Yaba Tumor Poxvirus Synthesis In Vitro

II. Adsorption, Inactivation, and Assay Studies

DAVID S. YOHN, FANNY R. MARMOL, VICTORIA A. HAENDIGES, AND
JAMES T. GRACE, JR.

Viral Oncology Section, Roswell Park Memorial Institute, Buffalo, New York

Received for publication 2 December 1965

ABSTRACT

YOHN, DAVID S. (Roswell Park Memorial Institute, Buffalo, N.Y.), FANNY R. MARMOL, VICTORIA A. HAENDIGES, AND JAMES T. GRACE, JR. Yaba tumor poxvirus synthesis in vitro. II. Adsorption, inactivation, and assay studies. *J. Bacteriol.* 91: 1953-1958. 1966.—Means to increase the efficiency of the Yaba tumor poxvirus assay in BSC-1 cell cultures were sought. A method was devised wherein 0.4 ml of each virus dilution was layered onto BSC-1 cells in 80-mm Leighton tubes and permitted to adsorb at 25 C for 18 hr prior to incubation at 35 C. The diluent found most reliable was medium 199 containing 50% bovine amniotic fluid adjusted to 2.0 mM calcium and 1.0 mM magnesium at pH 7.0. These modifications yielded highly reproducible titrations with a greater than twofold increase in assay sensitivity. Irreversible adsorption of Yaba virus by BSC-1 cells proceeds comparatively slowly at 25 to 33 C. Although the process is more rapid at 35 or 37 C, the increased thermal lability of the virus at these temperatures results in lower titers than with virus adsorbed at 25 C for longer periods of time. Yaba poxvirus appears to be 5 to 10 times more sensitive to thermal inactivation than vaccinia poxvirus.

In a previous report (7), we described an in vitro assay for Yaba tumor poxvirus in the continuous cercopithecus kidney cell line, BSC-1. Subsequent studies have led to certain alterations in the original methodology. These modifications have developed through analysis of Yaba virus adsorption by BSC-1 cells and of Yaba virus inactivation kinetics in various media at laboratory temperatures. The present report describes these studies.

MATERIALS AND METHODS

The virus employed in these studies titered 4.0×10^7 focus-forming units (FFU) per ml in BSC-1 and represented a pool of three saline-Genetron extracts of tumors from a 3-year-old *Macaca mulata* (rhesus) monkey, no. 10. The extraction procedure for this virus stock has been described previously (9).

Monolayer cultures of BSC-1 cells were grown in either medium 199 or Eagle's basal medium (EBM) supplemented with 10% agamma fetal calf serum and maintained in medium 199 or EBM diluted 1:1 with bovine amniotic fluid (BAF) and adjusted to pH 7.4 with NaHCO_3 . For preparation of assay tubes, stock cultures were treated for 10 min with prewarmed trypsin (37 C) prepared according to the method of Wallis et al. (6). Approximately 1.5×10^6 cells contained in 2 ml of growth medium were added to 80-mm Leigh-

ton tubes (cell surface area, 50 mm²) and incubated at 37 C until confluent monolayers developed.

In the assay as originally described, virus dilutions were made in saline and 0.2 ml was added to four or more Leighton tubes per dilution, bringing the total volume to 1.7 ml. The tubes were incubated at 35 C with medium changes twice weekly. No agar overlay was employed. Counts of FFU were made at 19 to 21 days. The modifications now employed are related mainly to the adsorption procedure and will be described herein. The revised assay will be summarized in the Discussion.

For the inactivation studies, 1 ml of appropriately diluted virus was placed in rubber-stoppered vials and immersed in appropriate water baths. At each sampling time, a vial was removed and rapidly chilled to 0 C. Titrations for residual virus were performed immediately.

RESULTS

Determination of optimal assay and adsorption conditions. Assay of Yaba poxvirus in BSC-1 as originally described (7) required a 3-week incubation at 35 C before all primary foci were visible microscopically. Attempts to reduce this interval by incubating cultures at 37 C generally resulted in overgrown or sloughed cell sheets. As an alternative approach, it seemed likely that if

rapid virus adsorption could be achieved the time requirement might be reduced. The means analyzed to increased virus adsorption efficiency included: (i) selection of that minimal volume of fluid which when layered on Leighton tubes with a 50 mm² cell surface area would provide random distribution of foci; (ii) selection of a virus diluent which would yield maximal adsorption; and (iii) selection of that temperature which would yield maximal titers with minimal adsorption time.

