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Persistent Infection of Cells in Culture by Respiratory Syncytial Virus (39286)

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(Introduced by R. M. Chanock)

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It has been established that a carrier state can be induced in many cell lines by various viruses (4, 11, 12). In these situations, viruses that normally produce unequivocal cytopathogenic effects (c.p.e.) in a given cell line, may infect the same cell line without the production of c.p.e. and may persist and multiply concurrently with continued growth and survival of the cell culture. We describe in this report the development of a persistent infection of HEp-2 cells with respiratory syncytial virus (RS virus).

Methods. Two strains of RS virus were used in this study; the Long strain, obtained from R. M. Chanock, NIAID-NIH, Bethesda, Maryland, and a recent isolate which we have termed the St. Lukes strain obtained from M. Menegus, St. Lukes Hospital, New York, N.Y. The Long strain when received by us had been grown in KB cells for six passages, L cells for two passages, and HEp-2 cells for two passages. We continued propagation of this strain in HEp-2 cells. The St. Lukes strain was isolated on WI-38 (Human Diploid) cells and was maintained on this cell line in our laboratory. The identify of both strains was confirmed by neutralization with antisera obtained from the Reference Reagents Branch, NIAID-NIH, Bethesda, Maryland.

The HEp-2 cell culture was received in our laboratory at approx the 365th passage. The cells received were descendants of those held in the American Type Culture Collection (ATCC). These cells were propagated for three passages on Eagle's Minimal Essential Medium (EMEM) with 2% fetal calf serum and then were stored frozen in liquid nitrogen. Prior to use in these studies, cells were thawed and grown in EMEM containing 30% fetal calf serum for one passage and then passaged four times in EMEM containing 2% fetal calf serum. At this point the total passage history of these cells was 373. Syncytial formation occurred consistently at this passage level 48–72 hr after infection with either strain of RS virus.

Infectivity of the RS virus was measured by plaque-forming unit (PFU) titrations in monolayers of HEp-2 cells (2, 9). These were performed in triplicate on 60-mm plastic tissue culture dishes overlayed with 1% methylcellulose in EMEM. Forty-eight hours after incubation in a 5% CO_2 atmosphere, the plates were chilled, the methylcellulose removed by suction, and the monolayers stained with hematoxylin and eosin (H & E). Plaques were then counted with a hand lens and geometric means of the number of plaques present calculated.

To determine whether there were differences in the synthesis of actinomycin-D resistant RNA species following infection of HEp-2 cells at different passage levels we utilized the procedure reported by Hodes, Schauf, and Chanock (3). Three-day-old monolayer cultures of HEp-2 cells at either the 373rd or 393rd passage level were inoculated with 1.0 ml of RS-virus (Long strain) containing approx 1×10^6 PFU/ml giving a multiplicity of input (M.O.I.) of approx 1.0 PFU/cell. The inoculum was allowed to absorb for 90 min after which the cultures were overlayed with 10 ml of EMEM containing 2% fetal calf serum and were incubated at 37°. Either 24 or 48 hr after inoculation, actinomycin-D was added to final concentration of 5 μg/ml, and the cultures were reincubated for 90 min. Uniformly labeled [^{3}H]uridine (sp act 6.3 Ci/mmole) was then added to give a final concentration of 20 μCi/ml. After 2 hr at 37°, the cultures were washed 2× with phosphate buffered saline, pH 7.2 (PBS), the cell sheet was removed by scraping into fresh PBS and centrifuged at 2000 rpm for 10 min. RNA was extracted from the pellets by the method of Scherrer (7). Sedimentation

analyses were performed on RNA solubilized in 0.1 *M* EDTA, 0.05 *M* NaCl, and 5 μg/ml of polyvinyl sulfate (PVS). A 0.1-ml sample of this solution was layered onto preformed 5–20% sucrose in 0.01 *M* EDTA–0.05 *M* NaCl. The gradients were centrifuged at 40,000 rpm for 4 hr in an SW 40 Rotor in a Sphinco L_2 65B centrifuge. Twenty drop samples were collected from the bottom of the tubes, and the RNA was precipitated by the addition of 2.0 ml of cold 10% (W/V) trichloroacetic acid (TCA) (200 mg of yeast-cell RNA was added to each tube as a carrier). Precipitates were collected on 0.45-μm porosity membrane filters, dried, and the disintegrations per minute (dpm) were determined in a Searle-Nuclear Isocap liquid scintillation counter linked to a PDS-3 data reduction unit. Sedimentation coefficients were estimated by comparison to the patterns found in HeLa cell RNA (28 S, 18 S, 4 S) by Scherrer and Darnell (8). These estimates were based on OD_{260} measurements on RNA extracted from noninfected HEp-2 cells in the absence of actinomycin-D analyzed on parallel gradients.

