JOURNAL OF VIROLOGY, Dec. 1969, p. 872-878
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Vol. 4, No. 6
Printed in U.S.A

Effect of Cell Physiological State on Infection by Rat Virus

RAYMOND W. TENNANT, KENNETH R. LAYMAN, AND RUSSELL E. HAND, JR.
Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

Received for publication 22 July 1969

Infection by rat virus has been studied in cultures of rat embryo cells to evaluate the Margolis-Kilham hypothesis that the virus preferentially infects tissues with actively dividing cells. An enhancement of infection was seen in cultures infected 10 hr after fresh medium was added as compared to infection of stationary cultures (infected before addition of fresh medium). Since addition of fresh medium stimulates deoxyribonucleic acid (DNA) synthesis, the number of cells per culture synthesizing DNA at the time of infection was compared with the proportion of cells which synthesized viral protein. Cells were infected before the medium change and 10 or 24 hr after the medium change and were pulse-labeled with ^{3}H-thymidine at the time virus was added. The cells were allowed to initiate viral protein synthesis before they were fixed and stained with fluorescein-conjugated anti-rat virus serum. Fluorescence microscopy permitted both labels to be counted simultaneouly and showed that the greatest proportion of cells synthesizing viral protein were those which had incorporated ^{3}H-thymidine at the time of infection.

Rat virus (RV) is a minute (18 to 19 nm) deoxyribonucleic acid (DNA)-containing virus (3, 5, 18), which, in addition to size, is remarkable for the variety of pathological changes it can induce (4). Although these changes are minimal in the adult rat, infection of embryos or neonates can result in abnormalities of growth and development that reflect selective injury to proliferating tissues (5, 8–10). Effects of the virus in newborns include mongol-like congenital deformities, runting, peridontal disease, hepatitis, and cerebellar hypoplasia with ataxia. A detailed study of the cerebellar effects in particular led Margolis and Kilham (8) to conclude that RV has an affinity for actively dividing cells.

Presented here is a report of attempts to determine whether infection of embryonic rat cell cultures by RV is influenced by the physiological state of the cells at the time of infection. Since addition of fresh medium to confluent cultures stimulates physiological activity (including DNA synthesis and mitosis), we infected cultures before and about 10 hr after the medium change to test the effect of cell state. Infection was enhanced in the most active cultures, and we then attempted to ascertain whether infection is related to cell DNA synthesis. To determine cell DNA synthesis, the cultures were pulse-labeled with ^{3}H-thymidine (TdR) at the time virus was added and then were allowed to begin synthesizing viral protein in the absence of unincorporated ^{3}H-TdR before the cells were fixed. Fluorescence microscopy allowed the autoradiographically labeled cells to be seen simultaneously with cells stained with fluorescent anti-RV serum and showed a high correspondence between cells synthesizing DNA at the time virus was added and cells which synthesized viral protein.

MATERIALS AND METHODS

Virus and cell cultures. Strain 171 of RV was obtained from Lawrence Kilham (Dartmouth Medical School). All experiments were performed with samples from pools of virus prepared in primary and secondary cultures of Sprague-Dawley rat embryo cells. A pool of uninfected cells for mock infection of controls was prepared similarly. Cell cultures were prepared from 17- to 20-day decapitated embryos of rats reared and maintained in the Experimental Animal Facility in the Biology Division of the Oak Ridge National Laboratory. These rats were of cesarean-derived stock and were free from hemagglutination inhibitors to RV or Toolan H-1 virus. Trypsin-dispersed primary cell cultures were grown to confluence in Eagle basal medium in Earle saline solution (EBME) containing 10% bovine serum, 200 μg of streptomycin per ml, and 200 units of penicillin per ml. Cultures were plated in glass petri dishes at 2×10^5 to 5×10^5 cells/ml and were transferred after trypsinization to 60-mm plastic dishes (Falcon Plastic, Los Angeles, Calif.) for experiments. When appropriate, the cells were plated on glass cover slips in the plastic dishes. All plates were held in an atmosphere of 5% CO_2 and air at 37 C. Unless stated otherwise, the secondary cultures were con-

fluent (about 2×10^6 cells per plate) by 48 hr after plating and were used in experiments at that time.