The minimal volume which bathed the entire cell monolayer and provided random distribution of foci was 0.4 ml. Smaller volumes proved unsatisfactory, particularly with adsorption periods greater than 2 to 4 hr.

The most efficient diluent of 10 tested under four combinations of pH and temperature (Table 1) was prepared with equal volumes of medium 199 and BAF adjusted to 2.0 mM calcium and 1.0 mM magnesium. (This diluent, adjusted to pH 7.0, has been designated 199-BAF-CM, and is presently used for adsorption of Yaba poxvirus to BSC-1 monolayers.) The results shown in Table 1 reveal two other points. First, adsorption efficiency was greater with diluents prepared with medium 199 than with similar diluents prepared with phosphate-buffered saline (PBS) or EBM. Second, additional calcium and magnesium generally enhanced adsorption efficiency. The mechanisms responsible for these observations were not explored.

An unequivocally optimal adsorption time and temperature relationship was not established

(Table 2). Adsorption at 25 C, although proceeding at a slower rate than at 33 to 37 C, was continuous throughout the 18-hr period and produced the highest per cent adsorption. Adsorption at 33 C initially occurred more rapidly than at 25 C, but, owing to reduction of virus concentration by inactivation, yielded a lower titer after 18 hr. Inactivation also reduced apparent adsorption rates at 35 and 37 C. In addition, at 37 C a large proportion of virus, presumably adsorbed by 8 hr, was rendered non-infective with an additional 10 hr of incubation at 37 C. Further studies of this latter observation are currently underway.

These studies (Tables 1 and 2) have demonstrated that, when 0.4 ml of Yaba virus diluted

TABLE 2. *Influence of time and temperature on adsorption of Yaba poxvirus by BSC-1 monolayers*

Adsorption time <i>hr</i>	Avg* per cent of inoculum adsorbed at			
	25 C	33 C	35 C	37 C
2	10.8	15.7	15.2	18.8
4	18.2	30.3	28.5	27.0
6	27.3	38.5	32.7	33.5
8	38.5	44.8	34.3	36.2
18	77.0	65.3	42.3	12.0

* For each observation, 500 FFU of Yaba poxvirus contained in 0.4 ml of 199-BAF-CM was layered on each of six Leighton tubes.

TABLE 1. *Effect of various media on adsorption of Yaba poxvirus by BSC-1 monolayers**

Virus diluent	Avg† no. of FFU adsorbed in 18 hr at				Avg FFU adsorbed	Relative efficiency
	25 C, pH 6.8	25 C, pH 7.2	33 C, pH 6.8	33 C, pH 7.2		
Medium 199, 50% BAF, Ca, and Mg.....	81.0	87.3	68.4	71.4	77.0	100.0
Medium 199, 2% agamma calf serum.....	80.0	67.0	71.2	70.7	72.2	93.9
Medium 199, 50% BAF.....	62.2	59.2	64.3	56.2	60.5	78.8
PBS, 2% agamma calf serum, Ca, and Mg.....	64.0	62.8	67.5	52.7	61.8	80.3
PBS, 2% agamma calf serum.....	59.7	45.2	63.7	58.2	56.7	73.7
PBS, 10% agamma calf serum.....	41.2	56.0	42.3	48.4	46.9	61.0
PBS, 50% BAF.....	36.1	29.4	18.3	13.2	24.3	31.6
EBM, 50% BAF, Ca, and Mg.....	42.7	47.0	55.0	43.3	47.0	58.1
EBM, 50% BAF.....	49.7	47.2	39.6	37.3	43.5	56.5
EBM, 2% agamma calf serum.....	21.3	21.8	18.3	19.3	20.2	26.3
Average.....	53.8	52.3	50.8	47.1	51.0	—

* Abbreviations: FFU = focus-forming units; PBS = phosphate-buffered saline; BAF = bovine amniotic fluid; EBM = Eagle's basal medium; Ca and Mg = 2.0 mM calcium and 1.0 mM magnesium, final concentration.

† Average of counts from six tubes. All tubes were inoculated with 100 FFU contained in 0.4 ml.

in 199-BAF-CM was layered on Leighton tubes, approximately 80% of the inoculum was adsorbed in 18 hr at 25 C. Assays performed with this adsorption procedure yielded twofold or greater increases in infectivity titers (Table 3). This procedure did not reduce the incubation interval significantly, since approximately 18 days were required before all primary foci were visible microscopically.

