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Plaque Assay and Improved Yield of Human Coronaviruses in a Human Rhabdomyosarcoma Cell Line

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Propagation and plaque assay of human coronavirus prototypes were studied in two human cell lines: a diploid fetal tonsil (FT) and a heteroploid rhabdomyosarcoma (RD) cell line. Plaques, observed within 2 to 3 days on FT cell monolayers with both 229E and OC43 viruses, appeared as colorless areas after staining with neutral red or crystal violet, whereas neutral red staining was required for visualization of plaques on RD cells. The plating efficiencies were approximately equal between the two cell lines, but virus assay by plaque formation was 15- to 30-fold more efficient than tube dilution assay with 50% endpoints. The discrepancy between 50% endpoint and plaque-forming unit values was striking and appeared to result from the fact that killing of cells (particularly RD cells) by coronaviruses was not accompanied by visible changes in the cells but killing was detected by the failure of infected cells to stain with a vital dye. The latent phase in one-step growth curves was 5 to 6 h for both viruses in either cell line, but the maximum yield of intracellular virus was reached in 18 to 20 h for FT cells and 24 to 28 h for RD cells. Virus release also differed between the two cell lines: in FT cells, the maximum yield of extracellular virus was reached 2 to 3 h later than that of intracellular virus, whereas in RD cells, the difference was 5 h for 229E virus and 10 h for OC43 virus. Although both cell lines appear equally useful for plaque assay, RD cells would be preferred for mass virus propagation because yields (5×10^8 plaque-forming units per ml) were 10-fold higher than in FT cells, a finding true for both virus prototypes.

Human coronaviruses have been identified as etiological agents of acute upper respiratory tract infections (11, 13, 18, 22). Their role in respiratory illnesses of infants and children (20) and in disease of the lower respiratory tract (12) is undefined. No doubt, the extent of coronavirus infections is considerably greater than presently known. This gap in knowledge is related to two major problems in research on human coronaviruses: great difficulties in primary isolation, which must rely mainly on cumbersome methods of organ culture, and cell culture systems inadequate for mass virus propagation. Consequently, serological test procedures to detect infections are lacking for most human strains. Prototype 229E was isolated in cell culture and virus growth studies in several cell lines have been reported (1, 3, 4, 6, 10). In contrast, prototype OC43 was isolated only in organ culture, but subsequently was propagated in suckling-mouse brain (17) and cell culture (5). However, its growth characteristics in cell culture have not been adequately described. Furthermore, the two prototypes have not been studied extensively in the same cell line under identical conditions, and, to our knowledge, a sensitive virus assay system applicable to both prototypes has not been developed. We have, therefore, searched for sensitive cell lines and have found a human diploid cell line and a human heteroploid cell line in which virus yields appear favorable for further studies. This report describes the propagation and quantitation of human coronavirus strains OC43, 229E, and two closely related serotypes in these cell lines.

MATERIALS AND METHODS

Cells. Human diploid fetal tonsil cells (FT) and human heteroploid rhabdomyosarcoma cells (RD) were used. The diploid human fibroblastic cell line was established in our laboratory from a human embryonic tonsil. The rhabdomyosarcoma (16) line was received from R. L. Crowell, Hahneman Medical College and Hospital, Philadelphia, Pa. The cell lines were found free of mycoplasmata when tested regularly by culture (14) and uracil incorporation (15).

Virus strains. Coronavirus 229E/WI-38/P_{14} (13), OC43/BSC-1/P_5, and OC38/BSC-1/P_5 (17) were received from A. Z. Kapikian, National Institutes of Health, Bethesda, Md. Coronaviruses OC43 and OC38 appear to be serologically identical (17). Coronavirus

B814 (22) and LP (23), thought to be closely related to coronavirus 229E, OC43/suckling mouse brain/P_9, and OC38/suckling mouse brain/P_9, were received from W. R. Dowdle, Center for Disease Control, Atlanta, Ga. Virus strains used in our study were selected for rapid growth in fetal tonsil and rhabdomyosarcoma cells and then plaque purified six times in both cell lines.

