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Multiplication of Polyoma Virus

I. Use of Selectively Labeled (H^3) Virus to Follow the Course of Infection

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The course of polyoma infection in susceptible rodent cells was previously investigated by electron microscopy (Bernhard, Cancer Res. **20**:712, 1960), fluorescence microscopy (Sachs and Fogel, Virology **11**:722, 1960), and acridine orange staining (Williams and Sheinin, Virology **13**:368, 1961). The data presented in this publication introduce autoradiography for observing the process of polyoma infection in primary mouse embryo cultures. This technique involves the selective labeling of the polyoma virus. In one instance, the protein coat was labeled with H^3-valine; in the other, the nucleic acid was labeled with H^3-thymidine. These labeled viral preparations were used separately to follow the course of viral infection by autoradiography.

The respectively labeled virus was prepared in primary mouse embryo cultures which were prelabeled for 18 hr (3 μc/ml of medium), infected with the wild-type polyoma virus (obtained from S. Stewart, National Institutes of Health), and postlabeled with the respective isotopes (5 μc/ml of Eagles medium with 10% dialyzed horse serum). The cells were harvested when extensive cellular degeneration was observed. Virus was liberated from the infected cells by the method of Crawford (Virology **18**:177, 1962), followed by centrifugation at 8,000 $\times$ g in a Sorvall (SS_3 superspeed) centrifuge for 30 min to remove the cellular debris. The infected tissue culture fluid was concentrated by centrifugation in a Spinco (model L, SW 25 rotor) centrifuge at 22,000 rev/min for 3 hr. The viral pellet was resuspended (5% of original volume) in Hanks balanced salt solution and treated with deoxyribonuclease (30 μg/ml), ribonuclease (30 μg/ml), $MgCl_2$ (10^{-3} M), and trypsin (0.1 mg/ml) for 30 min at 37 C. The enzymes were removed by centrifugation at 35,000 rev/min in the Spinco (SW 39 rotor) for 3 hr. The viral pellet was resuspended in Hanks balanced salt solution (1% of original volume), and was dialyzed against Hanks balanced salt solution for 10 hr to remove unincorporated extraneous label.

The respective virus preparations obtained

TABLE 1. *Course of polyoma infection as determined by use of selectively labeled polyoma virus*[a]

Time	H^3-thymidine polyoma				H^3-valine polyoma			
	Cytoplasmic		Nuclear		Cytoplasmic		Nuclear	
	Cells with grains	Avg no. of grains	Cells with grains	Avg no. of grains	Cells with grains	Avg of no. grains	Cells with grains	Avg no. of grains
hr	%		%		%		%	
3	33	15	0	0	30	10	0	0
6	32[b]	12	7	6	30	9	0	0
9	34	10	15	8	35	7	0	0
12	20	7	22	12	32	8	0	0
15	7	4	33	12	30	4	0	0
18	4	3	40[c]	18	30	4	4[d]	5
21	3	3	38[d]	18	30	5	6[d]	5
24	11[e]	7	30	6	28[e]	8	5[d]	3
27	14	10	25	4	30[e]	18	5[d]	3
33	15	8	12	3	38[e]	20	8[d]	4

[a] Averages obtained on the basis of 200 cells counted.
[b] Many grains perinuclear.
[c] Mixture of large and small grains in the nucleus.
[d] Some small grains in the nucleus.
[e] Small grains observed in the cytoplasm.

