Differential Sensitivity of Normal and Transformed Human Cells to Reovirus Infection

MICHAEL R. DUNCAN,[†] SANDRA M. STANISH, AND DONALD C. COX[‡]*

Department of Botany and Microbiology, University of Oklahoma, Norman, Oklahoma 73019

Received for publication 5 July 1978

Normal and simian virus 40-transformed WI-38 cells exhibited a differential sensitivity to infection with type 3 reovirus. A progressive decrease in viability began 24 to 36 h after infection of transformed cells terminating in complete lysis of cultures by 96 h. Normal cells were productively infected and continued to produce and release virus for as long as 14 days after infection, but exhibited no detectable cytopathology. Inhibition of cellular DNA synthesis began 15 to 18 h after infection in transformed cells before development of cytopathology. No inhibition of DNA synthesis was detected in infected normal cells. No significant differences were noted in the adsorption or early replication characteristics of reovirus in normal and transformed cells. Virus replication and host cell DNA synthesis in normal and transformed human cells were compared to reovirus-infected L-929 mouse fibroblast cells.

Reovirus infection of continuous cell cultures such as mouse L-929 fibroblasts or HeLa cells results in cell lysis. However, Loh et al. have reported that human embryonic lung cells (WI-38) exhibit no cytopathology 24 h after reovirus infection, even though the cells support virus replication (15). Bell and Ross showed that reovirus can establish a latent persistent infection in human embryonic fibroblasts resulting in production and release of progeny virions without consequent modification of cell growth rate (2). Similarly, Taber et al. (18) have recently established a line of Chinese hamster ovary cells persistently infected with reovirus. Ahmed and Graham (1) have also established persistent infections of L cells by using heavily defective populations of reovirus.

These observations suggest that the response to reovirus infection may be dependent upon certain characteristics of the host cell and not of the virus. We have investigated the possibility that such a differential response to infection might occur as a result of transformation. This suggestion is supported by the recent evidence of Hashiro et al. (10) that reovirus is preferentially cytotoxic for certain transformed cell lines. The replication of reovirus and the cellular response to infection in normal and simian virus 40 (SV40)-transformed WI-38 cells is reported in this study. Reovirus mediates a lytic infection in

the transformed cells and a prolonged noncytotoxic infection in normal cells.

MATERIALS AND METHODS

Cells. Mouse fibroblasts, strain L-929, were grown in Eagle minimal essential medium (MEM) supplemented with 5% heat-inactivated fetal calf serum (FCS) as previously described (16). Human embryonic lung cells, strain WI-38, at passage number 13 were purchased from Cell Associates, Inc. and grown in MEM supplemented with 10% FCS. The cultures were passaged when monolayers reached confluency at a split ratio of 1:4 according to the procedure of Hayflick (11). WI-38 VA 13 2RA, SV40-transformed WI-28 cells (7), were kindly provided by A. J. Girardi. These cells were passaged as were the WI-38 cells, except that MEM was supplemented with 5% FCS, and the split ratio was 1:5 instead of 1:4.

Virus. Reovirus type 3, Dearing strain, was propagated in L cell suspension cultures. Virus for all experiments was used after no more than 4 passages after plaque purification.

Virus purification. Reovirus was purified essentially as described by Smith et al. (17). Infected cells were homogenized in a medium composed of 0.01 M Tris (pH 8.0), 0.25 M NaCl, and 0.01 M 2-mercaptoethanol. The homogenate was extracted repeatedly with Freon PCA (kindly provided by E. I. du Pont de Nemours & Co., Inc.) until the aqueous phase was clear. The final aqueous phase was layered over a preformed, 10-ml CsCl density gradient pad (1.20 to 1.40 g/ml) and centrifuged for 2 h at 24,000 rpm in an SW27 rotor. The virus band was collected and further purified by sucrose velocity and CsCl equilibrium centrifugation by the method of Smith et al. (17).

Virus titration. Virus was titrated on L-cell monolayers by a modification of the procedure of Shaw and

[†] Present address: The Samuel Roberts Noble Foundation, Biomedical Division, Ardmore, OK 73401.

[‡] Present address: Department of Microbiology, Miami University, Oxford, OH 45056.

