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DIFFERENTIAL SENSITIVITY OF NORMAL AND TRANSFORMED CELLS TO REOVIRUS INFECTION

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DIFFERENTIAL SENSITIVITY OF NORMAL AND

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TO KATHY

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DIFFERENTIAL SENSITIVITY OF NORMAL AND TRANSFORMED CELLS TO REOVIRUS INFECTION

CHAPTER I

INTRODUCTION

A significant amount of current cancer research is concerned with a comparison of normal and transformed cells grown in culture. Such studies are relevant because cultured transformed cells have been shown to retain at least some of those characteristics which seem responsible for the production of cancer in vivo (15, 26). For example, the uncontrolled growth of a tumor can be compared to the loss of contact inhibition and increased growth rate of transformed cells grown in vitro. In contrast, cell division in confluent monolayers of normal cells is inhibited; a process which appears analogous to the control of cell division in normal tissue. Cultured transformed cells also retain the ability to cause tumors when implanted in appropriate animals. Adding to the credibility of such work is our ability to transform normal diploid cells with certain viruses in culture (19, 32, 36, 59, 61). Comparison of normal cells and the corresponding transformed cells eliminates the possibility that observed differences are due to variance among cell types.

Many basic differences in characteristics of transformed cells,

as compared to their normal counterparts, have already been documented. A few examples include membrane changes as reflected in transport of certain metabolites (49) and cell-cell interaction (1, 6, 7, 13), changes in glucose metabolism (14), changes in the synthesis and presence of certain non-histone chromosomal proteins (37), and changes in chromosome number (26, 31, 36, 45).

Unfortunately, no observed differences between normal and transformed cells have been successfully exploited to selectively kill, or inhibit the growth of transformed cells. This is due in part to our lack of understanding of the molecular basis of transformation and the resulting changes in cellular characteristics. Indeed, even the mechanisms which control the progress of a normal cell through the cell cycle have yet to be fully elucidated. As our knowledge in these areas increases, the possibility of determining methods for selective inhibition of transformed cells should also increase.

Viruses have been widely used as tools in the study of molecular genetics (22, 30), cellular aging (29, 44), and the regulation of cellular macromolecular synthesis (2, 9, 10, 23, 35). In our laboratory, reovirus is being used in an attempt to define the mechanisms of regulation of cellular deoxyribonucleic acid (DNA) function. When cells are infected with low multiplicities of this virus, DNA synthesis is inhibited 8 hours later, and the cells are lysed by 24 hours post-infection. This inhibition of DNA synthesis appears to be a specific process because it occurs at a time when no cytopathology or inhibition of ribonucleic acid (RNA) or protein synthesis is apparent (9, 20). These observations have resulted from work performed

exclusively with continuous, or transformed, cell lines. In fact, most studies concerning the infectious cycle of reovirus have been performed using transformed cells.

In 1970, Loh et al. reported on the acceleration of cytopathology by cycloheximide in reovirus-infected cells. One of the cell lines used in this study was the normal, diploid, human embryonic lung cell, WI-38. These workers reported no cytopathology in infected WI-38 cells as late as 24 hours post-infection, even though these cells were synthesizing progeny virus (40). Since most transformed cells exhibit extensive cytopathology by 24 hours after reovirus infection, these findings suggested the possibility that normal cells might respond differently to reovirus infection. Further evidence supporting this possibility was provided by Bell and Ross who showed that reovirus type 3 can establish a persistent latent infection in human embryonic fibroblasts, with no cytopathology (4).

Because at least some normal regulatory mechanisms appear to be altered in transformed cells, it is not unreasonable to suspect a differential sensitivity to viral infection. It has been shown, especially with tumor viruses, that the course of infection in some instances is determined solely by cell type. For example, infection of certain monkey cells by simiam virus 40 (SV-40) always results in cell lysis, while infection of human cells may result in transformation (59). Further, some workers have reported selective destruction of transformed cells by certain viruses. Taylor et al. have shown that Sarcoma-1 or Ehrlich's ascites tumor cells which have been injected into mice are selectively lysed when the mice are injected with bovine

enterovirus (58). This report also showed that the virus lyses polyoma-transformed 3T3 mouse cells, while producing no cytopathology in normal 3T3 cells. In addition, Bennette isolated an unidentified virus from mouse ascites cells which selectively destroyed Krebs 2 ascites and Ehrlich's ascites tumors in mice (5).

It seems plausible, therefore, that viruses could be utilized in further attempts to characterize the molecular basis of transformation.

This dissertation describes experiments in which the sensitivities of normal and transformed cells to reovirus infection were compared. Of particular interest was the study of DNA synthesis in normal infected cells. Because the cultures reported by Bell and Ross (4) to be persistently infected with reovirus continued to grow at a normal rate, it appears that DNA synthesis in these cells would not be inhibited. It seemed possible, therefore, that reovirus infection might selectively kill transformed cells because of the susceptibility of these cells to DNA synthesis inhibition.

Initial experiments studied the characteristics of reovirus infection of WI-38 cells. These results were compared to the characteristics of infection of mouse L-929 fibroblasts (L-cells) by reovirus which have already been reported by this laboratory and others. Significant differences were observed. However, the possibility existed that these differences were due only to variation in cell type. To determine whether differences in sensitivity to reovirus infection were caused by transformation, experiments were performed using WI-38VA13 2RA cells. These are WI-38 cells which were transformed by SV-40 (19).

CHAPTER II

METHODS AND MATERIALS

Cells. Mouse fibroblasts, strain L-929 (50) (Flow Laboratories), adapted for growth in suspension culture were used for plaque assays and infectious center assays. Stock cultures of L-cells were cultivated in minimum essential medium (MEM) modified for suspension culture (Grand Island Biological Company) supplemented with 5% (v/v) heat inactivated fetal calf serum (FCS, Grand Island Biological Company). These cells were maintained in logarithmic growth by dilution with fresh prewarmed growth medium every 48 h to a cell concentration of $5x10^4$ cells/ml.

Human embryonic lung cells, strain WI-38 (28) were obtained from Cell Associates, Stanford University, at passage number 13. These cells were grown in monolayer in Falcon plastic flasks (75 cm^2 surface area) in MEM supplemented with 10% (v/v) FCS. The procedure used to passage cultures was essentially that of Hayflick (27,28). When the monolayers were confluent, the old growth medium was decanted and enough prewarmed trypsin solution containing 0.25% (w/v) trypsin (Grand Island Biological Company), 0.85% (w/v) sodium chloride, and 0.001% (w/v) phenol red, pH 7.4, was added to cover the cell sheet. After 5 min, the trypsin solution was removed. When the cell layer

began to slough, 10 ml fresh growth medium was splashed onto the cells. The cell suspension was vigorously pipetted 3 times through a small bore 10 ml pipette to break up clumps. The cells were then divided into 4 flasks and fresh, prewarmed growth medium added to each. This gave a 1:4 split ratio, and when the cells had grown to confluency, the passage number was raised by 2, since the cells had divided twice.

Human foreskin cells, strain CF-3, were kindly provided by the Samuel Roberts Noble Foundation. These cells were grown and passaged with the exact procedure used with WI-38 cells.

SV-40 transformed WI-38 cells, WI-38VA13 2RA, were kindly provided by Dr. A. J. Girardi. These cells were passaged according to the procedure used with WI-38 cells, except for the following changes: (1) growth medium was supplemented with only 5% (v/v) FCS; (2) during the splitting procedure the trypsin solution was removed after an incubation of 1 min or less, due to the ease of dispersion of these cells; (3) the split ratio used was 1:5.

Stock cell freezing. Confluent monolayers were dispersed with trypsin and resuspended in 2 ml of freezing medium containing MEM supplemented with the appropriate concentration of FCS and 10% (v/v) glycerol, at a concentration of approximately 2×10^6 cells/ml. This suspension was put into a small glass freezing vial and stored overnight at 4C. The vial was then stored in the REVCO freezer at -50C until needed.

When frozen cell stocks were needed, the suspension was quickly thawed in a 37C water bath. The cells were then added to a flask containing fresh warm growth medium. Cell stocks frozen in this

manner retained a high percentage of cell viability for up to six months.

