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Fate of Labeled Polyoma Virus DNA in Cell Cultures* (33811)

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The use of bacteriophage, labeled by radioactive tracers, has proven to be an efficient tool in determining the fate of viral components in a bacterial host (1, 2). This technique has been applied more recently to the determination of the intracellular fate of isotopically labeled polyoma virus capsid and nucleic acid employing autoradiography (3). The early events in adenovirus type 5 infection of HeLa cells have been studied employing ^{14}C and ^{3}H labeled capsid protein and deoxyribonucleic acid (DNA) (4). We are reporting the results of experiments designed to determine the fate of purified polyoma virus containing isotopically labeled DNA in infected cells by detecting radioactivity in specific fractions of the cells. This study employed primary mouse embryo tissue culture (METC) cells, the majority of which undergo a productive infection (5,6) and BHK/21 cell cultures, in which very few of the cells produce viral antigen (7).

Materials and Methods. Virus. The SE polyoma virus was donated by Dr. Bernice Eddy and was serially propagated in monolayer cultures of primary METC cells. The cells were established from 10 to 15-day-old mouse embryos and were propagated in a growth medium containing medium 199 supplemented with 10% fetal calf serum (FCS). The maintenance medium contained medium 199 supplemented with 2% FCS. The virus was harvested from infected cultures employing receptor-destroying enzyme (RDE) according to the method of Crawford (8). Virus assay was performed by the tube hemagglutination test using washed 0.5% guinea pig erythrocytes. Titrations were incubated over-

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night at 4° and virus titers were expressed as hemagglutining units (HAU) per milliliter.

Labeling. Labeled polyoma virus was prepared by propagating the virus in monolayer cultures of primary METC cells in the presence of tritiated (^{3}H) thymidine, which was added to the medium at the time of infection. The ^{3}H thymidine, which was labeled in the methyl position, had a specific activity of 6.0 Ci/mmole and was used at a concentration of approximately 10 μCi/10^6 cells. At the time of maximum cytopathic effect (CPE), which was 5–7 days after infection, the labeled virus was harvested as previously indicated.

Purification. The virus preparation was purified by successive cycles of adsorption to and elution from guinea pig erythrocytes. This procedure was performed by adding the RDE harvested virus to 4% guinea pig erythrocytes in a proportion of one to four. Adsorption was allowed to occur overnight at 4°. The adsorbed virus was centrifuged at 850*g* for 15 min at 4°. The supernatant fluid was decanted and a volume of sterile phosphate buffered saline pH 7.4 (PBS), equal to the original volume of virus, was added to the erythrocyte–virus mixture. Elution was effected by incubation at 37° for 2 hr. The supernatant fluid containing the eluted virus was separated from the erythrocytes by centrifugation at 850*g* for 15 min at room temperature, and a second cycle was initiated using fresh erythrocytes.

Results and Discussion. Proof that virus is labeled. The efficiency of this purification procedure was determined by comparing its effectiveness in removing ^{3}H labeled nonviral material. This labeled nonviral material was prepared by propagating primary METC cells in a medium containing 10 μCi of ^{3}H thymidine (methyl-labeled)/ 10^6 cells and treating the cells in the same manner as that used for harvesting polyoma virus. Thus, preparations which contained (i) ^{3}H labeled virus with ^{3}H labeled nonviral material, and (ii) ^{3}H labeled nonviral material, were subjected to adsorption–elution cycles and monitored for radioactivity and virus throughout the procedure. The amount of radioactivity associated with the virus was calculated after determining the amount of radioactivity present in the purified virus preparation that was due to nonviral material. This determination was based upon the proportion of radioactivity removed from the preparation containing only labeled nonviral material. The data from these experiments are presented in Table I. In two experiments this procedure yielded a virus preparation in which 86 and 93% of the label was associated with virus after two successive adsorption–elution cycles. A third experiment required three adsorption–elution cycles to produce a virus preparation in which greater than 99% of the label was associated with the virus. The specific activities of the virus in these three experiments were 2.7, 3.6, and 3.7 cpm/HAU, respectively. The DNA of ^{3}H la-

TABLE I. Purification of Polyoma Virus by Successive Adsorption Elution Cycles Employing Guinea Pig Erythrocytes.

Material[a]	HAU[b]	cpm × 10^3	Sp act.[c]	Virus RA[d] (%)
Expt. 1				
Crude virus	4096	889.0		
PV-2	4096	14.7	3.6	93
Crude NVM	—	1380.0		
PNVM-2	—	1.65		
Expt. 2				
Crude virus	20,480	1650.0		
PV-2	10,240	28.2	2.7	86
Crude NVM	—	373.0		
PNVM-2	—	0.855		
Expt. 3				
Crude virus	1024	13,000.0		
PV-1	640	21.0	36.0	35
PV-3	640	2.4	3.7	>99
Crude NVM	—	362.0		
PNVM-1	—	0.390		
PNVM-3	—	0		

[a] Crude virus = RDE-extracted virus; PV-2, PV-3 = purified virus after two and three adsorption elution cycles, respectively; crude NVM = RDE-extracted mock infected cells; PNVM-2, PNVM-3 = purified nonviral material after two and three adsorption elution cycles, respectively.