Cox (16). Approximately 3×10^6 spinner cells were added to plastic tissue culture dishes (60 by 15 mm). After 1 to 4 h of incubation at 30°C, the medium was removed, and 0.1 ml of each virus dilution was added to each culture. The cultures were incubated for 1 h at 39°C in an atmosphere of 5% CO₂ in air. A 5-ml amount of MEM containing 3% FCS, 1% agar, and 100 µg of kanamycin per ml was added to each plate and allowed to solidify. The cultures were then incubated at 39°C in 5% CO₂ in air for 72 h. At this time, a second overlay containing 0.005% neutral red was added, and incubation was continued for 24 h, after which plaques were counted.

Measurement of virus adsorption. Cells were inoculated into 25-cm² plastic flasks at a concentration of 5×10^5 cells per flask. Reovirus was added at a multiplicity of infection of 10 to 20 PFU/cell in 2 ml of growth medium. At selected intervals, duplicate cultures were washed three times with MEM without serum to remove unadsorbed virus. A 2-ml amount of MEM without serum was added to each flask, the cells were disrupted by sonic treatment, and the lysates were assayed for cell-associated virus, as described previously.

Virus growth studies. Cells were inoculated into 25-cm² plastic flasks at a concentration of 5×10^5 cells per flask. Reovirus was added at a multiplicity of infection of 10 to 20 PFU/cell in 2 ml of growth medium. The cultures were incubated for 4 to 5 h, after which the growth medium was removed and the cultures were washed with MEM without serum to remove unadsorbed virus. A 2-ml amount of growth medium containing 100 µg of kanamycin per ml was added to each culture. At selected intervals, duplicate flasks were disrupted by sonic treatment, and the lysates were assayed for virus.

Determination of cell viability. Cell viability was determined at times after infection by trypan blue exclusion. At specified intervals, infected cultures in 25-cm² flasks were selected, the growth medium was removed, and the cells were washed twice with 5 ml of MEM to remove all serum. A 2-ml amount of MEM without serum containing 0.4% trypan blue was added to each flask. The cultures were incubated for 5 min at 37°C, after which the solution was removed. Total and stained cells were counted in random fields to determine percent viability.

Infectious center assay. Monolayers of normal or transformed WI-38 cells were infected with 10 to 100 PFU/cell of reovirus. After an adsorption period of 5 to 6 h, the cultures were washed three times with MEM without serum. Antireovirus guinea pig serum, which had been previously titrated by plaque neutralization assay, was appropriately diluted in MEM and added to the cultures to neutralize unadsorbed virus. After 1 h at room temperature, the serum was washed from the cultures with MEM. The cells were removed by trypsinization, resuspended in growth medium, and counted. The cell suspension was diluted, and 0.1 ml of the suspension containing 30 to 300 cells was inoculated onto tissue culture dishes (60 by 15 mm each) with 1.5 ml of growth medium. The cells were allowed to attach for 4 h at 37°C, after which 2.5×10^6 L-929 cells were added to each culture. After an additional 2-h attachment period at 37°C, the growth medium

was removed and an agar overlay was added as described for the virus plaque assay. The final dilutions of the infected normal and transformed WI-38 cells were centrifuged to remove the cells, and 0.1 ml of each supernatant was added to L-929 monolayers and overlaid with agar to serve as control preparations. After incubation for 3 days at 37°C in 5% CO₂ in air, a second overlay containing 20% FCS was added to each culture. On the sixth day, a third overlay containing neutral red was added, and the plaques were counted.

Measurement of DNA synthesis. Monolayer cultures were infected at a multiplicity of infection of 100 PFU/cell in 2 ml of growth medium containing 100 µg of kanamycin per ml. At selected intervals, duplicate control and infected cultures were pulse-labeled for 30 min with 0.5 µCi of [³H]thymidine per ml. Labeling was terminated by removal of the growth medium and addition of 2.5 ml of cold MEM without serum. The cultures were washed twice with cold MEM followed by the addition of cold 5% trichloroacetic acid. The cultures were disrupted by sonic treatment and stored at 4°C for at least 1 h. The trichloroacetic acid-insoluble precipitate was collected on membrane filters (Millipore Corp.) and air dried, and radioactivity was determined by liquid scintillation counting.

Isotopes. [³H]thymidine (19.9 Ci/mmol) was purchased from New England Nuclear Corp.