Virus. Reovirus type 3 was used in this study and was kindly provided by Dr. P. J. Gomatos. Plaque-purified virus was prepared by picking a large, well isolated plaque with a pipette. The agar plug was eluted at 4C for 24 h in 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.5 (SSC). The eluted virus was then placed onto a confluent monolayer of L-cells (25 cm^2 surface area) and incubated at 37C for 36 h. A lysate of the infected cells was made by sonication. This was considered one passage and was repeated by infecting 10 confluent monolayers of L-cells (75 cm^2 surface area) with the lysate from passage one.

Virus stocks for purification were prepared by concentrating L-cells to $1x10^7$ cells/ml in MEM without serum. The cells were infected with 10-20 plaque forming units (PFU)/cell and allowed to adsorb at 34C for 1 h. The infected cells were then diluted to $1x10^6$ cells/ml with growth medium. After 24 h incubation at 34C, the cultures were chilled and the cells and virus collected by centrifugation at 5000xg for 15 minutes. The pellets were resuspended for purification.

Virus purification. A modification of the procedure of Smith et al. (55) was used to purify reovirus. The final separated aqueous phase was layered onto a 10 ml CsCl density gradient (1.2 g/ml-1.4 g/ml) in a 38 ml centrifuge tube. This was centrifuged for 1 h at 76,000 x g in the SW 27 rotor using a Beckman L2-50. These viruses band at a density of 1.37 g/ml and the band was collected cleanly with a syringe

and needle. The CsCl was removed by exhaustive dialysis against cold SSC or by pelleting the virus by centrifugation for 1 h at 82,500 x g in the SW 27 rotor. The virus was layered onto a 20-40% (w/v) linear sucrose gradient and centrifuged for 1 h at 82,500 x g in the SW 27 rotor. The virus band was collected and dialyzed exhaustively against cold SSC. This was a purified stock suspension and was stored at 4C.

Virus assay. Reovirus was assayed on confluent monolayers of L-cells as previously described (54). Cells were added to small petri plates (60 x 15 mm, Falcon Plastics) at a concentration of $3x10^6$ cells/plate. After the cells had attached to the plates, the medium was removed and 0.1 ml of each virus dilution was pipetted onto the monolayer. The plates were incubated for 1 h at 39C in an atmosphere of 5% (v/v) CO_2 in air. Five ml of autoclavable MEM (Auto Pow, Flow Laboratories) containing 3% (v/v) FCS, 1% (w/v) agar (Difco Laboratories), and 100 μg/ml kanamycin, was added to the plates and allowed to solidify. The plates were incubated at 39C in an atmosphere of 5% (v/v) CO_2 in air for 72 h. At this time a second overlay containing 0.005% (w/v) neutral red was added. Plaques were visible within 24 h.

Virus adsorption curves. Cells were seeded into small plastic flasks (25 cm^2 surface area) at a concentration of $5x10^5$ cells/flask. Reovirus was added at a multiplicity of infection (MOI) of 10-20 pfu/cell in 2 ml of growth medium. At timed intervals, duplicate monolayers were washed 3 times with MEM (no serum) to remove unadsorbed virus. Two ml MEM (no serum) were added to the flasks, the cultures were

sonicated, and the lysates assayed for cell-associated virus by the plaque method on L-cells.

Virus growth curves. Cells were seeded into small plastic flasks at a concentration of $5x10^5$ cells/flask. Reovirus was added at a MOI of 10-20 pfu/cell in 2 ml of growth medium. The flasks were incubated for 4-5 h to allow for adsorption. The unadsorbed virus in the growth medium was removed, and 2 ml fresh growth medium containing 100 μg/ml kanamycin was added to the monolayers. At timed intervals duplicate flasks were sonicated and the lysates assayed for virus by the plaque method on L-cells.

Cellular DNA and protein synthesis assay. Monolayers of cells were seeded at $5x10^5$ cells/flask. Virus was added to half of the monolayers at a MOI of 10 or 100 pfu/cell in 2 ml growth medium containing 100 μg/ml kanamycin. The other half of the flasks were used as controls and were treated identically except that no virus was added. At timed intervals, duplicate control and infected cultures were pulse labeled with either ^{3}H-thymidine (19.9 Ci/mM) or ^{3}H-amino acids (32 Ci/mM) at 0.5 μCi/ml for 30 min. Labeling was stopped by removal of the growth medium and addition of 2.5 ml of ice cold MEM (no serum). This was removed and the monolayers washed again in cold MEM (no serum). Then 2 ml of cold 5% (w/v) trichloroacetic acid (TCA) was added and the monolayers were sonicated and stored at 4C. At least 1 h was allowed for precipitation. The precipitate was collected on millipore filters (pore size 1.2 μ). The filters were air dried and counted in a Beckman DPM-100 liquid scintillation spectrophotometer using Beckman Cocktail D

(5 g diphenyloxazole, 100 g naphthalene, 10 ml water, to 1 liter with 1-4 dioxane) as the fluor.

Determination of cell viability. Cell viability at times after infection was determined by exclusion of trypan blue (42, 43, 51, 53). At timed intervals infected monolayers were selected, the growth medium removed, and the cell sheet washed twice with 5 ml MEM (no serum) to remove all serum. Then 2 ml of MEM (no serum) containing 0.4% (w/v) trypan blue was added to the flask. Incubation was for 5 min at 37C. The solution was removed and the cell sheet examined by light microscopy. Total cells and cells stained blue were counted in random fields to determine percent cell viability.

Fluorescent antibody techniques. Cells were seeded onto coverslips (9mm x 35mm) in Leighton tubes at a concentration of $5x10^4$ cells/tube in 2 ml growth medium. After the cells had attached, the medium was removed and the monolayers infected with reovirus in 1 ml of growth medium containing 100 μg/ml kanamycin. At intervals after infection, the coverslips were removed and washed with SSC. The cells were fixed and stained by the indirect method described by Pope and Rowe (46). Antiserum was obtained by inoculating a guinea pig by the intranasal route with 0.1 ml of virus suspension in each nostril. The total inoculum was 10^9-10^{10} pfu. Serum was collected approximately 10 days after inoculation and was titered by a hemagglutination inhibition test (50). Fluorescein-conjugated anti-guinea pig serum was kindly provided by Dr. P. Bartels. The slides were observed with a Zeiss fluorescent microscope.

Infectious center assay. Monolayers of cells were infected with reovirus at 10-100 pfu/cell. After a period of 5-6 h to allow for adsorption, the monolayers were washed 3 times with MEM (no serum). Guinea pig serum with anti-reovirus antibodies was diluted in 2 ml MEM and added to the monolayers to inactivate unadsorbed virus. Incubation was for 1 h at room temperature. This serum was washed from the monolayers and the cells were removed by trypinsization, resuspended in growth medium, and counted. The cell suspension was then diluted so that 0.1 ml contained 30-300 cells. The cells were put into small petri plates at this concentration with a total of 1.5 ml growth medium. The cells were allowed to attach for 4 h; then 2.5×10^6 L-cells/plate were added. After an additional 1-2 h attachment period, the growth medium was removed, and the agar overlay used in the plaque assay was added. The final cell dilutions were centrifuged to pellet the cells, and 0.1 ml of each supernatant was added to the L-cell monolayers, and then overlayed with agar. These plates served as controls. After 3 days incubation, a second agar overlay, containing 20% (v/v) FCS, was added to the plates. On the sixth day, a third agar overlay containing neutral red was added. Plaques continued to appear for 3 days following the final overlay.

Antiserum used in this procedure was titered by a plaque neutralization technique. Serial 2-fold dilutions of the serum were prepared in MEM. A known concentration of reovirus was added to each dilution, the tubes were shaken, and incubated for 1 h at room temperature. A plaque assay was then performed from each dilution tube. The end point was arbitrarily chosen as the highest dilution of serum

causing a 95% reduction in the plaque-forming titer. This dilution was used in the infectious center assay.