Media. Cells were grown in Eagle minimum essential medium with 10% fetal calf serum, 6.6 mM sodium bicarbonate, 100 μg of streptomycin per ml, and 100 U of penicillin per ml. For virus propagation, the serum concentration was reduced to 1%, and HEPES buffer (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid), pH 7.2, at 33°C was added to a final concentration of 20 mM.

Overlay medium consisted of Eagle minimum essential medium with 1% fetal calf serum, 12 mM sodium bicarbonate, antibiotics, and 0.3% agarose (Sea Kem; Bausch and Lomb, Inc., Rochester, N.Y.). Virus was diluted in medium containing 0.2% bovine serum albumin instead of serum.

Virus propagation. Virus strains were propagated in tube cultures. Screw-capped tubes (16 by 125 mm) were inoculated with 250,000 cells contained in one ml of cell growth medium. FT cells were incubated for 48 h, and RD cells were incubated for 24 h at 37°C. Growth medium was then removed, and each tube was infected with 0.1 ml of virus fluid. After virus adsorption for 1 h at 33°C, 1 ml of virus growth medium was added to each tube, and incubation was carried out on a roller drum at 33°C. Endpoints were calculated by the Reed-Muench method (21).

Plaque assay. Cell monolayers were prepared by inoculating 60-mm plastic petri dishes with 1.0×10^6 FT cells or 1.2×10^6 RD cells in 5 ml of growth medium per plate. After an incubation period of 48 h at 37°C in 2.5% CO_2 for FT cells and 24 h for RD cells, the medium was removed and 0.2 ml of suitably diluted virus suspension was added to each plate. Virus adsorption was carried out for 1 h at 33°C in 2.5% CO_2. A 5-ml amount of overlay medium was added to each plate, and the medium was allowed to solidify at room temperature. Plates were incubated at 33°C in 2.5% CO_2. In FT cells, crystal violet or neutral red stain was used to visualize plaques. Cell monolayers were stained with crystal violet after fixation with Formalin on day 3 or 6 after infection with 229E, B814, and LP or OC43 and OC38 viruses, respectively. In neutral red staining, 0.5 ml of a 0.01% dye solution was added to each plate on day 2 after infection with 229E, B814, or LP virus. Plates were incubated overnight in the dark and then exposed to light for 1 h to allow for maximum differentiation of the unstained foci (plaques) from the background-stained monolayer. With OC43 and OC38 virus, neutral red staining was carried out 1 day later. In RD cells, plaques of all virus strains were developed with neutral red stain 1 day later than in FT cells.

Virus adsorption and penetration. Adsorption and penetration of coronavirus OC43 were studied on RD cell monolayers prepared in plates as described for plaque assay. At assay time, the cell monolayers were washed once with cold (4°C) Hanks balanced salt solution and then infected with 0.2 ml of suitably diluted virus to yield about 120 plaques per plate. Virus adsorption was carried out at 33°C in 2.5% CO_2 with one set of plates and at 4°C with another. At the end of each adsorption period, the cell monolayers were washed three times with cold Hanks balanced salt solution. Agarose overlay medium was immediately added to the control plates, and 1.5 ml of antiserum was added to the test plates. After virus neutralization at room temperature (23°C) for 1 h, the antiserum was removed, the cell monolayers were washed three times with Hanks balanced salt solution, and overlay medium was added. All plates were then incubated at 33°C in 2.5% CO_2. Plaques were developed with neutral red stain as described for the plaque assay.

Virus growth studies. One-step growth curves were carried out in replicate cell monolayers prepared in tubes as described for virus propagation. The multiplicity of infection used was 50 plaque-forming units (PFU) per cell, and adsorption was carried out for 2 h at room temperature. After virus adsorption, the cell monolayers were washed six times with Hanks balanced salt solution, 1 ml of growth medium was added, and incubation was carried out in stationary phase at 33°C. At sampling times, supernatant fluids from four tubes were pooled and centrifuged to remove debris. The cell sheets were washed six times, 1 ml of virus growth medium was added, and virus was released by three freeze-thaw cycles. Debris was removed by centrifugation, and the fluids were pooled. Supernatant virus samples were also subjected to the freeze-thaw procedure to maintain uniformity in the assay. The effect of freeze-thawing on infectious virus was tested with virus stocks of known infectivity titers. Samples were stored at −70°C until tested by plaque assay.

