that could be produced by contaminating nucleated cells can be calculated. The percentage of nucleated cells is assumed to be

0.1%, the upper limit of the range we have observed in this experiment. The upper limit at which contaminating nucleated cells produce virus is that of the nucleated counterparts which experience only moderately increased gravitational fields. By using these two upper limits, only 22% (210 IU) of the reported yield of A59V multiplication in enucleated cells could be contributed by contaminating nucleated cells. Similarly, less than 1% of the yield of JHMV (3 IU) and 1.5% of the yield of mengovirus (5×10^4 IU) could have been contributed by nucleated cells. It is unlikely that contaminating nucleated cells are as productive as control cells, due to the additional trauma they have endured.

To confirm this result, 17CL-1 cells were

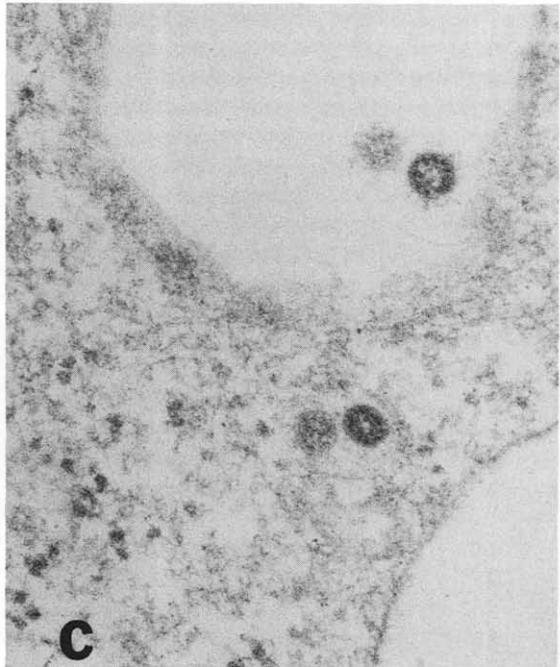
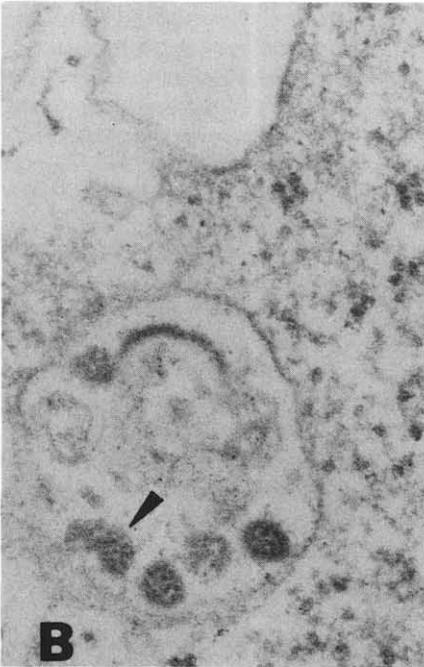
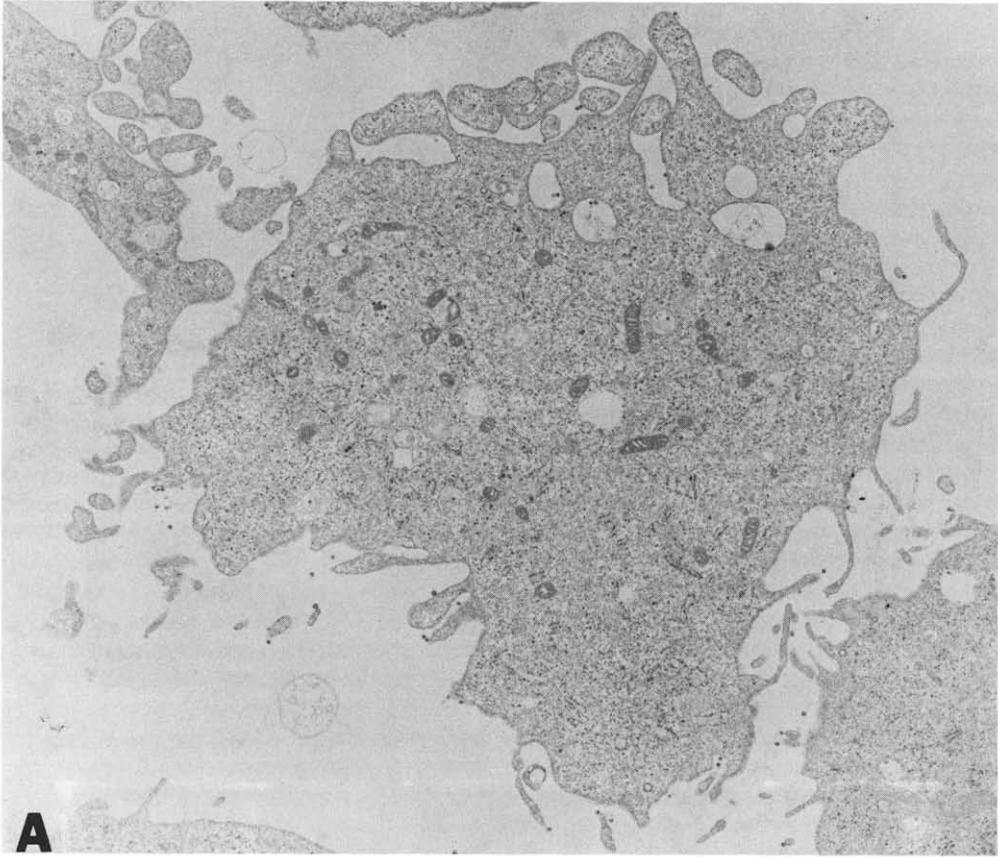