Virus assay. Plaque assays were performed in Fischer strain embryo cells prepared either from rats obtained from Charles River Farms or in cell cultures purchased from Microbiological Associates, Inc. Confluent cultures were exposed to 0.2 ml of virus suspended in phosphate-buffered saline (PBS; *p*H 7.2) and incubated at 37 C for 2 hr. The plates were overlaid with 5 ml of medium containing equal volumes of 1.8% agar (Difco, Noble Agar) in distilled water and 2× EBME with 10% fetal bovine serum and 400 units of penicillin per ml, 400 μg of streptomycin per ml, and 100 units of polymixin B sulfate per ml. An additional 5 ml of the above medium was added to the plates 4 days later. Eight days after inoculation, they were overlaid with the same medium containing neutral red (final concentration, 1:36,000). Plaques generally were counted on the 10th or 11th day. (Although virus replication in Sprague-Dawley strain embryonic rat cells leads to extensive cytopathic effects, plaques do not develop. Conversely, we have found that plaques do develop in cells prepared from Fischer strain cells. Since the Sprague-Dawley rats are free from antibody to RV and since viral hemagglutinins are produced under the agar overlay, we have no explanation for the absence of plaque development in the Sprague-Dawley cultures.)

Preparation of antiserum. Antibody against RV was prepared in Sprague-Dawley rats by using RV-infected Sprague-Dawley embryonic rat cell cultures as the antigen. The virus was prepared as a 10× cell-pack (16) by scraping infected cells from plates, sedimenting by low-speed centrifugation, and disrupting the packed cells by sonic treatment. Rats were given an intraperitoneal injection (0.5 ml) of antigen, followed in 2 weeks by 0.5-ml injections, into each flank, of antigen mixed with an equal volume of Freund's complete adjuvant (Difco). Before exsanguination, the animals were test-bled, and the hemagglutination-inhibition antibody titer was determined against 8 hemagglutinin units of RV.

By using techniques described previously (12), this serum was found to be free from antibody to other indigenous murine viruses, including Toolan H-1 virus, which bears many similarities to RV and which, with minute virus of mice, probably belongs to the same taxonomic group, named the picodnaviruses by Mayor and Melnick (11); pneumonia virus of mice; Theiler's GDVII; polyoma; Sendai; reovirus type 3; mouse adenovirus; and mouse hepatitis viruses.

Fluorescent-antibody staining. The antiserum was conjugated with fluorescein isothiocyanate (Sylvana Co., Milburn, N.J.). Before use, the conjugate was absorbed with powdered extract of whole, normal rat embryo. The powdered extract was prepared by homogenization of embryos followed by three extractions with acetone, air-drying, and pulverization. The absorption was performed by mixing 1 g of powder for each 5 ml of conjugate, incubating the mixture at 37 C for 1 hr, and collecting the fluid after centrifugation at $800 \times g$ for 20 min to remove debris.

For assay of infected cells by the fluorescent-antibody technique, cell cultures were grown on acetone-washed cover slips. At intervals after infection and pulse-labeling with ^{3}H-TdR as indicated below, the cultures were washed with PBS and fixed with cold acetone at room temperature for 20 min. The cover slips were air-dried at room temperature and then stored at −20 C. The cover slips were stained on plastic racks in a moist chamber (Bellco Glass Co., Vineland, N.J.). After incubation at 37 C for 1 hr, the samples were gently washed for 10 min at room temperature in an automatically agitated bath of PBS. The preparations were then washed for 2 min in distilled water and held in 95% ethyl alcohol for 20 min at room temperature to fix the conjugate (7).

Autoradiography. For autoradiography, the coverslip cultures were given a 0.5- or 1-hr pulse of ^{3}H-TdR (0.5 or 1 μc/ml) in basal medium (Eagle) with Hanks balanced salt solution (H-BME) at various intervals after infection. The nucleotide precursor had a specific activity of 2 c/mmole and was purchased from the New England Nuclear Corp., Boston, Mass. The washing of cells and fixation in cold acetone used for the fluorescent-antibody technique are also effective in removing the unincorporated precursor from the cell pool, since grain clusters are confined to nuclei.