Inactivation kinetics. The foregoing adsorption studies had provided presumptive evidence that Yaba poxvirus was relatively unstable at normal laboratory temperatures. A preliminary study of Yaba virus thermostability for 24 hr at 37 C in four diluents resulted in biphasic inactivation kinetics in three of the four diluents (Fig. 1). These kinetics, characterized by an initial shoulder of variable duration followed by a more rapid inactivation rate, occurred when the virus was diluted in PBS with 10% fetal calf serum, in medium 199, and in medium 199 containing 25% BAF. Only when PBS alone was employed as diluent were first-order kinetics observed.

Biphasic inactivation curves also occurred with Yaba virus diluted in 199-BAF-CM. These experiments were performed in triplicate at 25, 33, 35, and 37 C (Table 4). The parameters followed included the inactivation rate as expressed by the velocity constant k , the half life (IT_{50}) and the duration of the initial inactivation phase. Velocity constants, k , were calculated from the relationship $\log N/N_0 = -0.4385 kt$; half lives or 50% inactivation time, IT_{50} , were calculated from the derived relationship $IT_{50} = 0.6909/k$. The degree of variability encountered between replicates was lessened somewhat by averaging the values for the 24-hr observation period. However, a plot of the average 24-hr $\log k$ values against $1/K^\circ$ failed to give a straight line. Thus, a reliable analysis of the inactivation

FIG. 1. Survival of Yaba virus at 37 C. Yaba virus was diluted to 10^6 focus-forming units per ml in the diluents listed above. Samples were titrated for residual virus after the intervals of incubation at 37 C shown on the abscissa.

kinetics of Yaba virus with 199-BAF-CM as diluent was precluded.

The final inactivation experiment employed PBS as the diluent and included five temperatures from 30 to 40 C. In each instance, first-order kinetics (Fig. 2) were followed during the 10-hr experimental period. A plot of $\log k$ versus $1/K^\circ$ produced essentially a straight-line relationship (Fig. 3). The major deviation of mean values from the fitted line occurred at 37 and 40 C. This deviation is also reflected by the differences between observed and predicted mean IT_{50} values shown in Table 5. However, predicted values fell within the range of observed values at both these temperatures.

The marked differences in velocity constants and half-life values between virus diluted in 199-BAF-CM and virus diluted in PBS (compare Tables 4 and 5) at 33 and 35 C exemplifies the influence of medium components on virus thermostability. At 37 C, the protective effects of 199-BAF-CM are less apparent after the first inactivation phase; inactivation kinetics during the second phase in 199-BAF-CM are comparable to those observed with virus diluted in PBS.

TABLE 3. Comparison of Yaba poxvirus assay methods

Virus pool	Avg FFU per ml as determined by		Revised/ original
	Original procedure	Revised procedure*	
Gx M-10-1	3.6×10^6	7.9×10^6	2.19
Gx M-10-2	7.1×10^7	1.6×10^8	2.25
Gx M-10-3	6.8×10^6	1.6×10^7	2.35
Gx M-36-1	2.8×10^5	5.3×10^5	1.96
Gx M-36-2	5.1×10^7	1.2×10^8	2.35
Gx M-36-3	6.4×10^7	1.5×10^8	2.33
Gx M-36-4	3.9×10^7	7.9×10^7	2.03

* Virus diluted in 199-BAF-CM, 0.4 ml layered per tube, and adsorbed for 18 hr at 25 C.

TABLE 4. Inactivation values for Yaba poxvirus diluted in 199-BAF-CM

Temp	Replicate no.	First phase			Second phase		Mean values for 24 hr	
		Duration	IT ₅₀	<i>k</i>	IT ₅₀	<i>k</i>	IT ₅₀	<i>k</i>
<i>C</i>		<i>hr</i>						
25	1	8.0	16.5	.042	7.6	0.091	9.4	0.074
	2	10.0	18.0	.038	6.8	0.102	9.0	0.077
	3	12.0	20.6	.034	6.7	0.103	9.8	0.071
33	1	13.0	10.8	.064	7.0	0.099	8.4	0.082
	2	12.0	11.0	.063	7.5	0.092	8.0	0.086
	3	10.0	14.2	.049	5.8	0.120	7.5	0.092
35	1	4.0	5.8	.120	2.5	0.276	2.7	0.253
	2	2.5	8.2	.085	2.1	0.330	2.3	0.308
	3	4.0	7.2	.097	2.3	0.304	2.7	0.259
37	1	1.5	5.6	.123	0.58	1.191	0.66	1.041
	2	3.5	4.5	.154	0.61	1.133	0.77	0.898
	3	2.0	5.2	.133	0.72	0.960	0.84	0.826

FIG. 2. Thermal inactivation of Yaba poxvirus diluted in PBS (pH 7.2). Virus diluted to 10⁶ focus-forming units per ml.