RESULTS

Reovirus replication in normal and transformed cells. It has been reported that normal WI-38 cells support reovirus replication which does not result in observable cytopathology within 24 h after infection (15), a time when reovirus-infected L cells are lysed as a result of infection (5). However, the time course of the infection in WI-38 cells was not reported. If normal and transformed cells respond differently to reovirus infection, it is important to characterize virus replication in each cell type.

There was a progressive association of reovirus to both normal and transformed WI-38 cells (Fig. 1). Maximum virus association to both cell types occurred approximately 3 h after the addition of virus. This corresponds to the time of maximum adsorption observed during reovirus infection of L cells (8).

The time course of reovirus replication and the virus yield were similar in normal and transformed WI-38 cells but were markedly different in L-929 cells (Fig. 2). Infectious progeny appeared in L-929 cells 8 to 10 h after infection. The eclipse phase was significantly longer in both normal and transformed cells, and infectious progeny were not detectable until 16 to 18 h after infection. In addition, the rate of virus production was greater in L-929 cells than in the normal and transformed human cells.

During the course of these experiments, cultures of reovirus-infected normal WI-38 cells

FIG. 1. Adsorption of reovirus to normal (WI-38) and transformed (2RA) human embryonic lung cells. Cell-associated PFU are presented at selected intervals after infection as an average of duplicate samples.

were passaged as described above. The medium was periodically assayed for released virus. It was found that the cells continued to release virus for as long as 14 days after infection. Virus titers in the medium reached maximum levels at 26 h after infection and remained stable at a mean titer of 1.3×10^7 PFU/ml. During this time, the cells exhibited little cytopathology. In contrast, infected cultures of L-929 cells were lysed by 24 h after infection, and infected transformed WI-38 cells were all lysed by 96 h after infection. The transformed cells continued to produce virus until lysis. The mean virus concentration in the medium stabilized at 5×10^7 PFU/ml.

Viability of infected cells. Because observation of infected cells indicated that transformed WI-38 cells were more sensitive to reovirus infection than normal WI-38 cells, the viability of infected cells at selected intervals after infection was determined by trypan blue exclusion. No significant change in the viability of infected, normal WI-38 cells was detected by 96 h after infection (Fig. 3). However, infected transformed cells were reduced to 50% viability by 48 h after infection. This progressive decrease in the viability of infected transformed cells continued until 96 h after infection, when the cultures were completely lysed.

Infectious center assay. Because cultures of normal WI-38 cells which had been exposed

FIG. 2. One-step replication cycles of reovirus in L cells and normal (WI-38) and transformed (2RA) human embryonic lung cells. Total PFU are presented at selected intervals after infection as the average of duplicate samples.

to reovirus exhibited little cytopathology, it was necessary to determine whether a significant proportion of these cells were actually infected. It was possible that only small numbers of the normal cells were infected and were as sensitive as the transformed cells to infection. The effects of infection might have been obscured by the surviving uninfected cells. The proportion of productively infected normal and transformed cells was determined by infectious center assay. Results of a typical experiment are given in Table 1. Averages of three assays indicated that 93% of the transformed cells were infected and released virus, whereas 60% of the normal cells were productively infected after an initial exposure to virus.

DNA synthesis in infected cell. Reovirus infection of L cells results in an inhibition of cellular DNA synthesis which occurs early in the virus replication cycle before the appearance of detectable cytopathology (5, 16). Since the characteristics of virus replication and development of cytopathology varied in reovirus-infected L cells and normal and transformed human cells, DNA synthesis was measured in each cell type at selected times after virus infection. Inhibition of DNA synthesis was first detected in L cells between 8 and 12 h after infection (Fig. 4a), which corresponds to the time of appearance of the first progeny virions (Fig. 2). DNA synthesis in normal WI-38 cells was not inhibited at 16 to 18 h after infection, the time of the ap-

FIG. 3. Viability of normal (WI-38) and transformed (2RA) cells at selected times after infection. Cultures were infected with approximately 100 PFU of reovirus per cell. At intervals after infection, the monolayers were washed and then stained with trypan blue, and total and nonviable cells were counted. Viability is expressed as percentage of control at selected times after infection.