Antisera. Reference 229E immune guinea pig antiserum and OC43 immune mouse ascitic fluid were received from the Center for Disease Control, Atlanta, Ga. Rabbit antisera to 229E and OC43 were prepared by the method of Cooney and Kenny (7), with immunogens (1.5×10^{10} PFU/rabbit) prepared in RD cells from plaque-purified virus strains and partially purified in sucrose gradients (25 to 55%, wt/vol).

Virus confirmation. The identity of strains 229E and OC43 was confirmed by virus neutralization with reference and hyperimmune rabbit antisera. No cross-reaction was observed by plaque reduction or kinetic neutralization between 229E and OC43. All virus strains showed typical coronavirus morphology by electron microscopy.

RESULTS

Virus propagation. Virus strains were selected for rapid growth in FT cells. Cytopathic effects (CPE) proceeded slowly for two to three passages with all virus strains and were confined to only a few cells on the entire monolayer over incubation periods of 10 to 14 days. At the third or fourth passage, CPE appeared in focal areas 4 to 5 days after infection and eventually spread over the entire cell sheet. After several additional passages, complete CPE was observed in cells infected with 229E and LP after 2 days and with OC43, OC38, and B814 after 3 days. Two

distinct types of CPE were observed: spindling and detachment of cells in 229E infection and swelling and extensive vacuolation of cells in OC43 and OC38 infection. Cytopathic effects of B814 and LP closely resembled that of 229E infection, but mild vacuolation was also observed (Fig. 1).

Three additional passages of these selected strains were required for good growth in rhabdomyosarcoma cells. The distinguishing features of both types of CPE were also observed, but in contrast to those in the fetal tonsil cells they were less pronounced and particularly difficult to detect with OC43, OC38, and B814 in their early stages. However, virus titers of all strains were in the range of 5×10^8 to 8×10^8 PFU/ml, about 10- to 15-fold higher than in the fetal tonsil cells, although the same number of cells were infected. Characteristic cytoplasmic inclusions were not observed with these coronavirus strains.

Plaque assay. In FT cells, plaques of 229E, LP, and B814 virus were observed microscopically on day 2 or 3. They appeared as clear, unstained areas when cell monolayers were stained with crystal violet on day 3. Visualization of plaques of OC43 and OC38, on the other hand, required 3 to 4 more days of incubation (total of 5 to 7 days), and therefore a second agarose overlay was employed before crystal violet staining. However, plaques could be observed by neutral red staining on day 3 or 4. In contrast, development of CPE and cellular destruction in rhabdomyosarcoma cells were found to proceed more slowly, and plaques were rarely observed microscopically and could not be seen adequately by crystal violet staining, although two additional agarose overlays were employed. However, plaques were clearly visible with neutral red (Fig. 2) when staining was carried out 1 day later than for FT cells. Plaques were not seen in either cell line in the absence of viral inocula. Both cell lines provided good confluent monolayers without interruptions which might be confused with plaques. The assay was simple in both cell lines and did not require complex overlay media or multiple overlays when neutral red staining was employed. However, the use of agarose was essential. Most importantly, plaque numbers were proportional to the relative virus concentration of the inocula (Fig. 3). The reproducibility and the sensitivity of plaque assay in different batches of cells from the two cell lines were compared (Table 1). Each assay was started independently from virus stocks known to yield about 40 plaques per plate at the final dilution. The assay was found to be reproducible for all virus strains, and the sensitivity of both cell lines was about the same. However, the FT cells were found unsatisfactory after the 30th passage.

Virus adsorption and penetration. Adsorption and penetration of coronavirus OC43 were studied on RD cell monolayers (Table 2). At 33°C, maximum virus attachment was reached after 1 h of adsorption. At that time, 53.5% of the attached virus could still be neutralized by hyperimmune rabbit antiserum. The calculated, theoretical neutralizing efficiency of the antiserum against virus in suspension would result in 90% neutralization in 15 min. Attached virus was no longer neutralizable after 3.5 h. At 4°C, virus adsorption was found to be less efficient than at 33°C and attached virus remained 100% neutralizable during 6 h of adsorption.