Transformation of CF-3 cells by SV-40. CF-3 cells were seeded in large plastic flasks (75 cm^2 surface area) at a concentration of $1x10^6$ cells/flask. Both high passage, 54, and low passage, 18, were used. These cultures were infected with high multiplicities of SV-40, $10^{6.8}$ Tissue Culture Dose 50/flask, in 5 ml MEM containing 10% (v/v) FCS. A stock of SV-40 was kindly provided by Dr. M. K. Patterson. After a 5 h adsorption period, an additional 20 ml of growth medium was added. Control cultures at both passage levels were treated identically except for addition of the virus. After infection, all cultures were maintained and passaged according to the procedure for normal CF-3 cells. Control and infected cultures were routinely frozen for stocks.

When the infected cultures began to enter the "crises" stage (19), growth medium was replaced daily to remove cellular debris. After the remaining infected cells showed characteristics of transformation, they were grown in growth medium supplemented with only 5% (v/v) FCS, and the split ratio was changed to 1:5.

CHAPTER III

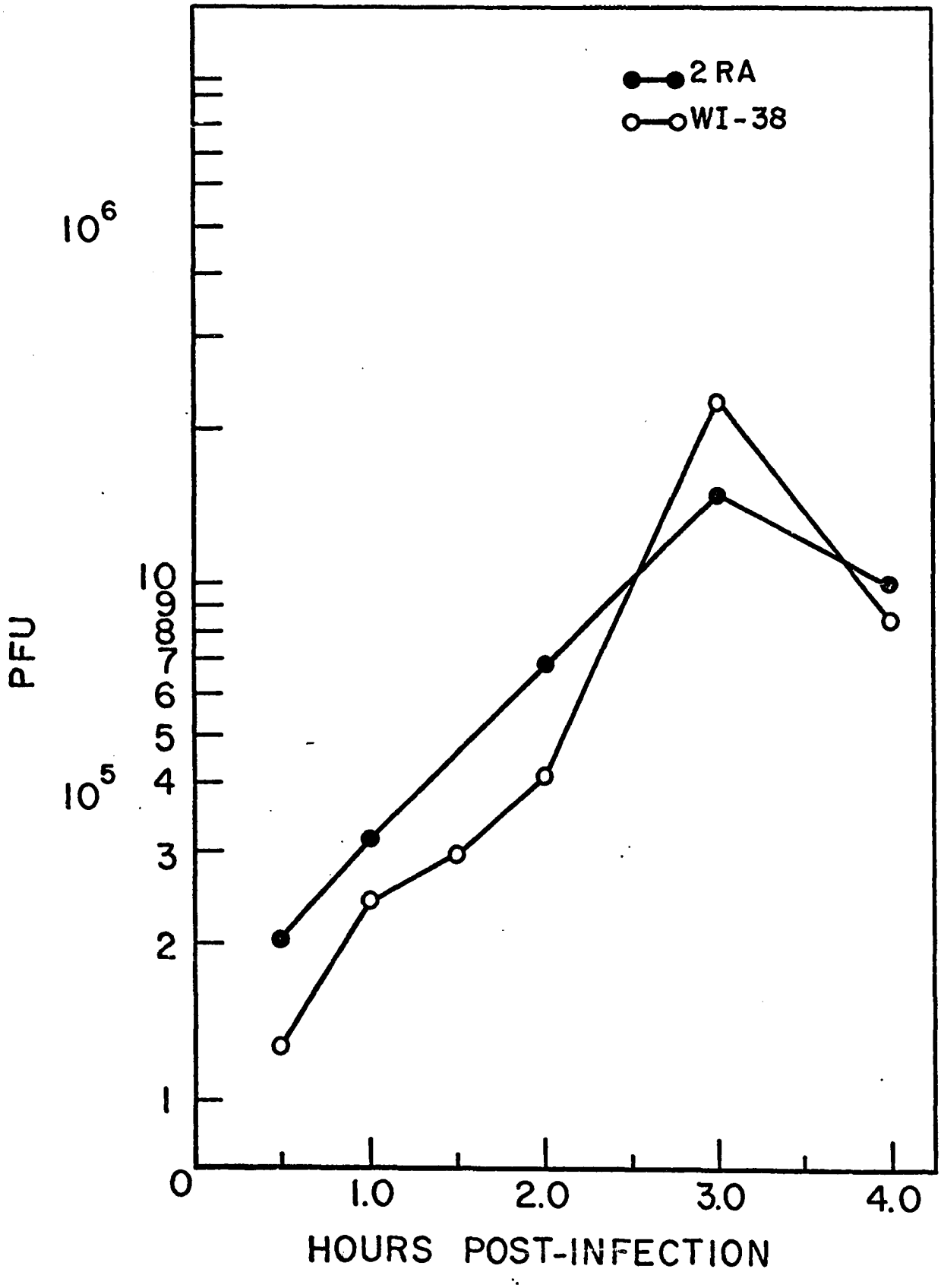
RESULTS

Adsorption of reovirus onto normal and transformed cells. The efficiency of viral replication is initially related to successful virus-cell interaction. Selective destruction of transformed cells by a virus could occur by failure to adsorb to normal cells. As an initial step in the study of the replication of reovirus in normal and transformed cells, the adsorption of the virus to each cell type was studied. Monolayers of cells were infected with virus and at intervals the cell sheets were washed free of unadsorbed virus. The monolayers were then sonicated and the lysates assayed for cell-associated virus. Results of these experiments are shown in Figure 1.

There was a progressive attachment of reovirus to both normal and transformed human cells. Peak adsorption occurred on both cell types at about 3 h after the addition of virus. This corresponded to the time of peak adsorption observed during reovirus infection of L-cells (21). It appeared that the virus was taken into the cells and uncoated rapidly, since cell-associated infectivity decreased rapidly after 3 h.

Reovirus replication in normal and transformed cells. It has been reported that normal WI-38 cells supported the synthesis of

Figure 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.


2 RA
WI-38
10^6
10
9
8
7
6
5
4
10^5
3
2
1
0
PFU
1.0
2.0
3.0
4.0
HOURS POST-INFECTION

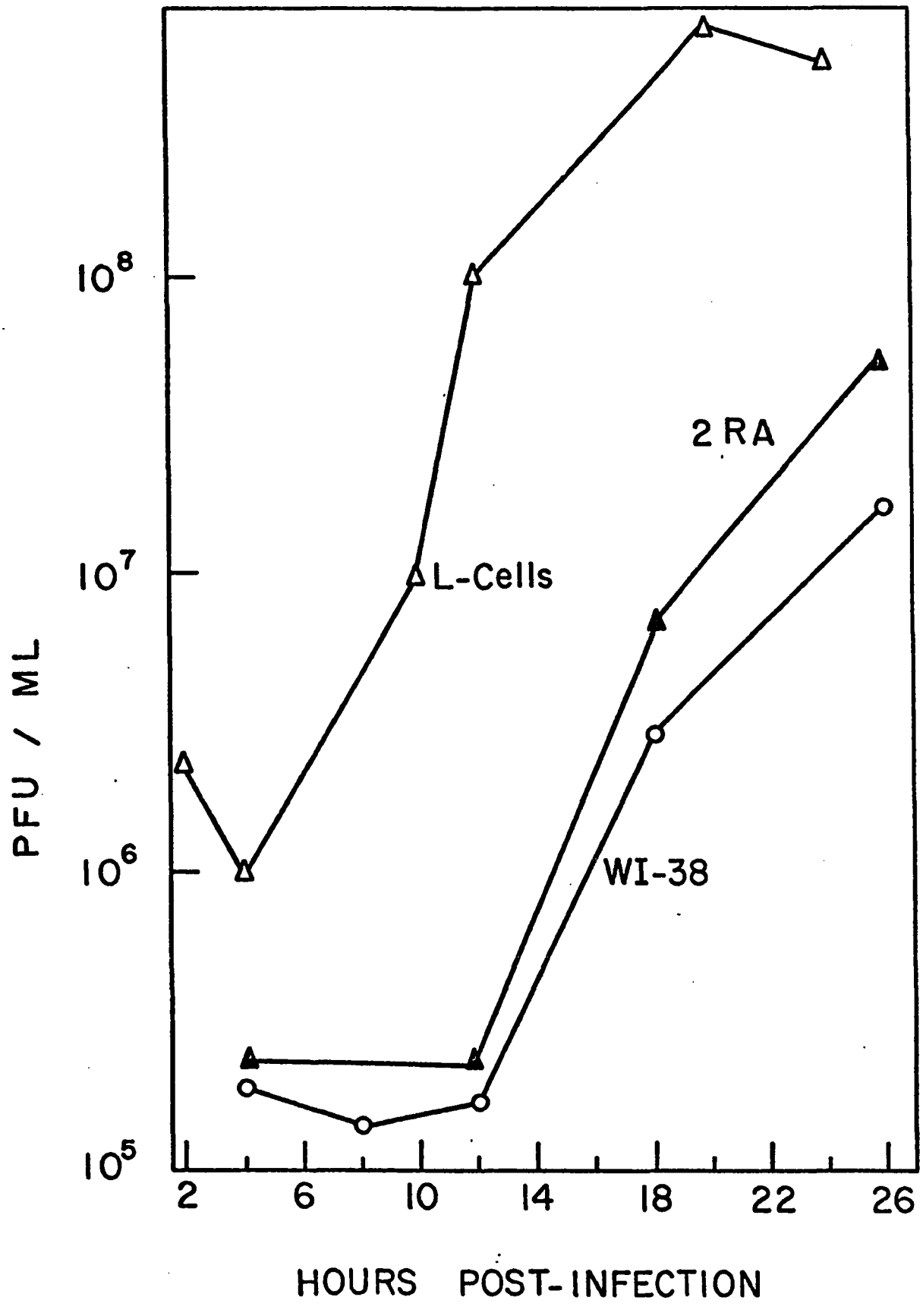
progeny reovirions (40). However, the time-course of this infection has not been reported. It was of interest to study the growth of reovirus in normal cells because cytopathology was not observable by 24 h (40), a time when infected L-cells were lysed by the virus. Also, it was important to determine if normal and transformed cells would support different reovirus replication characteristics.