Results. In the first experiment (Table I) HEp-2 cell monolayers in 60-mm plastic tissue culture dishes were prepared such that there were approx 5×10^5 cells/culture growing as monolayers 48 hr after preparation. These cultures were then inoculated with 100 PFU of virus, after 90 min absorption at room temperature (25°), 6 ml of EMEM containing 1% methylcellulose was added to each culture. Incubation was continued at 37° for 48 hr. Following the incubation period, cultures were chilled to 4° and the overlaying medium was removed. The monolayers were stained with H & E and the plaques were counted (9). There was a sudden drop in numbers of syncytia formed between the 383rd and 393rd passage of the cell line. This phenomenon occurred with both the Long and St. Lukes strains of RS virus.

In another series of experiments HEp-2 cells at passage levels of 373, 383, and 393 were grown as monolayers on 60-mm plates as before, yielding 5×10^5 cells/plate. These cells were inoculated with 1.0 ml of either Long or St. Lukes RS virus containing approximately 5×10^5 PFU/ml, thus yielding an M.O.I. of approx 1 PFU/cell. After a 90-min absorption at room temperature (25°), the cultures were covered with 6 ml of EMEM containing 2% fetal calf serum and incubated under 5% CO_2 for 72 hr at 37°. The medium was removed, and the cells were washed 2× with Hanks Balanced Salt Solution (HBSS). The cells were removed from the plates with 2 ml of 0.25% trypsin. One-half milliliter of fetal calf serum was added to the cell suspension to inhibit further trypsinization. The cells were counted in a hemocytometer and then subjected to sonic disintegration for 1.0 min at 4°. The amount of extracellular virus in the medium and the amount of cell-associated virus was determined by plaque assay as described previously (2, 9). Plaque assays were done using HEp-2 cells at the 373rd passage. Table II illustrates the results of these experiments. The amount of extracellular virus obtained dropped precipitously between the 383rd and 393rd passage of the HEp-2 cell line with both strains of RS virus. Conversely, there was an increasing amount of cell-associated virus detectable with increasing passage levels.

Infection of HEp-2 cells at either the 373rd or 393rd passage level with RS virus showed increased production of material incorporating [^{3}H]uridine (Fig. 1A–D). Twenty-four hours after infection there was a clearly defined peak at approx 10 S and a

TABLE I. VARIATION IN NUMBERS OF PLAQUES OCCURRING IN HEp-2 CELL MONOLAYERS AT DIFFERENT LEVELS OF CELL PASSAGE FOLLOWING CHALLENGE WITH SIMILAR AMOUNTS OF RS VIRUS FROM TWO DIFFERENT STRAINS.

Number of passages of HEp-2 cells	Strain of RS virus	Plaque-forming units added	Plaques obtained 24 hr[a]
373	Long	100	91 ± 15
378	Long	100	118 ± 23
383	Long	100	87 ± 15
388	Long	100	12 ± 3
393	Long	100	2 ± 2
373	St. Lukes	100	127 ± 31
378	St. Lukes	100	111 ± 18
383	St. Lukes	100	92 ± 16
388	St. Lukes	100	18 ± 5
393	St. Lukes	100	6 ± 4

[a] Plaque counts are the geometric mean of five replicate determinations.

TABLE II. AMOUNT OF RS VIRUS FOUND IN THE MEDIUM AND IN THE CELLS SONICALLY DISINTEGRATED 72-hr AFTER INFECTION OF DIFFERENT PASSAGE LEVELS OF HEp-2 CELLS.

Passage level of HEp-2 cells	Strain	PFU/ml of medium (72 hr)	PFU/10^5 cells (72 hr)	PFU/Cell (72 hr)	(Extracellular virus/Cell-associated virus) × 100 (%)
373	Long	1.0×10^5	5×10^4	0.5	200
383	Long	1.5×10^5	8×10^4	0.8	188
393	Long	1.0×10^3	2.2×10^5	2.2	0.46
373	St. Lukes	1.0×10^5	4×10^4	0.4	250
383	St. Lukes	1.2×10^5	6×10^4	0.6	200
393	St. Lukes	8.0×10^2	1.8×10^5	1.8	0.44