[b] Total hemagglutinating units.

[c] cpm/HAU.

[d] Percentage of virus associated radioactivity.

beled polyoma virus purified in this manner was twice extracted with 85% phenol containing 2% sodium dodecyl sulfate and twice precipitated with cold 95% ethyl alcohol. The extracted DNA was hydrolyzed by heating at 70° for 30 min in 1.0 N $HClO_4$. The DNA hydrolysate was subjected to ascending paper chromatography which revealed a slight ultraviolet adsorbing peak at an R_f value corresponding to thymidylic acid. The chromatograph was cut in sections corresponding to half R_f values and their radioactive content determined by counting in a Packard liquid scintillation system (9). The results indicated that there was a single peak of radioactivity at an R_f value corresponding to thymidylic acid.

Fate of labeled virus during eclipse. The eclipse period was determined by infecting replicate cultures of primary METC cells with 2000 HAU/culture. After a 1-hr period of adsorption, the cultures were washed three times with sterile PBS and maintenance medium was added to each culture. At various time intervals between 2 and 48 hr, duplicate cultures were extracted for virus employing RDE and the resulting extracts were examined for the presence of hemagglutinin. The data from this experiment are presented in Fig. 1 and indicate that hemagglutinin was

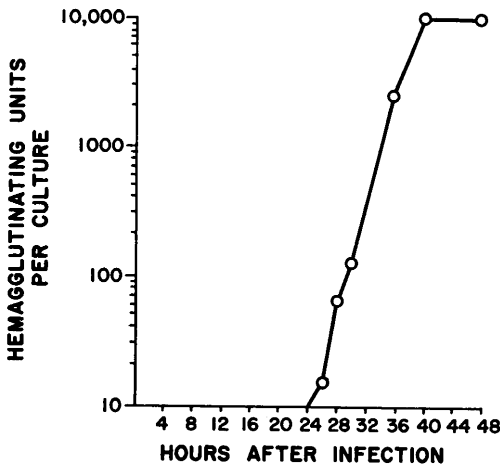

FIG. 1. The synthesis of polyoma hemagglutinin by infected primary METC cells: duplicate cultures were extracted for virus employing RDE and examined for hemagglutinin using washed guinea pig erythrocytes.

TABLE II. The Early Fate of Labeled Polyoma DNA in Primary METC and BHK/21 Cells.

After infection (hr)	% Adsorbed radioactivity in DNA extract[a]	
	METC[b]	BHK/21[b]
1	88	—
2	73	75
4	93	—
5	—	67
6	71	—
9	61	98
11	—	66
12	85	—
14	78	103
16	47	94
18	79	80

[a] The cells were extracted by a modified Ogur–Rosen $HClO_4$ technique. Radioactivity was absent in all acid soluble and RNA fractions.

[b] The percentages are the averages of quadruplicate cultures for METC experiments and from a pool of quadruplicate cultures for the BHK/21 experiment.

not detected until 26 hr after infection. The ^3H labeled purified polyoma virus was used to infect primary METC cells in order to determine its fate during the eclipse period. The virus preparation was allowed to adsorb to monolayers of primary METC cells for 1 hr at 37°. The monolayers were washed twice with PBS to remove unadsorbed virus and maintenance medium was added to each culture. At various time intervals, the cells were harvested and the nucleic acids were extracted according to a modified Ogur–Rosen technique (10). The amount of radioactivity in the resulting extracts was determined and is expressed as a percentage of the absorbed radioactivity. The results (Table II) indicated that as early as 1 hr after infection, the amount of radioactivity in the DNA fraction was at a maximum and that it remained at a relatively constant level throughout the period of observation. In addition, radioactivity was not found in the acid-soluble fraction (0.2 N $HClO_4$) and it would therefore appear that the virus DNA was not degraded during the eclipse period. These data support Khare and Consigli's interpretation (3) of

their autoradiographic data that polyoma DNA remained intact during the eclipse period, as well as the results of Borenfreund *et al.* (11) employing mitomycin C which indicated that chemically unaltered polyoma DNA was necessary to establish a productive infection. The fate of polyoma virus containing ^3H labeled DNA was determined in BHK/21 cells during the first 18 hr after infection. The experiment was performed in the same manner as the experiments which employed primary METC cells. The results (Table II) indicated that in BHK/21 cells, the DNA was also taken up rapidly and remained at a constant level during the first 18 hr after infection. It would appear that the virus DNA was not degraded due to the consistent absence of radioactivity in the acid-soluble (0.2 *N* $HClO_4$) fractions.