Relative sensitivity of 50% endpoint assay and plaque assay. Since plaque assay titers were higher than 50% endpoint titers, the sensitivity of both assays was tested with 229E and OC43 virus (Fig. 4). The 50% endpoint assay in fetal tonsil cells was about 10 times less sensitive than the plaque assay with both viruses even if corrected for the theoretical value of 0.7 PFU per 50% endpoint unit. Additionally, in the rhabdomyosarcoma cells, the 50% endpoint assay was found to be 15 times less sensitive with 229E virus and about 30 times less sensitive with OC43 virus. With B814, LP, and OC38 virus

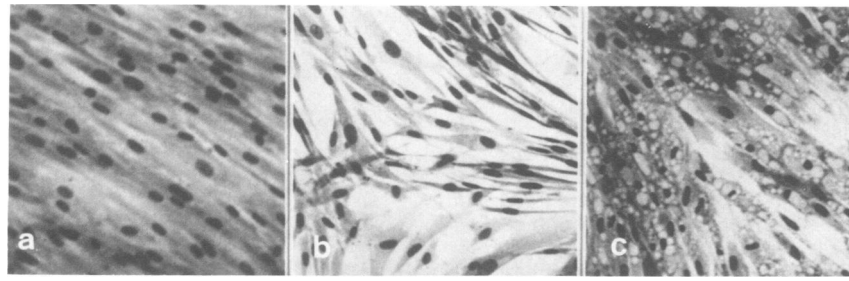

FIG. 1. *Cytopathic effect of coronavirus 229E and OC43 in FT cells. (a) Uninfected cells; (b) 229E-infected cells; (c) OC43-infected cells. May-Grünwald-Giemsa stain.*

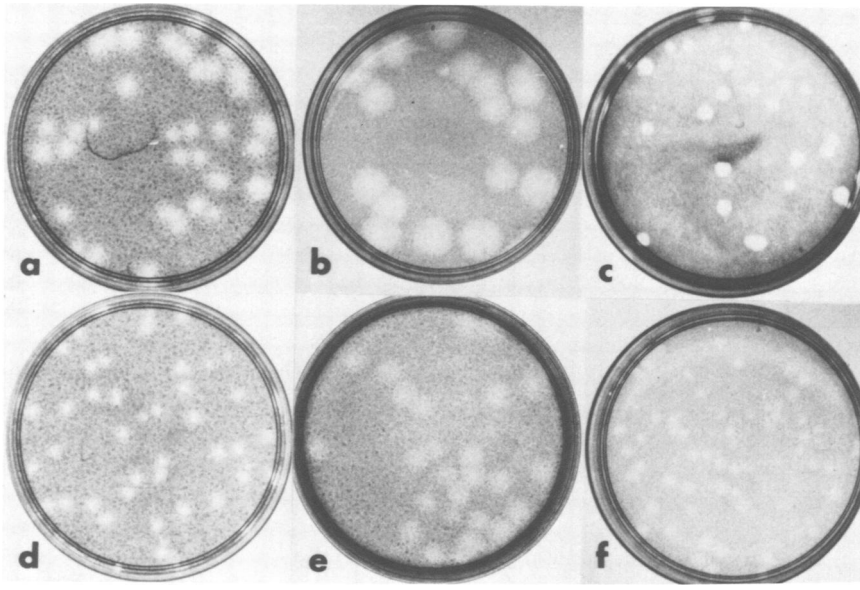

FIG. 2. *229E and OC43 virus plaques on two human cell lines at 4 days. (Top row) 229E virus plaques on: (a) FT cells, crystal violet stain; (b) FT cells, neutral red stain; (c) RD cells, neutral red stain. (Bottom row) OC43 virus plaques on: (d) FT cells, crystal violet stain; (e) FT cells, neutral red stain; (f) RD cells, neutral red stain.*