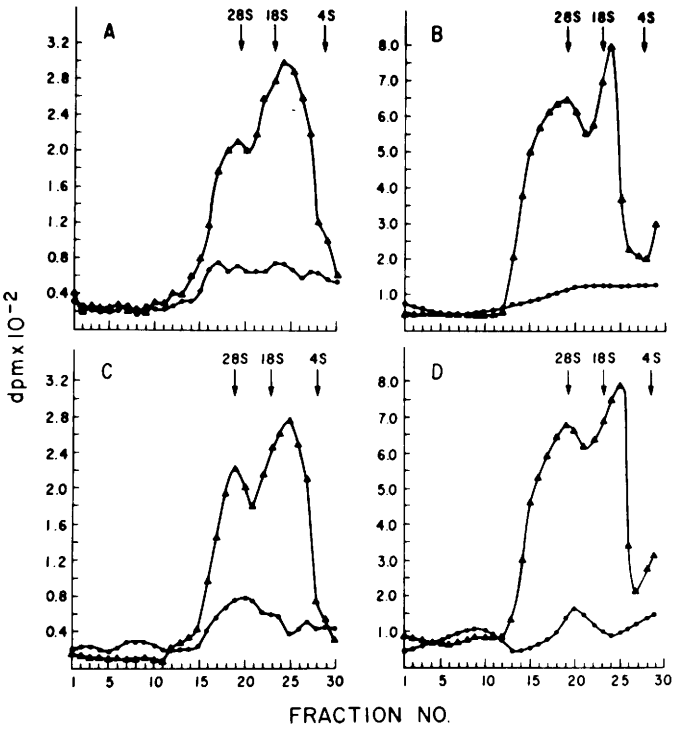

FIG. 1. (A) Incorporation of [^{3}H]uridine into HEp-2 cell culture (passage 373) inoculated 24 hr previously with 1×10^6 PFU (M.O.I. = 1.0) of Long RS virus. Culture was treated with 5 μg/ml of actinomycin D for 90 min, then labeled with [^{3}H]uridine (20 μCi/ml) for 2 hr prior to harvesting. The RNAs were extracted, and banded on preformed 5-20% sucrose gradients. Fractions were collected and precipitated with 10% TCA. Acid-precipitable ^{3}H-labeled material is shown by ▲——▲. An uninfected HEp-2 cell culture was submitted to the same procedure. The results of this control culture are shown by the graph labeled ●——●. Note: Syncytia were abundant in this culture. (B) Incorporation of [^{3}H]uridine into HEp-2 cell culture (passage 373), inoculated 48 hr previously with 1×10^6 PFU (M.O.I. = 1.0) of Long RS virus. Subsequent treatment identical with that detailed in Fig. 1A. Note: Syncytia were abundant in this culture. (C) Incorporation of [^{3}H]uridine into HEp-2 cell culture (passage 393), inoculated 24 hr previously with 1×10^6 PFU (M.O.I. = 1.0) of Long RS virus. Subsequent treatment identical with that detailed to Fig. 1A. Note: Syncytia were seen in this culture. (D) Incorporation of [^{3}H]uridine into HEp-2 cell culture (Passage 393), inoculated 48 hr previously with 1×10^6 PFU (M.O.I. = 1.0) of Long RS virus. Subsequent treatment identical with that detailed in Fig. 1A. Note: No syncytia were seen in this culture.

smaller peak at approximately 28 S; by 48 hr the intensity of both peaks was more defined. These findings are consistent with those of Hodes *et al.* (2) for RS virus, A2 strain grown in HeLa cell cultures. In spite of the gross differences seen in the amounts of extracellular virus produced, as well as the minimal or nonexistent c.p.e. seen in the

higher passaged cells, we were unable to demonstrate any differences between RNA species obtained by these methods in virus grown in either the lower 373rd passage or the higher 393rd passage HEp-2 cells.

In another experiment, HEp-2 cells infected at the 393rd passage with the Long strain of virus and demonstrating 1.0×10^3 PFU/ml in the medium, 8.0×10^5 PFU/10^5 cells (2.2 PFU/cell) and essentially no c.p.e. 72 hr after inoculation, were removed from the plates by trypsinization and transferred to new plates as serial cultures. Passage of these infected cells was repeated at 72-hr intervals for 10 passages. At each passage, extracellular and cell-associated virus was measured as before. As will be seen in Table III, there was a continued drop in the amount of extracellular virus detectable, while the amount of cell-associated virus remained at high levels through this period.