Absence of label in progeny. In similar experiments, the infected cultures were allowed to proceed past the eclipse period. The progeny virus was harvested as previously indicated and virus concentration and radioactive content were determined. The results (Table III) indicated that there was no detectable amount of label in the progeny virus 30 or 36 hr after infection. On the basis that polyoma virus DNA replicates semiconservatively (12), these data indicate that those DNA molecules containing a parental strand did not undergo encapsidation or that this event did not occur to the extent that detectable levels of radioactivity were present in the harvested virus population. The report of Kaplan (13) on pseudorabies virus indicated the presence of a pool of progeny DNA molecules of which only a small proportion undergo maturation during the early part of the infectious cycle. Should a similar situation be operative here, the possibility that the early harvested virus populations would contain parental DNA strands would be significantly reduced.

TABLE III. The Absence of Radioactivity in Progeny Polyoma Virus Produced from Parental Virus Containing ^3H Labeled DNA.

After infection (hr)	HAU[a] adsorbed	HAU of progeny	RA[b] of progeny
30	422	160	0
30	448	160	0
30	442	80	0
30	422	80	0
30	410	320	0
36	525	5120	0
36	800	2560	0
36	678	640	0
36	727	5120	0

[a] Hemagglutinating units.
[b] Radioactivity.

Summary. Experiments performed to determine the effectiveness of adsorption–elution cycles of polyoma virus purification techniques indicated that an excess of 90% of the label in purified virus preparations is associated with virus. This was corroborated by isolating, and identifying the radioactive component of virus DNA as a thymidylic acid moiety. Experiments employing labeled polyoma virus as a tracer in primary METC cells have indicated that the virus was rapidly adsorbed by the cells since the maximum amount of radioactivity was found in the DNA fraction as early as 1 hr after infection. The virus remained associated with the DNA fraction of the cells and probably was not degraded as indicated by the consistent absence of radioactivity in the acid soluble fractions. Label from parental virus DNA was not found in progeny virus in detectable amounts. It would seem, therefore, that at the time of harvest parental DNA did not undergo encapsidation and become a member of the progeny population. The fate of parental DNA during and after maturation remains to be elucidated. Experiments employing BHK/21 cells yielded similar results in that the labeled DNA was adsorbed rapidly by the cells, probably was not degraded, and remained at a constant level throughout the first 18 hr after infection.

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Analgesic Tolerance to Etorphine (M99)* and Morphine in the Mouse (33812)

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The new highly active morphine like drug, etorphine hydrochloride (M99 Reckitt), has been shown to have potent knock down properties for many animals, both wild as well as domesticated (1–5). Competitive antagonism

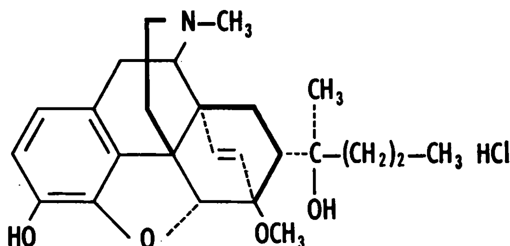

FIG. 1. 7α[1-(*R*)-hydroxy-1-methylbutyl]-6,-14-*endo*-ethenotetrahydrooripavine hydrochloride (M99).

between M99 and nalorphine (*i.e.*, nalorphine antagonizing the pharmacological effects of M99) has been shown to exist (6). Similar antagonism between M99 and cyprenorphine (M285), a new and highly potent morphine antagonist, has also been reported (6). While M99, like morphine, is a potent analgesic agent (7–9), no studies of tolerance to this drug have been published. The purpose of the present studies was to determine the relative degree of tolerance to the analgesic effects of M99 in the mouse and to study the time course of the development and persistence of this tolerance.

* Received M99 from Dr. Wayne H. Linkenheimer, Manager, Nutrition and Physiology Section, American Cyanamid Co., Princeton, N. J. 08540.

Methods. Young adult female Swiss Webster mice (Simonsen Laboratories, Gilroy, California) 18–27 g, were tested by means of the "caudal immersion" technique using a modification of the method of Ben-Bassat *et al.* (10). The water temperature used was 50 ± 0.2° with a 15-sec maximum immersion period after which the tail was removed from the water. A vented adjustable plexiglass tube was used to hold the mouse with tail extended into the water bath. Approximately four-fifths of the tail was immersed. Timing to 0.1 sec was by means of a stop watch. The end point was an oscillatory flick of the tail characteristic of attempted withdrawal. Drug dosage was by the subcutaneous route in Series I and by the intraperitoneal route in Series II and III. All drugs used were weighed and mixed in water, M99 as the hydrochloride and morphine as the sulfate.

While no ED_{50} determinations were made, the 15-min postinjection period utilized was shown to represent a period of near peak effect in a number of animals tested at these and other dosages (unpublished data). While potency ratios were not determined in Series I (Fig. 2), near equianalgesic doses utilizing these time intervals were estimated from the