TABLE 1. Results of typical infectious center assays for WI-38 and WI-38 VA13 2RA cells

Cell line	No. of cells inoculated	No. of plaques counted	Cells productively infected (%)
WI-38	160	101	63
WI-38 VA13 2RA	210	199	95

pearance of progeny virions (Fig. 2), and no inhibition of DNA synthesis was detected as late as 36 h after infection (Fig. 4b). However, inhibition of DNA synthesis began in transformed WI-38 cells at 15 to 18 h after infection (Fig. 4c). As in L cells, the beginning of the inhibition of DNA synthesis in transformed cells corresponded to the time of appearance of progeny virions (Fig. 2). It should be noted that inhibition of DNA synthesis began in transformed cells at a time when the infected cells exhibited no cytopathology, and the inhibition was nearly complete before significant cytopathology was detected (Fig. 3).

DISCUSSION

Reovirus mediates a selective destruction of transformed human cells. The oncolytic activity of certain other viruses has been related to the ability of the virions to adsorb to and replicate in the transformed but not the normal cell (19). Reovirus adsorbs to normal and SV40-trans-

formed human WI-38 cells and replicates in both cell types. However, the data presented here suggest fundamental differences in the characteristics of virus replication and modification of cell function in normal and transformed cells.

The yield of progeny virions in SV40-transformed WI-38 cells was two to three times that in normal WI-38 cells. This is probably the result of fewer normal cells being infected during the initial cycle of replication. This is supported by the infectious center assay, which indicated that infection was established in only 60% of the normal cells, versus 95% of the transformed cells, after a 6-h exposure to virus.

It is not clear from the results presented here whether normal WI-38 cells include a population resistant to reovirus infection or whether all cells eventually become infected after successive virus replication cycles in productive cells. The latter appears to be the most likely possibility. A very slight increase in the doubling time and decrease in cell size was noted in normal cells by 14 days after infection, indicating a progressive infection. Even though Bell and Ross (2) have reported the establishment of persistent latent reovirus infections in human embryonic lung fibroblasts, it seems that reovirus may produce prolonged lytic, rather than persistent, infections in many cell lines. We have recently found that reovirus infection of certain human diploid cell lines, including WI-38, results in the complete

FIG. 4. DNA synthesis in control and infected L cells and normal (WI-38) and transformed (2RA) human embryonic lung cells. Cells were infected with 100 PFU of reovirus per cell. Incorporation of [³H]-thymidine is expressed as acid-insoluble counts per minute (CPM) at selected times after infection and represents the average of duplicate cultures. (a) L cells; (b) normal WI-38 cells; (c) transformed (2RA) WI-38 cells.

destruction of cell cultures 40 to 60 days after infection (S. M. Stanish and D. C. Cox, manuscript in preparation). These studies also indicate that the time required for total destruction of cultures may be dependent on the population doubling level.

It is clear that inhibition of cell DNA synthesis is not a necessary consequence of reovirus replication, since no inhibition occurred in normal infected cells. Even though only 60% of these cells were initially infected, significant inhibition should have been detectable in the infected population. The inhibition also appears not to be the result of a loss of cell viability, since the inhibition of DNA synthesis in transformed cells preceded the decrease in viability by a significant interval of time. Others have suggested that the formation of dense perinuclear inclusions may modify the integrity of the nuclear membrane and its possible function in DNA replication (6). Such modifications may also alter nuclear-cytoplasmic interactions necessary for the

initiation and maintenance of DNA synthesis. This is supported by the observations that the formation of perinuclear inclusions and the inhibition of DNA synthesis were coincident and occurred only in L cells and the transformed human cells. Neither occurred in the normal human cells (unpublished data).

The data presented here suggest the possibility that the selective cytolytic effect of reovirus for transformed cells may, in part, be related to the virus-mediated inhibition of cellular DNA synthesis in those cells. This inhibition occurred in transformed cells before the appearance of detectable cytopathology and may indicate a primary biochemical lesion leading to cell lysis. Since reovirus has been shown to inhibit the initiation of DNA synthesis in L cells (4, 9), and since the regulation of initiation may be modified in transformed cells, reovirus may select for characteristics which control DNA synthesis in transformed cells. However, other changes in cellular macromolecular metabolism in normal and transformed cells have not been studied in sufficient detail to establish whether or not the inhibition of DNA synthesis is the initial virus-induced modification.