Infected cells pulse-labeled with ^{3}H-TdR and exposed to fluorescent antibody were prepared for autoradiography by mounting cover slips with the cell layer upright on glass slides with a drop of immersion oil underneath as "mounting medium." The photographic emulsion was Kodak NTB-2 diluted 1:2 with distilled water. The slides were dipped in the emulsion, allowed to drain, and then placed in a horizontal position for exposure. The radioautographs were developed after 1 to 3 days. Cells with clusters of 10 or more grains in the region of the nucleus were considered to be labeled, and at least 1,000 cells distributed over 10 fields on the slide were counted.

Fluorescence microscopy. Conjugate and ^{3}H-TdR-labeled nuclei were counted under ultraviolet light with a light microscope (Zeiss) by using a BG12(3-mm) exciter filter and alternately 410-, 440-, or 500-nm barrier filters. Although both fluorescent nuclei and reduced silver grains were visible with the 440-nm filter, the 410-nm filter permitted more accurate counting of all ^{3}H-labeled cells.

Analysis of DNA synthesis. The synthesis of DNA was also determined by following the incorporation of ^{3}H-TdR into trichloroacetic acid-insoluble precipitate by using the method of Regan and Chu (13). Infected cells were exposed to a 1-hr pulse of ^{3}H-TdR (1 μc/ml) in prewarmed H-BME. The medium was aspirated, and the cells were scraped immediately into 1 ml of PBS and quick-frozen. To determine the level of incorporation, the thawed samples were disrupted for 1 min in a Raytheon sonic oscillator (200 w, 10 kc). After disruption of cells, 100-μliter samples were plated on filter paper discs (Whatman; 3 mm by 2.3 cm). To determine trichloroacetic acid-insoluble counts, the discs were washed three times in cold 5% trichloroacetic acid, once with 95% ethyl alcohol and with acetone and then were air-dried. Radioactivity was determined by immersing the discs in toluene containing 5 g of 2,5-diphenyloxazole per liter and by counting in a Beckman scintillation spectrometer. All

samples were counted sufficiently to ensure a counting error of not more than 5%. Protein content of the samples was determined by the method of Lowry et al. (6).

RESULTS

Infection as a function of cell state. In the first experiments, we wanted to determine whether the physiological state of the cell cultures at the time of infection is related to their ability to initiate virus synthesis. A comparison was made between the infection of stationary-state, embryonic-rat secondary cultures and replicates of the same culture infected at intervals after the addition of fresh medium to stimulate cellular physiological activity.

We consider rat embryo cell cultures to be in a stationary state by 48 hr after subculturing (cell density about 2×10^6 cells/ml), when the level of DNA synthesis is constant or decreasing. The stimulation of cell physiological activity after addition of fresh medium was evidenced by an increase in the rate of DNA synthesis (Fig. 1) and subsequent cell division. Consequently, stationary-state cultures with cells grown on cover slips were divided into three groups. The first group (A) was infected with RV [about 15 plaque-forming units (PFU)/cell] before the addition of fresh growth medium. After the adsorption period (1 hr), fresh medium was added and the cultures were held for 13 hr before the cells were fixed. The other two groups (B and C) were given fresh medium at the time the cells in group A were infected. At 10 hr after the medium change, the group B cultures were infected with RV as above and fixed 13 hr thereafter. The group C cultures were treated similarly 24 hr after the medium change; the cells were fixed 13 hr after infection. The respective cover slips were stained with fluorescein-conjugated anti-RV serum, and the proportion of cells synthesizing viral protein was determined.

The cultures were held for 14 hr after infection, before the cells were fixed on the basis of the results of a separate experiment, the results of which are shown in Fig. 2. This figure presents the pattern of RV protein synthesis, detected with the fluorescein-conjugated anti-RV serum, in confluent cultures. Cells synthesizing viral protein (presumably primarily capsid protein) could first be detected by 9 hr after infection, and the number began to plateau by 21 hr. The fluorescence was confined to the nucleus throughout the infectious cycle. Therefore, the 14-hr period was chosen as the interval to fix the cells in the cell state experiment, since it represented an early period in the replication cycle of RV when many cells synthesizing viral protein could be detected. Further, it represented a period when cells which immediately began to synthesize viral protein after infection would be preferentially detected.