One-step growth curve experiments were performed using both normal and transformed human cells. At intervals, the infected monolayers were sonicated and the lysate assayed for progeny virus. This technique assayed both released and mature, intracellular virus. Figure 2 illustrates reovirus replication characteristics in L-cells, normal WI-38 cells, and transformed WI-38 cells (2RA).

The time course of reovirus replication was approximately the same in normal and transformed human cells. It appeared that transformed cells produced 2 to 3 times more progeny per cell than the normal cells by 26 h post-infection. Reovirus synthesis in L-cells differed markedly, however, from that observed in normal and transformed WI-38 cells. In L-cells, infectious progeny appeared 8-10 h after infection. The eclipse phase was much longer in the human cells; infectious progeny were not detectable until 16-18 h after infection. Also, the rate of production of new virus in L-cells is greater than that observed in the 2 human cell lines.

In addition to the one-step growth curves, infected, normal WI-38 cells were grown and passaged according to the procedure for uninfected stock cells. The growth medium on these cells was periodically removed and assayed for released virus. It was found that these

Figure 2. A comparison of reovirus replication in L-cells, normal WI-38, and transformed WI-38 (2RA) cells. Total pfu are presented at selected intervals after infection as an average of duplicate samples.


10^8
10^7
10^6
10^5
PFU / ML
L-Cells
2 RA
WI-38
2
6
10
14
18
22
26
HOURS POST-INFECTION

cells slowly released virus for up to 14 days after infection. During this time the cells exhibited little cytopathology.

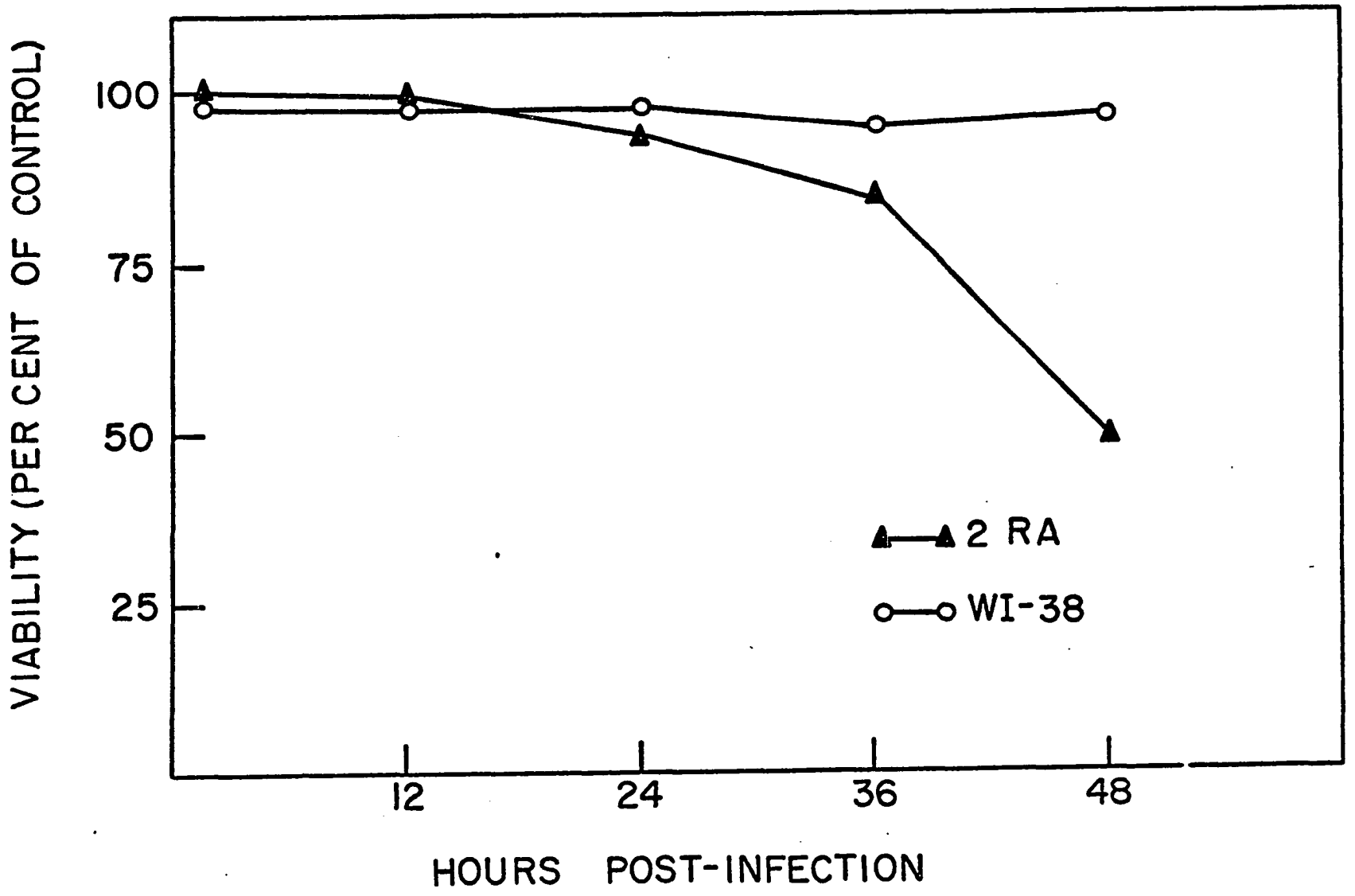
In contrast, L-cell and transformed WI-38 cultures produced a maximum amount of virus, as illustrated in Figure 2, and then the cells were lysed as a result of the infection.

Viability of infected cells. Because observations of infected cultures indicated that transformed cells were more sensitive to reovirus infection than normal cells, the viability of infected cells at times after infection was studied. Trypan blue was chosen as a vital stain. Living cells have been shown to exclude this dye, while dead cells are stained a deep blue (42, 51, 53).

Infected normal and transformed cells were stained with trypan blue and observed microscopically. The percentage of viable cells was determined for infected and control cultures. Figure 3 illustrates results from these experiments. No significant change in the percentage of viability of infected, normal WI-38 cells occurred by 48 h post-infection. Infected cultures of transformed WI-38 cells, however, were reduced to 50% viability by 48 h after infection. This progressive decrease in viability of infected transformed cells continued for 96 h when the cultures were completely lysed. During this time, infected normal WI-38 cells retained a high (95%) percentage of viability, and little cytopathology was observed.