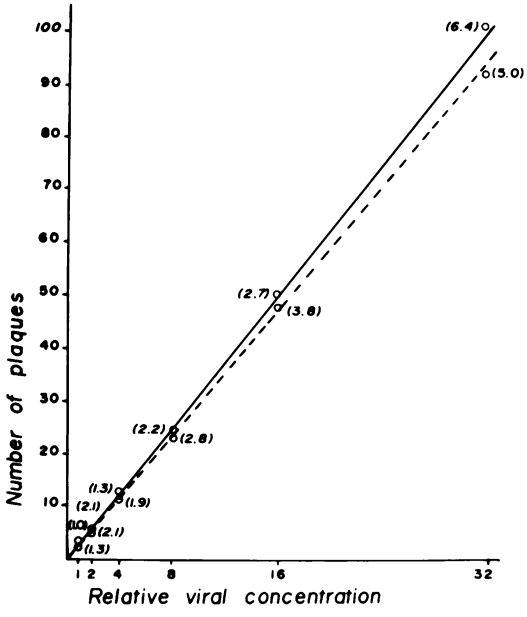

FIG. 3. *Correlation of number of plaques with virus concentration. Symbols: ——, 229E virus; ----, OC43 virus. Data are plotted as the mean number of plaques derived from 10 plates of each concentration of virus. Standard deviations from the mean are shown.*

strains, the relative sensitivity of FT cells resembled that for 229E virus, and OC43 virus in rhabdomyosarcoma cells.

TABLE 1. *Reproducibility of plaque assay of coronavirus strains in two cell lines*

Virus	FT cells	RD cells
229E	39.3 ± 0.77[a]	39.0 ± 1.24
B814	39.8 ± 0.92	40.1 ± 1.30
LP	40.1 ± 1.06	39.8 ± 1.10
OC43	38.9 ± 1.20	38.6 ± 1.41
OC38	39.1 ± 1.10	39.4 ± 0.92

[a] Mean number of plaques per plate derived from eight assays of each of 10 plates over a period of 9 months. Virus concentrations of all stock cultures were adjusted to yield about 40 plaques per plate at final dilution.

Virus growth studies. The growth of 229E and OC43 virus was studied in both cell lines on cell monolayers under conditions where at least 95% of the cells could be infected. With 229E virus, cell-associated virus in fetal tonsil cells increased rapidly, and maximum virus titers were reached at 18 h after infection, although CPE was not observed at that time (Fig. 5). Maximum extracellular virus titers were observed at 28 h when slight CPE could be seen. The titers of both cell-associated and released virus decreased as CPE became more evident. Extensive CPE was observed at 42 h. In rhabdomyosarcoma cells, cell-associated virus increased in parallel with that in FT cells. Maximum virus titers were reached in 24 h and were 10-fold greater than the highest levels obtained in FT cells.

TABLE 2. *Adsorption and penetration of coronavirus OC43 on RD cell monolayers*

Adsorption time (h)	33°C		4°C	
	% of maximum virus adsorption[a]	% of attached virus neutralizable[b]	% of maximum virus adsorption[a]	% of attached virus neutralizable[b]
0.25	92	89	73.5	100
0.5	94.5	64	72	99.5
1.0	100	53.5	73	100
2.0	100	9	71	100
2.5	100	6	71.5	100
3.0	100	3.5	72.5	100
3.5	100	0	70.5	100
6	98	0	68.5	100

[a] 120 plaques ± 4.2 represent maximum values (100%).

[b] Percentage of virus neutralized calculated against total virus attached (i.e., 11% of the attached virus was not neutralized at 0.25 h). Each plate was treated with 1.5 ml of antiserum diluted 1:32 to yield 200 neutralizing units per ml. The antiserum had a *K* value of 1,100.

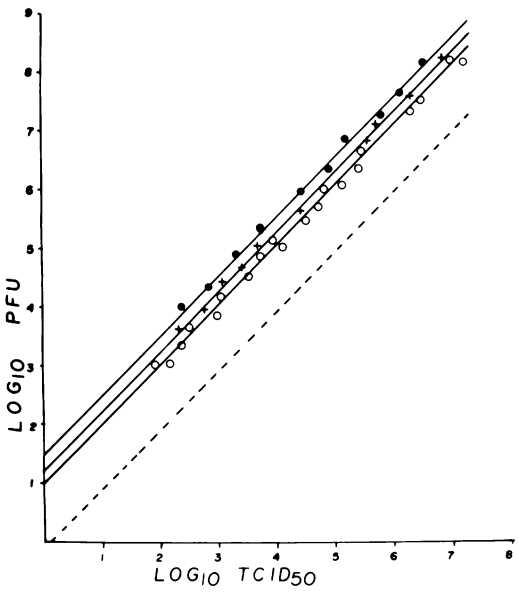

FIG. 4. *Ratio of 50% endpoint to plaque assay endpoint. Symbols: ----, 0.7 PFU = 1.0 50% tissue culture infective dose ($TCID_{50}$); ○, FT cells: 229E, OC43; +, RD cells: 229E; ●, RD cells: OC43.*