As shown in Table 1, the efficiency of infection was lowest in cells in the stationary state (group A) at the time virus was added. The highest efficiency was seen in cultures infected 12 hr after the medium change (group B). Those infected 24 hr after the medium change were in an intermediate position. These effects are not nutritional in the strict sense (that is, absence of precursors), since fresh medium was added to all cultures after the

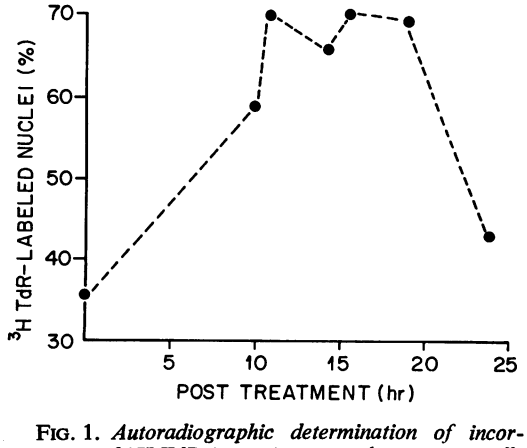

FIG. 1. *Autoradiographic determination of incorporation of ^{3}H-TdR in stationary embryonic rat cells after addition of fresh medium.*

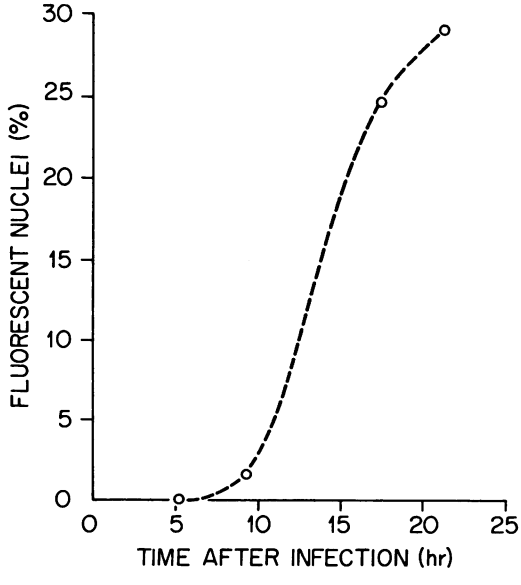

FIG. 2. *Synthesis of RV protein after infection of stationary embryonic rat cells.*

virus adsorption period. These results, therefore, indicated that infection by RV might be related to some cellular synthetic function(s).

Viral protein synthesis as a function of cell DNA synthesis. To assess RV infection as a function of cellular synthesis, we chose to monitor DNA synthesis because it is a discrete event in the cell cycle which can be detected with relative ease. It seemed possible to follow cellular DNA synthesis by giving the cells a pulse-label of ^{3}H-TdR at the time of infection, since it was quite unlikely that viral DNA synthesis could be initiated. To determine whether the labeled precursor was probably incorporated only into cell DNA, an experiment was done to follow the fate of the label. Confluent secondary cell cultures were divided into two groups: group I was given ^{3}H-TdR immediately, and group II was given fresh medium and then the precursor 12 hr later. Both cultures were infected after the pulse period; virus was allowed to adsorb, and the cells were washed once with PBS before fresh medium was added. Cultures were harvested (i) immediately after the pulse and (ii) after infection and the PBS wash. Although some soluble precursor remained in the cell pool at the end of the pulse period, the soluble pool was depleted within 2 hr (Table 2). The percentage of ^{3}H-labeled nuclei, likewise, was the same in both groups. Since it is unlikely that much viral DNA is synthesized within 2 hr after adsorption, the ^{3}H label should be a valid indication of cell DNA synthesis.

TABLE 1. *Efficiency of infection at periods after addition of fresh medium*

Time infected	Group	Fluorescent cells/total cells counted	Fluorescent cells
			%
Before medium change	A	38/770	4.9 ± 2[a]
At 10 hr after medium change	B	260/889	29 ± 3
At 24 hr after medium change	C	277/1,823	15 ± 2

[a] Standard error of the mean: the differences between A/B and B/C are statistically significant at a probability of 0.01.