DNA synthesis in infected cells. Reovirus infection has been shown to inhibit DNA synthesis in L-cells. This inhibition occurred early in the infectious cycle, before cytopathology appeared. It was

Figure 3. A comparison of percent viability of normal and transformed (2RA) WI-38 cells at times after infection. Cultures were infected with reovirus with approximately 100 pfu/cell. At intervals after infection, the monolayers were washed, stained with trypan blue, and total and dead cells counted. Viability is expressed as percent of control at selected times after infection.


VIABILITY (PER CENT OF CONTROL)
100
75
50
25
12
24
36
48
HOURS POST-INFECTION
2 RA
WI-38

of interest to compare rates of DNA synthesis in infected normal and transformed WI-38 cells. Control and infected cultures were pulse-labeled with ^{3}H-thymidine for 30 min and radioactivity incorporated into acid-insoluble material was measured. Figure 4 illustrates results of these experiments using L-cells and WI-38 cells. L-cell DNA synthesis began to be inhibited by 8 h post-infection. The time of the beginning of this inhibition corresponded to a time in the growth cycle when mature progeny first appeared. DNA synthesis in infected WI-38 cells was not inhibited at the time of appearance of progeny virus (16-18 h) and no inhibition was seen as late as 36 h after infection. Figure 5 illustrates the results of similar experiments using transformed WI-38 cells. DNA replication in these cells began to be inhibited at about 15-18 h post-infection. Again, this corresponded to a time when infectious progeny first appeared in these cells. This inhibition appeared to be complete by 36 h after infection. It is significant to note that the inhibition began at a time when the infected cells exhibited no cytopathology and the percent viability was equal to that of control cultures (Figure 3).

Protein synthesis in infected cells. It has been suggested by some workers that cessation of cellular protein synthesis is the cause of DNA synthesis inhibition in reovirus-infected L-cells (34). Total protein synthesis in normal and transformed WI-38 cells was studied by pulse-labeling control and infected cultures with ^{3}H-amino acids and assaying incorporation into acid-insoluble material. Results of these experiments are illustrated in Figure 6. No significant

Figure 4. A comparison of DNA synthesis in control and infected cultures of L-cells and normal WI-38 cells. Cells were infected with reovirus (L-cells 10 pfu/cell; WI-38 cells 100 pfu/cell), and rates of DNA synthesis determined as described in Materials and Methods. Radioactivity in acid-insoluble material is presented as counts per min at selected times after infection, for duplicate cultures.

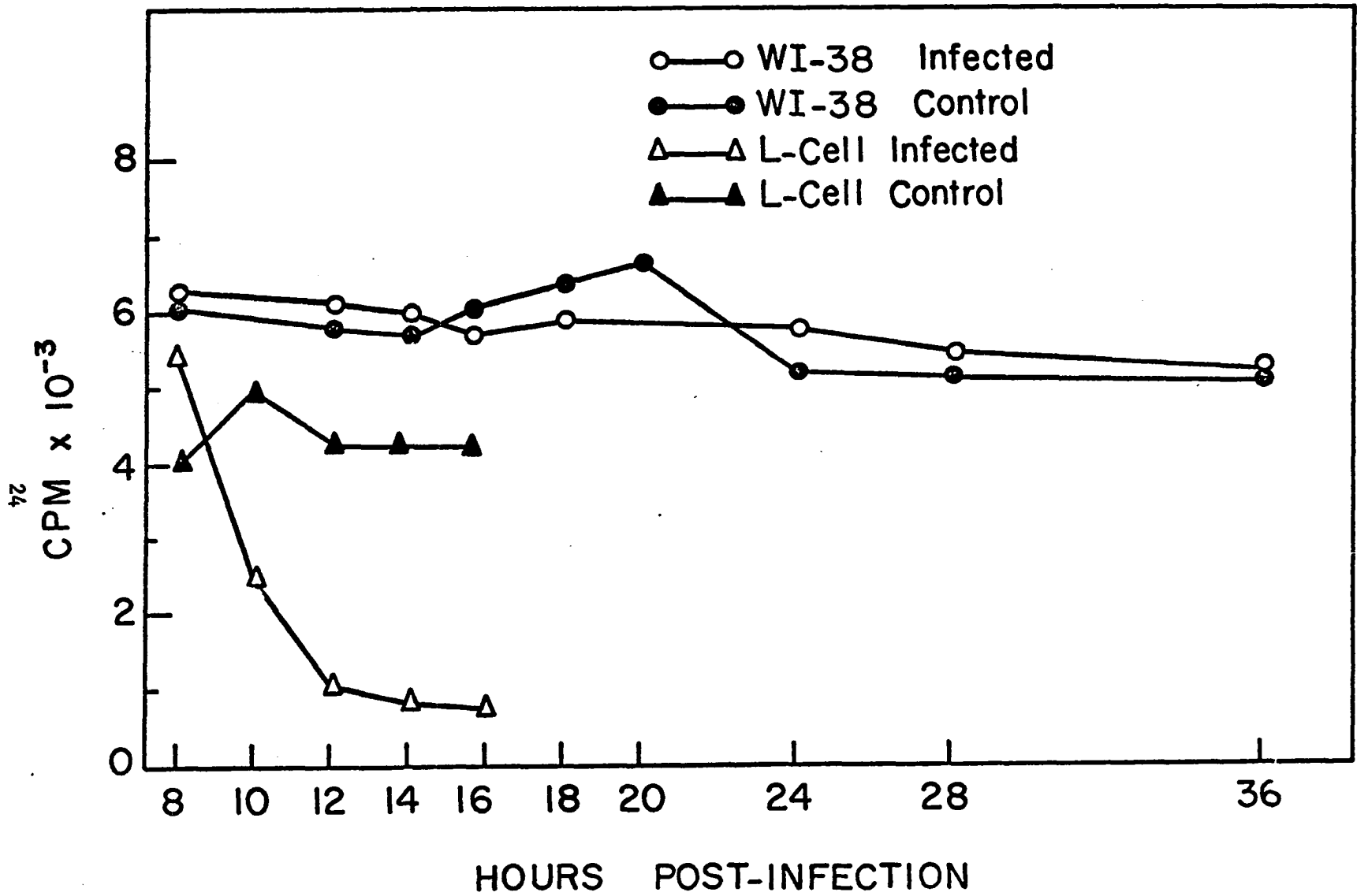
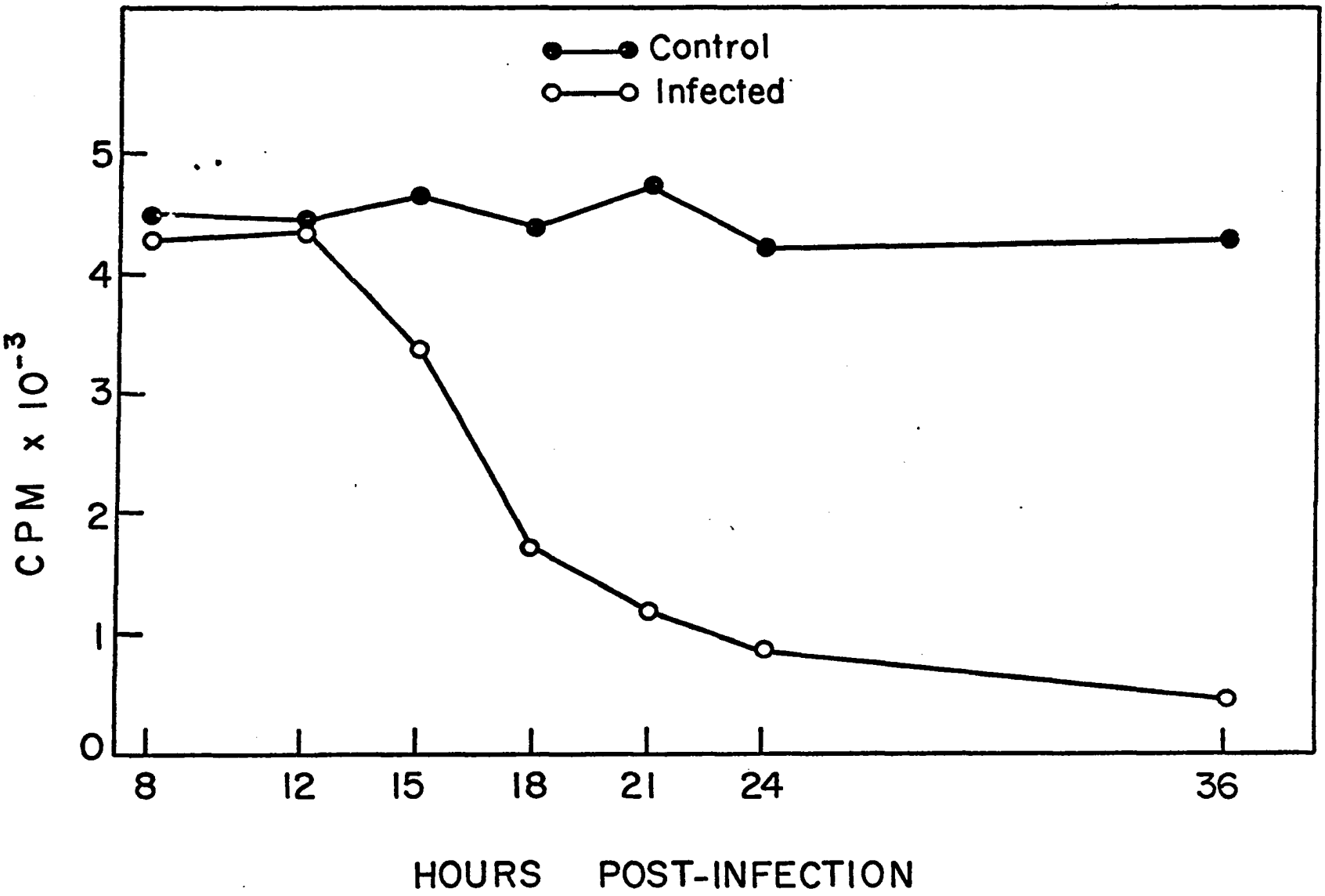