Discussion. The ability of RS virus to cause syncytium formation in cell lines varies not only with the cell line but also with sublines of the same cell line (1). In the present study we have shown that resistance to syncytium formation developed rapidly (15–20 passages) under the conditions used in our laboratory. Decrease in the sensitivity of the cells to form syncytia was not restricted to a single strain of the virus but occurred in parallel with the two strains used. Why the sensitivity decreases at such a rapid rate is unclear. The HEp-2 cell line used was derived from that of the ATCC and was stored frozen prior to initiating the cell cultures used in these studies. It is possible that the storage followed by rapid passage served as a selective mechanism for the development of a subline resistant to syncytium formation; however, we have no direct evidence that this is the case.

TABLE III. RATIO OF EXTRACELLULAR TO CELL-ASSOCIATED RS VIRUS IN SERIAL PASSAGES OF HEp-2 CELLS SHOWING NO SYNCYTIAL FORMATION AFTER INFECTION.

Passage	Cell-associated virus (PFU/10^5 cells)	Extracellular virus (PFU/ml)	(Extracellular virus/Cell-associated virus) × 100
0	0	5×10^5	–
1	7×10^5	1×10^3	0.143
2	6.2×10^5	4×10^2	0.065
3	6.5×10^5	2×10^2	0.031
4	7.5×10^5	1.2×10^2	0.016
5	5.5×10^5	9×10^1	0.016
6	6.5×10^5	8×10^1	0.012
7	1.2×10^6	8×10^1	0.007
8	5×10^5	7.5×10^1	0.015
9	4.5×10^5	7×10^1	0.016
10	4.5×10^5	6.5×10^1	0.014

One of the parameters of the cell-virus relationship investigated was the ratio of extracellular virus to cell-associated virus at cell-passage levels that varied in sensitivity to syncytium formation. These studies (Table II) indicate that as sensitivity to syncytium formation decreases there is a decrease in extracellular virus and a concomitant increase in cell-associated virus. We were unable to demonstrate any measurable difference between the RNA species obtained in cells forming syncytia (373rd passage) or those resistant to syncytium formation (393rd passage) (Fig. 1A–D).

While none of these findings explains the rapid loss of sensitivity to syncytium formation, they do point out that the HEp-2 cell line used in these studies, while developing a resistance to c.p.e. of the virus, remains essentially unchanged in its ability to support viral replication. The only essential change in the cell-virus relationship that we can measure appears to be a significant decrease in the amount of virus released from the infected cell.

We have used the above findings to develop a covertly infected subline of HEp-2 cells. Cells infected with RS virus at a passage level at which c.p.e. was minimal were serially passed at 72-hr intervals for 10 passages (Table III). The decrease in amount of extracellular virus with continued passage, along with the relatively constant amount of cell-associated virus, in the presence of essentially no c.p.e., suggests the evolution of a carrier state of the RS virus in these HEp-2 cells.

Previously described carrier cultures of paramyxovirus-infected cell lines have been more dependent upon the strain of virus used than the cell lines or their passage level (10). Rustigan (5) has reported on the development of HeLa cell sublines, persistently infected with measles virus. In these studies it appeared that the development of carrier cell lines was due to either virus

alteration, cellular selection, or both. In further studies (6), he reported on a cell line which produced incomplete virus after passage under specific antibody. In comparing the results reported here to those of Rustigan it would seem that the probability of alteration (mutation) of the RS virus in these experiments is minimal and that the factor or factors responsible for the development of the covert infection in this specific instance are most probably cell-related.

The continued presence of infected virus in covertly infected cells on serial passage demonstrates, that under the conditions used, RS virus is capable of infecting HEp-2 cells without the production of c.p.e., and that on continued passage the cell line remains infected with the virus. This development of an *in vitro* covert infection in the model used suggests the possibility that RS virus in nature might produce covertly infected cells in the respiratory tract resulting in the persistence of residual virus or viral genome in such tissue.

Summary. The virus-cell relationship of RS virus and the HEp-2 cell line has been examined. The production of cytopathic effect (c.p.e.) on HEp-2 cells has been found to be dependent upon the passage level of the cell line. Cells at lower passage levels exhibit c.p.e. in the form of syncytium formation, while those at higher passage levels no longer exhibit this effect. Cells infected at higher passage levels are covertly infected and continue to produce large amounts of infectious virus which remains cell-associated. On continued passage, these cells remain infected with virus but show no c.p.e. and release little if any infectious virus into the medium.

Examination of the RNA species present in infected cells revealed that similar species are present in both the overtly and covertly infected cells.

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