TABLE 2. *Evidence for incorporation of the ^{3}H-TdR pulse into cell DNA only*

Group	Time after addition of pulse (0.5 hr)	Trichloroacetic acid-insoluble counts/min (μg of protein)	Soluble counts/min (μg of protein)	^{3}H-labeled cells
				%
A	Immediately after pulse	451	85	23
	After virus adsorption (2 hr)	664	0	24.5
B	Immediately after pulse	236	76	22
	After virus adsorption (2 hr)	383	3	22

To determine whether viral protein synthesis occurs preferentially in cells synthesizing DNA at the time of infection, an experiment was performed with infected cells labeled with ^{3}H-TdR at the time virus was added. Secondary cultures were planted, and when the cells were confluent (48 hr later) they were divided into two groups. After the used medium was removed, the group A cultures, which represented a stationary level of cell synthetic activity, were infected with RV (about 15 PFU/cell) and held for adsorption. The cultures were then given a 1-hr pulse of ^{3}H-TdR (3 μc/plate), washed with PBS, and then given fresh medium. Twelve hours later the cells were fixed. At the time the A group cultures were inoculated, the group B cultures, which represented actively synthesizing cells, were given fresh medium and held for 10 hr to increase the level of DNA synthesis. The group B cultures were then infected, pulse-labeled, washed, and given fresh medium; these cells were also fixed 12 hr after infection. After all respective cover-slip cultures were harvested, the cells were stained with fluorescein-labeled anti-RV serum and then processed for autoradiography. They were then examined by fluorescence microscopy to determine the proportion of cells synthesizing DNA at the time of infection simultaneously with those which subsequently synthesized detectable viral protein.

Figure 3 shows the fluorescent staining and ^{3}H-labeling processes viewed separately and simultaneously. In frame *a*, nine nuclei are distinctly stained with fluorescent antibody. The same cells in frames *b* and *c* show the ^{3}H label. Nucleus number 1, for example, is labeled with both flourescent antibody and ^{3}H, whereas the cells on either side are not stained with antibody but are lightly labeled with ^{3}H. In the case of nucleus number 2, the fluorescent stain is apparent but, as shown in frame *c*, the ^{3}H label is absent. Nucleus number 2 is the only fluorescent nucleus in this field without the ^{3}H label. Results of the counting are tabulated in Table 3 and support the following conclusions. (i) A 1.4-fold increase in cells synthesizing DNA (1.8-fold increase in total incorporation; B/A) at the time of infection resulted in about a 6-fold increase in cells synthesizing viral protein (B/A). (ii) Approximately 95% of the