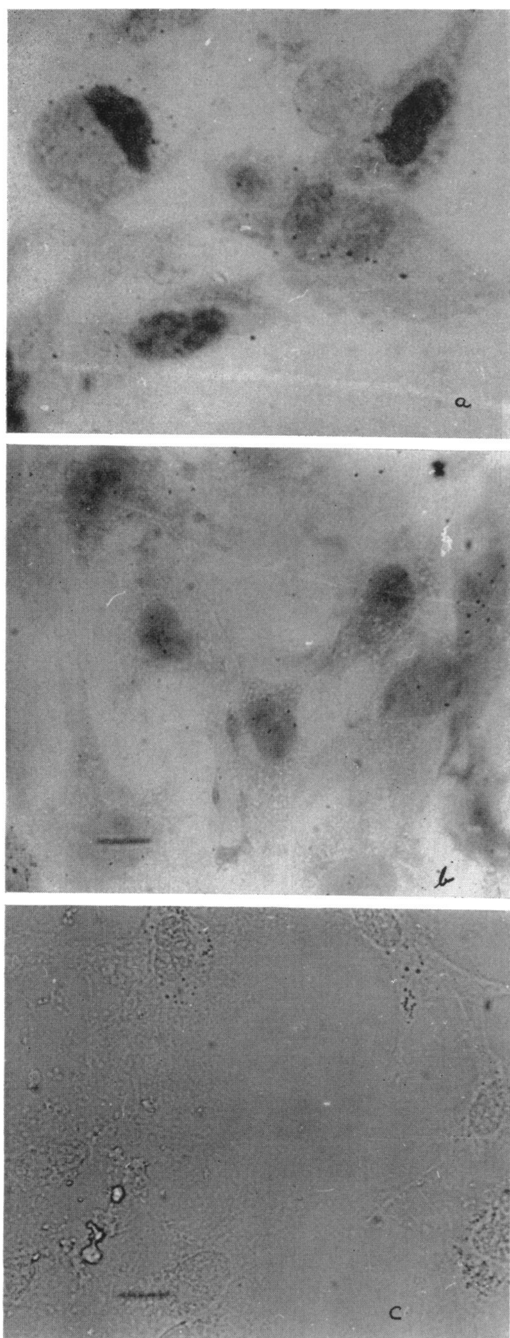

FIG. 1. (*a*) *H_3-thymidine-labeled virus 9 hr postinfection, demonstrating intracytoplasmic and intranuclear grains. Stained with hematoxylin and eosin.* × *675.* (*b*) *H^3-thymidine-labeled virus 18 hr postinfection, demonstrating predominance of intranuclear grains. Stained with hematoxylin and eosin.* × *675.* (*c*) *H^3-valine-labeled virus 15 hr postinfection, demonstrating intracytoplasmic grains. Unstained.* × *500.*

were used to infect sparse primary mouse embryo cultures which had been previously grown on cover slips (22 mm^2) in Falcon petri dishes (35 by 10 mm). The cultures were infected with 500 plaque-forming units (PFU) per cell, with a specific activity of 12 counts per min per PFU. The virus was adsorbed at 37 C in a humidified CO_2 incubator for 3 hr. The unadsorbed virus was removed by washing three times with Hanks balanced salt solution, followed by the addition of Eagle's medium supplemented with 4 × vitamins, 4 × amino acids, and 10% horse serum, and was again incubated in the CO_2 humidified incubator. The respectively infected cultures were harvested sequentially, at 3 hr postinfection and every 3 hr thereafter for 33 hr. Cultures so harvested were washed six times in phosphate-buffered saline (0.01 M PO_4, *p*H 7.2, in physiological saline), air-dried, fixed in acetone for 5 min, and stored at 4 C. The dipping-coating method of Messier and Lebland (Proc. Soc. Exptl. Biol. Med. **96:**7, 1957) for autoradiography was followed. Eastman Kodak NTB nuclear track bulk emulsion was employed, and autoradiographs were developed in Microdol-X. The preparations were stained with hematoxylin and eosin for photography.

When H^3-thymidine-polyoma virus autoradiographs were examined, it was observed (Table 1) that the viral particles pursued the following course of infection. Grains were observed exclusively in the cytoplasm at 3 hr postinfection; at 6 hr, they were predominantly found at the nuclear membrane, with an initial observation of grains present in the nuclei. With the progression of time during infection, the location of the grains proceeded from the cytoplasm to the nucleus (Fig. 1a). This event was optimal at 18 hr (Fig. 1b), when 40% of the nuclei contained grains. After 24 hr, it was noticed that the grains reappeared in the cytoplasm (11%). However, it was also observed that the size of these grains appeared less discernible and smaller than those observed previously during the earlier phase of infection. Other experiments (*to be published*) indicated that these are the completed progeny virus re-entering the cytoplasm for subsequent release.

However, when the course of H^3-valine virus was investigated, a completely different picture was obtained (Table 1) in that the grains were found exclusively in the cytoplasm or in the juxta-nuclear position but never inside the nucleus from 3 to 15 hr after infection (Fig. 1c).

During this period, grains were observed to decrease in size and subsequently in number, indicating viral eclipsing. Loss of specific viral antigenicity was also observed by the loss of specific immunofluorescence during this interval (*unpublished data*). Subsequently, from 18 to 21 hr after infection, minute grains became visible in the nucleus, indicating the breakdown of the previously labeled protein coats and recycling of the H^3-valine into the formation of new viral antigens (*unpublished data*). At 24 hr after infection, minute grains were observed both in the cytoplasm and in the nucleus, indicating the release of completed virus. This was substantiated by the fluorescent-antibody technique and will be reported in a subsequent publication.

From these experiments it becomes evident that polyoma uncoating occurs in the cytoplasm at 6 hr or shortly before, as determined by following the course of H^3-thymidine-labeled virus entering the nucleus (DeHaven et al., Federation Proc. **24**:309, 1965) along with exclusion of H^3-valine-labeled virus in the companion culture. It was also demonstrated that the nucleus was the site of polyoma synthesis. Further experiments with the above technique will be presented in a subsequent publication

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