WI-38 Infected
WI-38 Control
L-Cell Infected
L-Cell Control
CPM x 10^-3
24
0
2
4
6
8
8
10
12
14
16
18
20
24
28
36
HOURS POST-INFECTION

Figure 5. DNA synthesis in control and infected (100 pfu/cell) transformed WI-38 cells. Acid-insoluble radioactivity is presented as counts per min at selected times after infection, for duplicate samples.


Control
Infected
CPM x 10^{-3}
0
1
2
3
4
5
8
12
15
18
21
24
36
HOURS POST-INFECTION

differences in the rates of total protein synthesis were detected in infected cultures of normal or transformed cells.

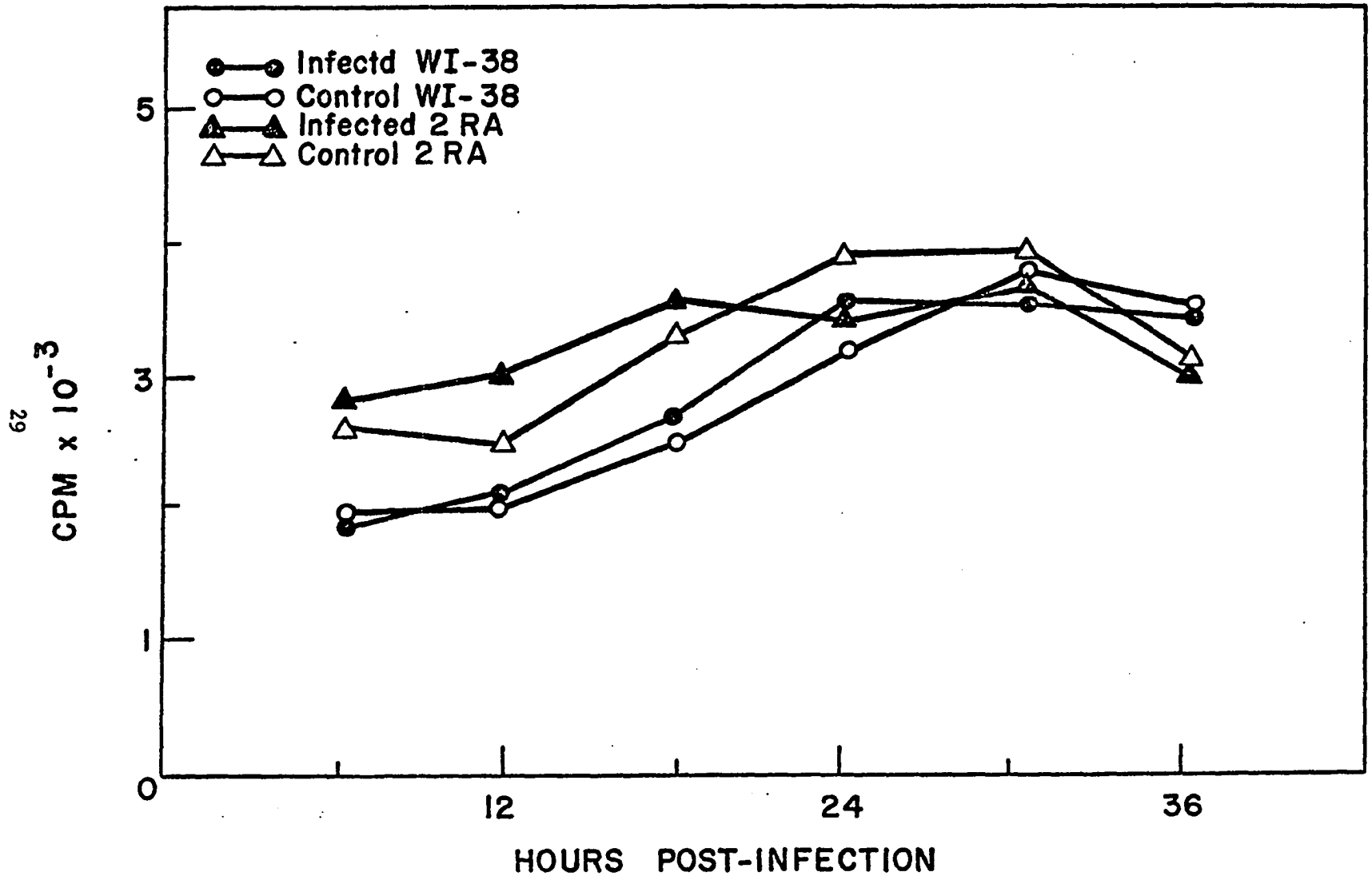
In infected L-cells, total protein synthesis began to decline, presumably due to cell death, by 12 h post-infection. This corresponded to a time when the inhibition of DNA synthesis was completed. It is of interest to note that at a time when DNA synthesis inhibition was completed in infected transformed WI-38 cells (36 h) total protein synthesis in these cells remained unaltered.

Infectious center assay. Because DNA synthesis in infected normal WI-38 cells was unaltered and these cultures showed little cytopathology, it was necessary to determine if a significant number of these cells actually became infected. It has been reported by other workers that cells in which reovirus had established a persistent infection were not all productively infected (38). It was possible, therefore, that only a small percentage of normal WI-38 cells became infected, and that these cells were just as sensitive to the infection in terms of DNA replication inhibition and cytopathology as the transformed cells.

Immunofluorescent staining of infected cells was performed to determine the percentage of cells which became infected and supported the synthesis of viral antigen. This technique, however, yielded inconsistent results in regards to the number of infected cells.

Infectious center assays, as described in Materials and Methods, were performed using normal and transformed infected cells. The results of these experiments indicated that almost 100% of the transformed cells were infected and released virus during the 7 days

Figure 6. A comparison of rates of total protein synthesis in infected and control cultures of normal and transformed (2RA) WI-38 cells. At the indicated times, duplicate cultures were pulse-labeled with ^{3}H-amino acids and assayed for incorporation into acid-insoluble material.