DISCUSSION

These studies were undertaken to develop methods for increasing the efficiency of the Yaba poxvirus assay in BSC-1 monolayer cultures, to explore certain problems related to the assay, and to investigate the properties of Yaba poxvirus associated with these problems. Although the modifications in assay methodology which

FIG. 3. Relationship of temperature to thermal inactivation rate. Velocity constants, *k*, were calculated as described in the text. Time factor is in hours. Mean values of log *k* with latitude of observations at each temperature are shown.

evolved from these studies increased assay efficiency, as shown by greater sensitivity, these changes failed to reduce the total incubation interval appreciably; at least 18 days are required before all primary foci are visible microscopically.

TABLE 5. *Inactivation values for Yaba poxvirus diluted in phosphate-buffered saline (PBS)*

Temp	Velocity constant (<i>k</i>)		Mean IT ₅₀ (hr)	
	Range	Mean	Observed	Predicted*
C				
30	0.0720-0.1535	0.1156	5.98	5.87
33	0.2539-0.3371	0.2956	2.34	2.30
35	0.4558-0.6586	0.5624	1.23	1.29
37	0.9926-1.3552	1.1231	0.62	0.75
40	1.6056-2.6844	1.9491	0.36	0.28

* Calculated from graph shown in Fig. 3.

However, their presence is detectable at 12 to 14 days by immunofluorescence methods (9).

It was found that reproducibility of the assay required rather precise conditions. It was necessary that the BSC-1 monolayers grown in 80-mm Leighton tubes be fully sheeted but not overgrown prior to use. Adsorption of virus proceeded most efficiently when virus was diluted in 199-BAF-CM and 0.4 ml was layered onto each cell sheet from which the medium had been removed. Adsorption overnight (18 hr) at 25 C proved most reliable. After adsorption, a minimal maintenance medium, such as EBM-50% BAF at pH 7.2 to 7.4, and incubation at 35 C produced an environment wherein cell proliferation was minimal and viral synthesis with cytopathic effect was favored. Use of highly nutritive media or incubation at 37 C, or both, increased cell proliferation and resulted in a masked or even aborted infection.

The observation that a comparatively long adsorption period, 18 hr, was required to obtain maximal titers indicates that the infectivity rate is limited at some initial stage. Dales (1) published a convincing review of the evidence that after viral attachment vaccinia poxvirus penetrates the cells by active cell phagocytosis. The viral protein coats are degraded, presumably by lysosome hydrolases (2), and the viral genome is released after digestion of the core membrane by specific uncoating enzyme (4). With vaccinia virus, these processes in HeLa or L cells require 3 hr or less (1, 4). Detailed analyses of these respective processes with Yaba and vaccinia poxvirus in BSC-1 have yet to be reported. However, preliminary observations in this laboratory have demonstrated that, under identical conditions, the vaccinia infectious cycle in BSC-1 is at least four to six times more rapid than the Yaba cycle. Since it is difficult to conceive that BSC-1 cells would differentially phagocytize vaccinia virus more rapidly than Yaba virus, it is suggested that the rate of irre-

versible adsorption of Yaba virus by BSC-1 cells is limited at 25 C by one or more steps associated with coat degradation. In support of this contention, it has been noted that BSC-1 cells, infected with Yaba virus at a multiplicity of 2.0 for 18 hr at 25 C, rinsed five times with PBS, and then placed at 35 C, released 24 to 32% of the cell-associated infectious inoculum over the next 6 hr (8). Similar data are not available concerning release of cell-associated virus during adsorption at higher temperatures.

The inactivation studies demonstrated that Yaba poxvirus is relatively labile in media devoid of protein and divalent cations. Extrapolation of the Arrhenius plot predicts a half-life of approximately 1 min (48 sec) at 50 C. In a study of heat inactivation of vaccinia virus, Kaplan (5) presented data from which it could be calculated that the IT₅₀ of a temperature-sensitive portion of the Lister strain of vaccinia virus was 5 min at 50 C. His data also indicated that the W. R. strain was only slightly more resistant. The lack of significant differences in heat sensitivity between these two vaccinia strains suggests that the greater sensitivity of Yaba virus is a point of differentiation between these two poxviruses.