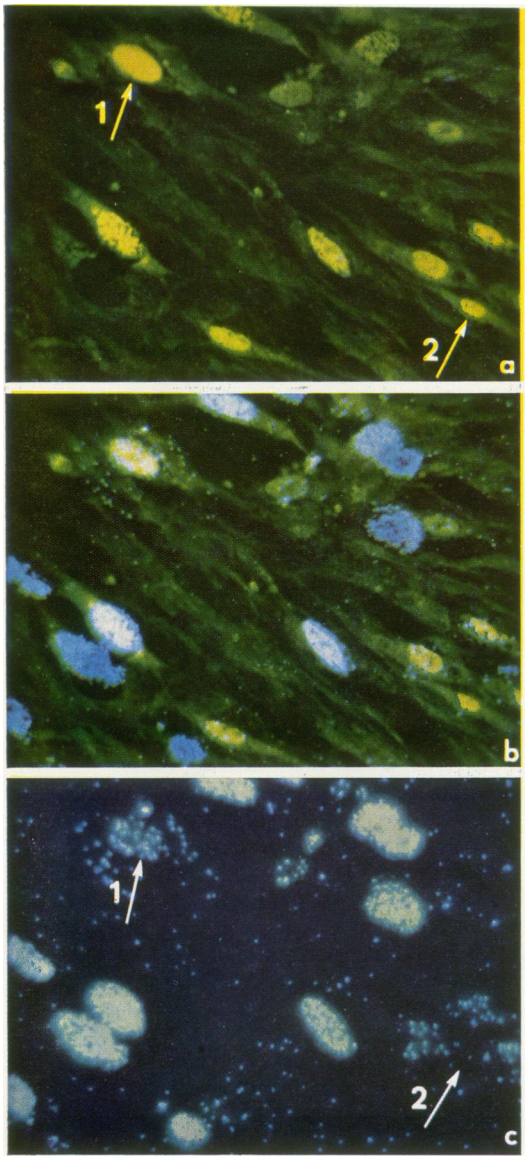

FIG. 3. *Simultaneous fluorescence microscopy of RV-infected cells stained with fluorescein-conjugated anti-RV serum and cells pulse-labeled with ^{3}H-TdR. Cells were pulse-labeled with ^{3}H-TdR at the time of infection and fixed and stained with fluorescent antibody 14 hr after infection. Frame a shows cultures seen with the 500-nm barrier filter; the cells are labeled with fluorescent antibody. Frame b shows cultures seen with the 470-nm filter; both the ^{3}H- and antibody-labeled cells are seen. Frame c (no filter) shows only cells labeled with ^{3}H-TdR.*

cells synthesizing viral protein were those which had incorporated ^{3}H-TdR at the time of infection. Although not all cells incorporating ^{3}H-TdR at the time virus was added became infected, the vast majority of cells which became infected were those which were synthesizing DNA. The high proportion of cells which did not become infected, even at a multiplicity of 15 PFU/cell, probably represent a high proportion of cells in these cultures not susceptible to RV. Of those which are susceptible, the efficiency of infection is then influenced by some cellular functions.

To determine more conclusively that the ^{3}H label represents cell and not viral DNA synthesis, an experiment was performed in which the pulse was added before infection. The experimental design was the same as above; that is, group A received the pulse-label and virus before the medium change, whereas group B was pulse-labeled and then infected at 12 hr after the addition of fresh medium. A 1.2-fold increase (B/A) in the number of cells synthesizing DNA at the time of infection resulted in a 1.6-fold increase in cells stained with fluorescent antibody (Table 4). We cannot readily explain the higher percentage of cells synthesizing viral antigen in this experiment as compared to that shown in Table 3, since the overall level of DNA synthesis was about the same in both experiments. It is possible, however, that this group of primary cultures contained a larger proportion of susceptible cells. In this experiment (Table 4), 8% of the cells in group A stained with fluorescent antibody in the absence of the ^{3}H label, and, in group B, about 29% were fluorescent without the ^{3}H label. However, since more cells were entering DNA synthesis at 12 hr after the medium change (Fig. 2) and since the 0.5-hr pulse was given before infection, it would be expected that a large proportion of cells would begin DNA synthesis after the pulse was removed. Further, the difference between the fluorescent and ^{3}H-labeled cells and the fluorescent cells without the ^{3}H label was significant, with a probability of 0.01 (i.e., such an event might occur by chance only in 1 of 100 observations). Therefore, these data support the conclusion that the increased efficiency of infection of cultures active in synthesis may be related to some event associated with cell DNA synthesis.

DISCUSSION

Margolis and Kilham (8, 9) studied some pathological effects of RV in neonatal hamsters and concluded that the pronounced cerebellar hypoplasia, involving extensive cell destruction, resulted from the preferential infection of actively dividing cells. The cerebellum was the principal area of neural tissue in which development, involving extensive cell division, was taking place at this postpartum period. In other studies with H-1 virus (15), it was shown that susceptibility to

TABLE 3. *Viral protein synthesis as a function of cell DNA synthesis*

Time infected	Group	^{3}H-labeled cells		Fluorescent cells[a]				Mean counts/min of ^{3}H-TdR (μg of protein)
				With ^{3}H[b]		Without ^{3}H		
		Total count[d]	Per cent	Total	Per cent	Total	Per cent	
Before medium change	A	1,704/3,842	44 ± 2[c]	15/704	2 ± 0.7	1/704	0.1 ± 0.1	823
At 10 hr after medium change	B	2,165/3,539	61 ± 4	110/879	12.5 ± 1	7/879	0.7 ± 0.2	1,484
At 24 hr after medium change	C	1,431/3,603	40 ± 2	73/807	9 ± 0.8	2/807	0.3 ± 0.3	869

[a] No fluorescent nuclei were ever seen in the control cultures, although thousands of cells were examined.
[b] Probability A > B = 0.01; B > C = 0.02.
[c] Standard error of the mean.
[d] Number of cells with label/total cells counted.