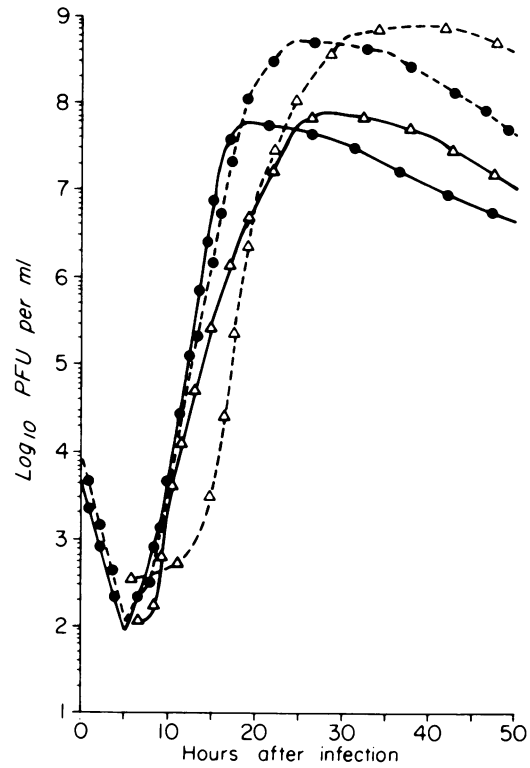

FIG. 5. *One-step growth curves of coronavirus 229E in two human cell lines. Symbols: ——, FT cells; ----, RD cells; ●, cell-associated virus; △, supernatant virus.*

Release of virus from RD cells appeared to be slower than from FT cells as judged by the time of appearance of extracellular virus both in the early growth phase and at final maximum titers which were reached at 40 h. Only slight CPE was observed at 40 h in RD cells, whereas CPE was marked at 48 h when both cell-associated and extracellular virus titers were declining. With OC43 virus, maximum cell-associated virus titers were observed in FT cells 20 h after infection, at which time CPE could not be seen (Fig. 6). Highest extracellular virus titers were reached at 32 h, but only slight CPE was present. Virus titers declined with increasing CPE. In RD cells, maximum cell-associated virus titers were seen without CPE at 28 h. Highest extracellular titers and slight CPE were observed at 42 h. Good CPE was present at 56 h, when both cell-associated and extracellular virus titers were declining. Release of OC43 virus appeared delayed in RD cells by 8 h (over the entire curve)

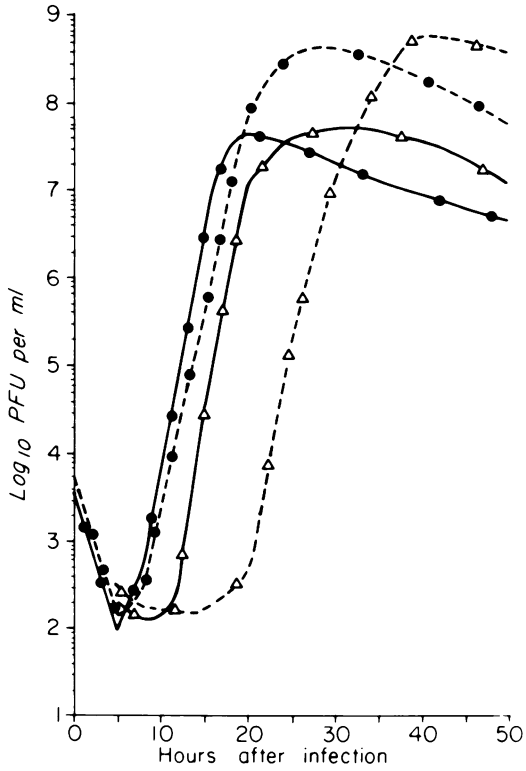

FIG. 6. *One-step growth curves of coronavirus OC43 in two human cell lines.* ——, *FT cells;* ----, *RD cells;* ●, *cell-associated virus;* △, *supernatant virus.*

when compared to release in FT cells. However, cell-associated titers rose at nearly the same rate in both cell lines. Virus titers were higher in RD cells than in FT cells with both viruses. The freeze-thaw procedure was found to decrease infectious virus titers four- to fivefold. Accordingly, total virus yield is underestimated by at least that factor.