TABLE 1
MULTIPLICATION OF MURINE CORONAVIRUSES AND MENGOVIRUS IN ENUCLEATED 17CL-1 CELLS

Virus	Virus yield ^a			
	Untreated cells	Nucleated cells ^b treated with cytochalasin B	Enucleated cells ^c	Input virus ^d not neutralized
A59V	5.0×10^5	2.1×10^5	1.3×10^3	<8
JHMV	5.0×10^3	2.8×10^3	4.1×10^2	<8
Mengovirus	3.5×10^7	5×10^7	3.5×10^6	34

^a All cells were infected at a multiplicity of 5.0 and incubated at 37° for 30 min. The virus inoculum was removed and unadsorbed virus neutralized with antiserum (titer 1:1280) for 1 hr at 37°. Coverslips were then washed five times with DEB 2 and placed in 35-mm petri dishes with 2 ml of DEB 2. Petri dishes containing a coverslip of infected cells were then frozen at -40° and the remaining coverslips incubated for an additional 10 hr, then frozen at -40°. Samples were thawed, disrupted with sonic oscillation and clarified, and the virus yield was determined by an endpoint dilution assay (8). The results are expressed in infectious units per coverslip. All determinations are averages of duplicate coverslips.

^b Treated as described in Fig. 1, but allowed to attach for 120 min and centrifuged at 3000 rpm.

^c Produced as described in Fig. 1.

^d Enucleated cells were frozen at -40° 1.5 hpi after being washed free of antiserum.

enucleated, infected with A59V, and prepared for electron microscopy at 9 hr post-infection. An enucleate cell containing coronavirus particles is shown in Fig. 3.

The nucleus is apparently not required for A59V and JHMV multiplication or viral-specific antigen production. The role of the intranuclear antigen(s) observed by Robb and Bond (8) is not explained by these experiments. Nuclear antigen(s) has also been observed during respiratory syncytial virus, a paramyxovirus, multiplication and yet this virus can also replicate in enucleated cells (14, 15). If the MHV-specific nuclear antigen(s) has a role in virus replication, there is not an absolute requirement for this antigen(s).

ACKNOWLEDGMENTS

This work was supported in part by USPHS Grants NS 0708, NS 13898, NS 12383, and NS 15211 from the NINCDS and a grant from the National Multiple Sclerosis Society. Dr. Leibowitz is the recipient of Teacher/Investigator Award NS 00418 from the NINCDS. We would like to thank Robert Garrett for performing the electron microscopy.

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FIG. 3. Virus maturation in a 17CL-1 cytoplasm. 17CL-1 cells were enucleated as described in Fig. 1, infected with A59V at a multiplicity of 5, and incubated at 37°. At 9 hpi the cells were removed from the coverslip by a brief treatment with 0.1% trypsin, 0.1% EGTA in Puck's saline, diluted with DEB 10, collected by centrifugation, washed with phosphate-buffered saline, pelleted by centrifugation, and fixed in 2.5% buffered glutaraldehyde. The cell pellets were postfixed in osmium tetroxide, embedded in Araldite, and prepared for electron microscopy. An infected 17CL-1 cytoplasm is shown in panel A (final magnification 7000×). Higher magnification (panel B, 72,000×, panel C, 85,000×) of portions of this cytoplasm demonstrate typical intracellular coronavirus particles in cytoplasmic vacuoles. A budding virus particle is seen in panel B (arrow).

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