TABLE 4. *Viral protein synthesis in cells pulse-labeled before infection*

Time infected	Group	^{3}H-labeled cells		Fluorescent cells			
				With ^{3}H		Without ^{3}H	
		Total count[a]	Per cent	Total	Per cent	Total	Per cent
Before medium change	A	805/1,531	52.5 ± 3	189/1,045	18 ± 2	15/1,045	1.4 ± 0.8
At 10 hr after medium change	B	1,793/3,196	56.5 ± 2	369/1,252	29 ± 3	106/1,252	8 ± 1
At 24 hr after medium change	C	1,691/3,320	51 ± 3	128/768	17 ± 4	26/768	3 ± 0.1

[a] Number of cells with label/total cells counted.

virus-induced hepatitis in neonatal rats could be induced in resistant adult rats by partial hepatectomy and attendant reparative cell division.

The studies reported here attempted to pursue the Margolis and Kilham hypothesis by examining the interaction of RV and embryonic rat cells in culture. We attempted to establish first whether a relationship exists between viral infection and the state of the cells at the time they are exposed to virus. Evidence was obtained that infection of cells at a period after addition of fresh medium, when an increased proportion of cells are synthesizing DNA, results in a larger number of cells synthesizing viral protein. Therefore, it appears that infection of cells at certain periods of the cell cycle at least decreases the time required to initiate viral protein synthesis. Stated in another manner, it appears that a function associated with some stage of the cell cycle participates in the replication of RV. Support for this hypothesis was obtained in the experiments in which we attempted to measure viral protein synthesis as a function of cell DNA synthesis occurring at the time of infection. These experiments were based upon the assumption that a label of ^{3}H-TdR given at the time virus is added, when analyzed autoradiographically, would be specifically indicative of cell DNA synthesis. Although we have shown no data on the kinetics of viral DNA synthesis, we feel that this is a valid assumption for the following reasons. (i) The replication cycle of RV, based upon staining of cells for viral protein, shows that most of the synthesis occurs between 12 and 18 hr after infection. (ii) The pulse-labeled precursor is effectively incorporated within 2 hr after the precursor is removed, so that no soluble label is available for incorporation into any viral DNA synthesized between 1 and 2 hr after the pulse. (iii) It is possible, but quite unlikely, that any appreciable viral DNA is synthesized within 2 hr after infection. If viral DNA synthesis was initiated in this time period, an increase in the number of cells labeled in this period would be expected, as tested in the experiment shown in Table 2, but no increase was observed. (iv) It is unlikely that any of the labeled precursor was reutilized, since in other experiments (R. W. Tennant, *unpublished observations*) no evidence was obtained for the degradation of cell DNA after infection with RV.

On the basis of experiments in which cells were pulse-labeled at the time of infection and sub-

sequently examined for synthesis of viral protein, we found evidence that RV preferentially infected cells that were synthesizing DNA. Although these experiments do not permit a distinction between a causal or merely an incidental relationship between cell DNA synthesis and viral protein synthesis, they do show a clear association between RV infection and the cell physiological state. It is possible that these effects are related to the replication of RV DNA, since there is evidence that the DNA of RV (14), and the similar minute virus of mice (1, 2), is a single-stranded molecule. In the case of the small, single-stranded DNA bacterial viruses, in which the amount of information potentially contributed by these viruses for the replication process is also quite limited, evidence has been presented for the involvement of host cell functions (17). First, the frequency of recombination between phage S13 mutants has been shown to be affected by host mutations affecting bacterial recombination. Second, the conversion of the single-stranded DNA ring of ϕX174 to a double-stranded replicative form appears to be performed by preexisting host cell enzymes, and the replicative form must become associated with a specific cellular site. Third, by use of Bonhoeffer mutants, it has been shown that host functions have a role in replication of the replicative form. The studies which we have reported provide a basis for investigating similar interactions between mammalian cell functions and the replication of the mammalian single-stranded DNA viruses. Such studies may also provide new information on cellular synthetic activities.

ACKNOWLEDGMENTS

We thank Conrad B. Richter for guidance in photography, Paul Nettesheim for advice on the fluorescence microscopy technique, and William Gude for preparation of the autoradiograms.

This investigation was supported by the U.S. Atomic Energy Commission under contract with the Union Carbide Corp.

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