DISCUSSION

Human coronaviruses, on the basis of cultural methods used in primary isolation, can be divided into two groups: cell culture strains and organ culture strains. The propagation, growth characteristics, and quantitative assays of several cell culture strains which appear to be closely related, but not identical, have been described (1–4, 6, 10, 19). On the other hand, only two organ culture strains, which appear to be identical, grew in cell culture (5). Quantitative assays and detailed growth studies in cell culture, to our knowledge, have not been reported. We have successfully selected virus strains of both groups for growth in two different human cell lines and developed a sensitive plaque assay which enabled us to study the growth of two virus prototypes in these cell lines.

In both cell lines, initial virus propagation was difficult. Eventually, good viral growth was obtained in both cell lines with all virus strains when pH was controlled effectively. Our plaque assay can be used with both cell lines and meets the criteria for a useful plaque assay (8), the most important one being a linear relationship between relative virus concentration and number of plaques. The assay is simple and practical, since it does not require complex media or multiple overlays. Importantly, the assay was also found applicable to and equally sensitive for all virus strains tested. The use of agarose, as has been reported for rhinoviruses (9) and herpesviruses (24), was found to be essential.

When results obtained in the two cell lines were compared, striking differences were observed. In the diploid cell line, distinct and characteristic CPE was easily recognizable. Nevertheless, plaque assay was 10-fold more sensitive than 50% endpoint assay. Virus plaques, with all strains tested, could be observed microscopically or visualized on cell monolayers by either crystal violet or neutral red staining. In contrast, in the heteroploid cell line, detection of early CPE and accurate determination of endpoints in tube dilution assay were found to be difficult with most strains because of slight CPE, and titers were at least 10-fold lower than titers measured by plaque assay. Only a few plaques were observed microscopically, and crystal violet staining did not reveal additional plaques. On the other hand, plaques could be developed with neutral red stain, and surprisingly, the sensitivity of the assay was equivalent to assay in diploid cells. This result suggests that RD cells are killed as judged by neutral red staining, but that their morphology is not changed. Accordingly, neither CPE nor plaques are visible.

Virus growth studies with strain 229E in diploid cell monolayers (1, 6, 10) and 229E and LP in heteroploid cell suspension culture (3) have been reported. A comparison of these results as well as with ours is difficult due to large differences in experimental conditions. In our studies, growth cycles of plaque-purified coronavirus prototypes 229E and OC43 were compared in two different cell lines under identical conditions. As has been seen by others, maximum cell-associated virus titers were reached before maximum released virus titers in both cell lines. Intracellular growth cycles of both viruses were closely similar in the two cell lines; however, higher titers were obtained in rhabdomyosarcoma cells. Release of virus was slightly delayed in rhabdomyosarcoma cells with 229E virus but

was substantially delayed with OC43 strain. This difference is probably not due to levels of infection since nearly all the cells were infected. However, slower and more prolonged release of virus was observed in RD cells, especially with OC43, over a wide range of multiplicies of infection in other experiments. These findings seem to correlate with slower development of plaques in RD cells in contrast to faster and more extensive cellular destruction in FT cells.

Although the sensitivities for primary virus isolation have not been determined, both cell lines appear to be useful in the study of human coronaviruses. In our studies with laboratory virus strains, easily recognizable CPE and ability to maintain tube cultures on roller drums for extended periods of time without cellular deterioration appear to be desirable features of FT cells. On the other hand, sensitivity declined rapidly after the 30th passage. Special properties could not be attributed to our FT cell line, since three other cell lines derived from different embryos were also found useful. In contrast, only one of two other RD cell lines tested, RD A-673 (HEM Research, Inc., Rockville, Md.), was found satisfactory. The usefulness of RD cells was not influenced by cell passage, large quantities of cells were more easily obtainable, and, most importantly, virus yields were significantly higher. These characteristics appear to be most desirable for further studies.

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