Infectd WI-38
Control WI-38
Infected 2 RA
Control 2 RA
CPM x 10^-3
0
1
3
5
12
24
36
HOURS POST-INFECTION

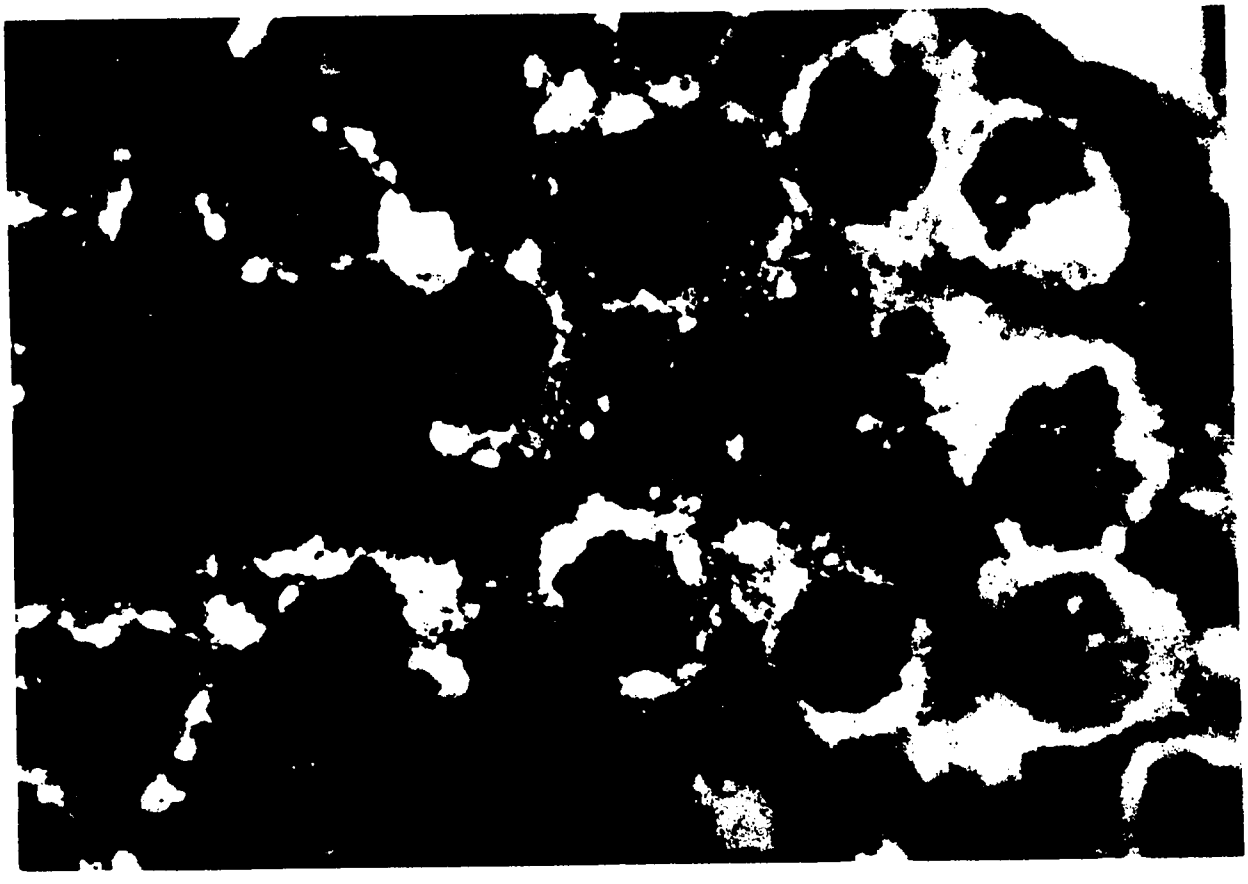
of the assay. Approximately 60% of normal cells were infected and released virus during the same period.

Immunofluorescent studies. Although the method of immunofluorescent staining was not used for the determination of the percentage of cells which actually became infected, this technique gave reproducible results regarding the development of viral antigen within the cells. These observations were important because of the possibility of differences in viral development in normal and transformed cells.

The development of viral antigen in infected L-cells, normal WI-38 cells, and transformed WI-38 cells was studied by fluorescent antibody staining at intervals after infection. Such studies have been performed using reovirus-infected L-cells and HeLa cells (12, 18, 46, 56, 57). Our results using infected L-cells were in agreement with those published. Viral antigen in these cells appeared in a finely particulate form by 2-4 h after infection. By 6-8 h post-infection viral "factories" appeared arranged in dense perinuclear inclusions. Figure 7 illustrates these perinuclear inclusions. The inclusions spread into the cytoplasm shortly before cell death. Because the perinuclear inclusions appeared at the time of the beginning of DNA synthesis inhibition, it has been proposed that an alteration of nuclear membrane could be responsible (17).

Viral antigen developed in normal and transformed WI-38 cells in a manner similar to that seen in L-cells. Dense inclusions did not appear in these cells until approximately 18 h post-infection, however. This observation corresponded with the slower production of progeny

Figure 7. Photograph of reovirus-infected L-cells stained with fluorescent antibody at 8 hours post-infection. Perinuclear arrangement of viral antigen is evident.



virus seen in these cells. Perinuclear inclusions were observed in the transformed cells by 18-20 h after infection. This is illustrated in Figure 8a. In normal WI-38 cells, however, no perinuclear inclusions were observed as late as 24 h post-infection (Figure 8b). Dense inclusion packets in normal cells were observed, but they remained scattered throughout the cytoplasm and even extended into narrow cellular processes.

Transformation of CF-3 cells by SV-40. To obtain other normal-transformed cell systems for future experiments, an attempt was made to transform human CF-3 cells with SV-40. These cells were infected with a high MOI with SV-40 and passaged identically to control cultures. Two days after infection, approximately 10% of the cells were lysed. After the debris was removed, no growth or morphological differences were observed in these cultures for a period of approximately 10 weeks.

During this period, the passage number of the cultures had increased by 7. After this time, sudden cell lysis was observed in the infected cultures. Cells in these cultures continued to lyse for 3-4 days. This corresponded to the "crisis" stage reported by Girardi in the SV-40 transformation of WI-38 cells (19).

The remaining cells began to proliferate at an increased rate and exhibited the following characteristics of transformed cells in culture: (1) loss of contact inhibition; (2) the cells grew quickly from a 1:5 split ratio using growth medium supplemented with only 5% FCS; and, (3) cells dispersed very quickly after the addition of trypsin.

Figure 8. Photographs of reovirus-infected transformed (a) and normal (b) WI-38 cells stained with fluorescent antibody at 24 hours post-infection. Viral antigen is in perinuclear arrangement in transformed cells, but scattered throughout the cytoplasm in normal cells.

a.

b.

High passage (54) and low passage (18) CF-3 cells were transformed. The transformation process required an identical time period for cells of each passage level. The crisis stage was observed on the same day in each passage level culture.

CHAPTER IV

DISCUSSION

The selective destruction of certain transformed cells by bovine enterovirus reported by Taylor et al. was shown to be caused by the inability of this virus to adsorb to the normal cells tested (58). Such a mode of differential infectivity might be expected because of membrane changes in transformed cells. In our experiments the efficiency of adsorption to normal WI-38 cells appeared very similar to that which occurred with transformed WI-38 cells, however. Adsorption to these cells was not as efficient as that reported for reovirus on L-cells in suspension culture (21, 33). The fact that both normal and transformed WI-38 cells were grown in monolayer, however, may account for this decrease in adsorption efficiency. The time of peak adsorption to normal and transformed WI-38 cells was the same as that observed for L-cells.

The synthesis of progeny reovirions in normal and transformed WI-38 cells followed a similar time course. The slow production of virus by both cell lines may be a characteristic of cells of human origin. Studies with reovirus type 1 infections of other human cell lines supported this possibility (48).