It is apparent that certain features of reovirus replication and the resulting effects on the host differ in normal and transformed human cells and continuous cell lines which possess neoplastic characteristics. This lytic response appears to be selective for other transformed cells, and suggests reovirus may prove to be a useful probe in characterizing differences in certain normal and transformed cells. The therapeutic value of such oncolytic selectivity has been suggested in earlier studies (3, 14). In addition, reovirus has recently been shown to possess significant therapeutic value in mouse tumor systems (12, 13). This effect appears to be mediated by immune enhancement. Both oncolysis and antigenic modification could result from the ability of reovirus to selectively alter transformed cells.

ACKNOWLEDGMENT

This investigation was supported by grant VC-108 from the American Cancer Society.

LITERATURE CITED

1. Ahmed, R., and A. F. Graham. 1977. Persistent infections in L cells with temperature-sensitive mutants of reovirus. *J. Virol.* 23:250-262.
2. Bell, T. M., and M. G. R. Ross. 1966. Persistent latent infection of human embryonic cells with reovirus type 3. *Nature (London)* 212:412-414.
3. Bennette, I. F. 1960. Isolation of a non-pathogenic tumor-destroying virus from mouse ascites. *Nature (London)* 187:72-75.
4. Cox, D. C., and J. E. Shaw. 1974. Inhibition of the initiation of cellular DNA synthesis after reovirus infection. *J. Virol.* 13:760-761.

5. **Ensminger, W. D., and I. Tamm.** 1969. Cellular DNA and protein synthesis in reovirus-infected L cells. *Virology* **39**:357-360.
6. **Ensminger, W. D., and I. Tamm.** 1969. The step in DNA synthesis blocked by reovirus infection. *Virology* **39**:935-938.
7. **Girardi, A. J., D. Weinstein, and P. S. Moorhead.** 1966. SV40 transformation of human diploid cells. *Ann. Med. Exp. Biol. Fenn.* **44**:242-254.
8. **Gomatos, P. J., I. Tamm, S. Dales, and R. M. Franklin.** 1962. Reovirus type 3. Physical characteristics and interaction with L-cells. *Virology* **17**:441-454.
9. **Hand, R., and I. Tamm.** 1974. Initiation of DNA synthesis in mammalian cells and its inhibition by reovirus infection. *J. Mol. Biol.* **82**:175-183.
10. **Hashiro, G., P. C. Loh, and J. T. Yau.** 1977. The preferential cytotoxicity of reovirus for certain transformed cell lines. *Arch. Virol.* **54**:307-315.
11. **Hayflick, L., and P. S. Moorhead.** 1961. The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25**:585-621.
12. **Kollmorgen, G. M., D. C. Cox, J. J. Killion, J. L. Cantrell, and W. A. Sansing.** 1976. Immunotherapy of EL-4 lymphoma with reovirus. *Cancer Immunol. Immunother.* **1**:239-244.
13. **Kollmorgen, G. M., W. A. Sansing, D. C. Cox, J. J. Killion, and J. L. Cantrell.** 1976. Induction of immunity with immunotherapy, p. 25-38. *In* R. G. Crespen (ed.), *Neoplasm immunity: mechanisms*. ITR Press, Chicago.
14. **Lindenmann, J., and P. Klein.** 1967. Immunological aspects of viral oncolysis, p. 1-86. *In* *Recent results in cancer research* 9. Springer-Verlag, New York.
15. **Loh, P. C., H. R. Oie, and R. M. T. Ratnayake.** 1970. Accelerated cytopathology in HeLa cells induced by reovirus and cycloheximide. *Infect. Immun.* **2**:705-712.
16. **Shaw, J. E., and D. C. Cox.** 1973. Early inhibition of cellular DNA synthesis by high multiplicities of infectious and UV-inactivated reovirus. *J. Virol.* **12**:704-710.
17. **Smith, R. E., H. J. Zweerink, and W. K. Joklik.** 1969. Polypeptide components of virions, top component and cores of reovirus type 3. *Virology* **39**:791-810.
18. **Taber, R., V. Alexander, and W. Whitford.** 1976. Persistent reovirus infection of CHO cells resulting in virus resistance. *J. Virol.* **17**:513-525.
19. **Taylor, M. W., B. Cordell, M. Souhrada, and S. Prather.** 1971. Viruses as an aid to cancer therapy: regression of solid and ascites tumors in rodents after treatment with bovine enterovirus. *Proc. Natl. Acad. Sci. U.S.A.* **68**:836-840.