Kaplan also noted that a portion of vaccinia virus was inactivated at a rate slower than the temperature-dependent major portion and that the relative amount of this portion decreased with increase in temperature from 50 to 60 C. The temperature-independent portion was detected by carrying out the study to zero infectivity. In the present study, inactivation was not carried out to zero infectivity. Thus, it is possible that additional inactivation phases may have appeared with further incubation of Yaba virus. The single logarithmic inactivation rates obtained from 30 to 40 C for 10 hr with Yaba virus diluted to PBS suggest that only a single inactivation process was involved during this interval. The protective effects of serum protein, divalent cations, or other components of medium 199 suggest that the inactivation process involved permeability changes or denaturation of the viral capsid protein, or both. Kaplan interpreted his two-component inactivation curves as indicative of a virus population of heterogeneous heat sensitivity. Hiatt (3), in a discussion of virus inactivation kinetics, suggested a dual reaction scheme as a more plausible explanation of Kaplan's data. Since Kaplan's data and that of others (*see* 3) indicate that virus inactivation is not the result of a single reaction at all temperatures, it is readily conceivable that one reaction may involve viral capsid protein denaturation, whereas another may involve structural changes in the genome. These reactions would proceed at different

velocities and produce nonlinear plots of $\log N/N_0$ versus t .

Our demonstration of variable biphasic inactivation kinetics at 37 C with virus in diluents other than PBS should not be interpreted as evidence of a heterogeneous virus population or as representative of two (or more) inactivation mechanisms. These results merely represent an interference with an inactivation process shown to be first-order with virus diluted in PBS. Had the studies been carried to zero infectivity and been performed at higher temperatures, it seems unlikely that first-order kinetics would have persisted. This assumption is based on two expectations. First, heterogeneity of at least a physical nature should be present in the virus pool since it represented an extract from tumor cells with virus in various maturation stages, and the less mature particles would be expected to be most labile. Second, at higher temperatures molecular changes in both protein and nucleic acid structures should occur. Inactivation experiments at higher temperatures were not performed since it was not the principal purpose of these studies to present a full analysis of heat inactivation kinetics of Yaba virus. The temperatures selected were those employed during routine laboratory handling of the virus. The kinetics obtained are of immediate application to Yaba virus growth curve studies (8). In addition, they provide a basis upon which to explain the lower assay titers obtained when

Yaba virus was adsorbed at 33 to 37 C rather than at 25 C.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants CA-07998 and CA-07745 from the National Cancer Institute, and by the John A. Hartford Foundation.

LITERATURE CITED

1. DALES, S. 1965. Penetration of animal viruses into cells. *Progr. Med. Virol.* **7**:1-43.
2. DE DUVE, D. 1963. The lysosome concept. Ciba Found. Symp. Lysosomes.
3. HIATT, C. W. 1964. Kinetics of the inactivation of viruses. *Bacteriol. Rev.* **28**:150-163.
4. JOKLIK, W. K. 1962. The multiplication of poxvirus DNA. Cold Spring Harbor Symp. Quant. Biol. **27**:199-208.
5. KAPLAN, C. 1958. The heat inactivation of vaccinia virus. *J. Gen. Microbiol.* **18**:58-63.
6. WALLIS, C., R. T. LEWIS, AND J. L. MELNICK. 1960. Preparation of kidney cell cultures. *Texas Rept. Biol. Med.* **18**:194-197.
7. YOHN, D. S., J. T. GRACE, JR., AND V. A. HAENDIGES. 1964. A quantitative cell culture assay for Yaba tumor virus. *Nature* **202**:881-883.
8. YOHN, D. S., V. A. HAENDIGES, AND E. DE HARVEN. 1966. Yaba tumor poxvirus synthesis in vitro. III. Growth kinetics. *J. Bacteriol.* **91**:1986-1991.
9. YOHN, D. S., V. A. HAENDIGES, AND J. T. GRACE, JR. 1966. Yaba tumor poxvirus synthesis in vitro. I. Cytopathological, histochemical, and immunofluorescent studies. *J. Bacteriol.* **91**:1977-1985.