Reovirions in infected L-cells tended to remain cell-associated at a time when progeny formation had ceased (34). Release appeared

to depend in this case upon the complete disintegration of the cell. Because infected normal WI-38 cells appeared to release progeny virions at a time when no cytopathology was observable, a unique method of release was indicated. Reovirus type 1 has been shown to be released from human cells by a "bubbling" process in which large packets of virions were pinched off in areas of cellular membrane (57). If reovirus was released in a similar method from WI-38 cells, plaque assays from growth medium on infected cells might have yielded misleading results if large numbers of released virions were membrane-bound. To test this possibility, a fraction of growth medium from infected cultures of WI-38 cells was divided. One half was extracted with ½ volume of ether for 10 min, then diluted and assayed for infectious virus, while the other half served as a control and was assayed. The ether-extracted fraction did not show a titer above that of the control fraction, indicating that no infectious virions were membrane-bound. This experiment may have indicated, however, that "pinched off" membrane may not have completely enclosed the virions, or that the membrane quickly disintegrated after separation from the cell. It appeared that the method of release of reovirus from infected WI-38 cells may best be determined by electron microscopy.

The establishment of persistent latent infections by reovirus type 3 in human embryonic fibroblasts has been demonstrated by Bell and Ross (4). These experiments also showed a production and release of virus with no cytopathology, as long as the cells were actively dividing. Our observations of reovirus-infected normal WI-38 cells indicated the possibility of a slow, persistent infection in these cells.

Infected cultures (10 pfu/cell) were passaged for as long as 14 days with no observable cytopathology. If the infected normal WI-38 cultures were at low passage levels and growing rapidly, the growth rate of these cells was slowed by day 14 after infection, as compared to control cultures. The size of these cells also exhibited some decrease. However, the cells did not detach and still excluded trypan blue.

The establishment of persistent latent infections by reovirus was not always limited to normal cells, however. Levy et al. have reported that latent reovirus infections could be established in Burkitt's lymphoma cell lines, if the MOI was very low (38). The transformed cells used in our study, though, were lysed by 4 days after infection with reovirus.

Mouse L-cells have been used extensively in the study of the reovirus infectious cycle, and for the production of virus stocks, primarily because these cells supported a rapid synthesis of large numbers of progeny virions. The slow replication of this virus with minimal cytopathology in normal human cells, however, may more accurately reflect the virus' interaction with adult animals. Reovirus has been shown to be ubiquitous, commonly isolated from man and lower animals. The virus has never been associated with disease, however, in adult animals (50). Even very large inocula have failed to produce overt disease. Approximately 10^{10} pfu were periodically inoculated intranasaly into a guinea pig for the production of antiserum. The inoculated animal never exhibited symptoms of disease, even though reovirus reportedly replicates in the respiratory tract

of guinea pigs (50). In addition, experiments performed by Dr. M. Kollmorgan at the Oklahoma Medical Research Foundation testing the anti-tumor capacity of reovirus in mice, have shown that control animals inoculated with 10^{10} pfu intraperitoneally inhibited no adverse effects. These observations suggest that reovirus replication *in vivo*, like that *in vitro* in normal cells, caused little cytopathology.

The process by which reovirus inhibited the replication of cellular DNA remains unresolved. The use of infectious subviral particles in attempts to identify a viral component which could mediate this inhibition have not been successful (8). Also, various subcellular systems have been isolated from infected cells. These systems did not differ from those of control cells in their ability to participate in DNA synthesis *in vitro* (3).

Ensminger *et al.* have indicated that the inhibition of DNA synthesis in reovirus-infected cells may be caused by a decrease in the number of initiation sites (16). Inhibitors of protein synthesis have been shown to cause a similar type of inhibition of DNA replication. These and other observations (62) have led some workers to speculate that the inhibition of DNA synthesis may not be specific, and may be the result of a decreased level of cellular protein synthesis. It seemed reasonable, and has been suggested by other workers (17), that the dense perinuclear arrangement of viral antigen seen in reovirus-infected transformed cells could block or alter the nuclear membrane so that newly-synthesized proteins necessary for initiation of DNA synthesis would be prevented from entering the nucleus. Other "cytoplasmic factors" necessary for DNA synthesis (47) might also be

blocked from entering the nucleus. Several observations supported the possibility that perinuclear antigen might be involved in the inhibition of cellular DNA synthesis. First, perinuclear antigen was observed only in cells in which reovirus inhibited DNA synthesis (L-cells and transformed WI-38 cells). Second, the perinuclear arrangement, as seen with immunofluorescence, appeared at approximately the time of the beginning of the inhibition of DNA synthesis. Third, Bartkoski and Cox have shown that nuclei taken from reovirus-infected L-cells at a time when DNA synthesis is inhibited, synthesize DNA in vitro as efficiently as nuclei from control cells (3).

The observation that reovirus infection inhibits DNA synthesis in transformed WI-38 cells at a time when no inhibition is observed in normal WI-38 cells could introduce a useful tool in the study of the inhibitory process, as well as in the study of altered regulatory mechanism in transformed cells.

Because the efficiency of viral replication in normal WI-38 cells was similar to that seen in transformed WI-38 cells, it appeared that some basic differences in regulatory mechanisms concerning DNA synthesis must have existed in the transformed cells.

If the supposition is accepted that cessation of cellular protein synthesis was responsible for DNA synthesis inhibition in reovirus-infected cells, then altered regulation of protein synthesis at the ribosomal level could be indicated. It is possible that viral messenger RNA could be preferentially bound by ribosomes of transformed cells, while in normal cells the viral messenger had no such advantage. In this case, viral and cellular messenger in normal cells

would compete for ribosomes and this hypothesis could account for the slow production and release of virus, while normal cellular processes continued with little cytopathology, which was seen in the normal WI-38 cells. A ribosome "factor" which catalyses the binding of late adenovirus messenger RNA to ribosomes in SV-40 transformed cells has been reported (41). This study has shown that the corresponding normal cells did not produce this factor.

Whether or not the differential sensitivity to reovirus infection seen in normal and transformed WI-38 cells is characteristic of most normal-transformed cell systems remains to be determined. If transformation does render cells more susceptible to reovirus infection, the virus, or possibly a subviral component, could prove useful as an anti-tumor agent. In this respect, it will be of interest to determine if the adenine-rich oligonucleotides of reovirions selectively inhibit DNA replication in transformed cells. Also, the interaction of reovirus with cells transformed by agents or chemicals other than SV-40 would be of interest.

It is desirable that normal-transformed cell systems used for comparison studies be as closely related historically as possible. In this regard, the transformation of CF-3 cells in our laboratory was important for future experiments. The transformation of these cells by SV-40 generally corresponded to that reported with other human cells (19). However, some workers have reported a much quicker transformation in senescent, high passage cells whose growth rate had significantly slowed (32, 36, 61). Both high and low passage CF-3 cells required the same time for transformation to occur. The high passage

CF-3 cells, however, had not yet exhibited a drastic reduction in growth rate, which could account for this discrepancy.

CHAPTER V

SUMMARY

These experiments compared the growth of reovirus, and its effects in normal and SV-40 transformed WI-38 cells.

Reovirus adsorbed with equal efficiency to both cell lines. Viral replication in normal and transformed WI-38 cells was much slower than that which occurred in mouse L-cells. The eclipse phase in the human cells continued until 16 h post-infection. By 26 h after infection, the transformed WI-38 cells produced 2 to 3 times as much virus per cell as the normal WI-38 cells.

Cellular DNA synthesis in reovirus-infected transformed WI-38 cells began to be inhibited by 15-18 h after infection. At this time these cells exhibited no cytopathology and no inhibition of total protein synthesis. DNA synthesis in infected normal WI-38 cells remained unaltered as late as 36 h post-infection.

Cultures of transformed WI-38 cells were lysed by 96 h after infection with reovirus, while infected normal WI-38 cells showed no cytopathology for up to 14 days post-infection, although they continued to produce and release virus.

The development of viral antigen in normal and transformed WI-38 cells was observed by an immunofluorescent technique. Large

amounts of perinuclear antigen in infected transformed cells appeared at approximately the time of the beginning of DNA synthesis inhibition. No perinuclear antigen was observed in infected normal cells.

These results indicated that reovirus infection may selectively destroy transformed cells while establishing a slow persistent infection in normal cells. The data also suggest a role of perinuclear antigen in the inhibition of DNA replication in reovirus-infected transformed cells.

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