Yaba Tumor Poxvirus Synthesis In Vitro

III. Growth Kinetics

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ABSTRACT

YOHN, DAVID S. (Roswell Park Memorial Institute, Buffalo, N.Y.), VICTORIA A. HAENDIGES, AND ETIENNE DE HARVEN. Yaba tumor poxvirus synthesis in vitro. III. Growth kinetics. *J. Bacteriol.* 91:1986-1991. 1966.—The infectious synthetic cycle of Yaba poxvirus by BSC-1 cells at 35 C is described and discussed together with related observations obtained by immunofluorescence and electron microscopy. After adsorption of virus at 25 C, at least 6 hr of incubation at 35 C was required before viral eclipse was demonstrable. Infectious progeny appeared after an additional 48 hr of incubation at 35 C. Newly synthesized virus particles were seen at 68 hr but not at 44 hr by electron microscopy. Maximal maturation occurred between 100 and 120 hr. Maximal yields were obtained from the 5th through the 8th day. The differences in kinetics between Yaba and vaccinia poxviruses, as described for the latter in the literature, are discussed. Possible factors accounting for these differences are enumerated.

Previous descriptions of Yaba virus propagation in primary rhesus monkey kidney cells (2), in cercopithecus kidney cell line, BSC-1 (23, 24), and in primary human fetal kidney and adult grivet monkey kidney cells (14), provided presumptive evidence that Yaba virus replication occurred at a rate much slower than replication of other poxviruses such as vaccinia (5, 12, 15), rabbit poxvirus (3), and Shope fibroma virus (10, 11). The previous reports on Yaba virus were based largely on either microscopic observations of cytopathic effects alone or in combination with histochemical or immunofluorescent staining, or both. Analysis of the results obtained with the combined techniques indicated that, although 36 to 48 hr are required before Yaba virus antigen synthesis is detected in BSC-1 cells (24), the complete cycle requires 50 to 60 hr. The present studies were undertaken to establish more precisely the time relationships of Yaba poxvirus synthesis in BSC-1 monolayers. It is anticipated that the results will provide baseline information for detailed analyses of the biochemical events associated with Yaba virus synthesis in vitro.

MATERIALS AND METHODS

Virus and assay. The virus lot employed in these studies was obtained from saline-Genetron extracts of tumors obtained from a 3-year-old rhesus monkey, no. 10. Extraction of these tumors was described pre-

viously (24). The virus assay and titration methods also were described in detail previously (25). Briefly, virus was diluted in medium 199 containing 50% bovine amniotic fluid (BAF) adjusted to 2.0 mM calcium, 1.0 mM magnesium, and to pH 7.0. Amounts of 0.4 ml of each dilution were added to six 80-mm Leighton tubes per dilution and incubated for 18 hr at 25 C. A 1-ml amount of Eagle's basal medium (EBM) containing 50% BAF was added, and the tubes were incubated at 35 C for 19 to 21 days. Fresh maintenance medium was added every 3rd or 4th day.

Cell cultures. BSC-1 monolayer cultures were grown in EBM containing 10% agamma calf serum and were maintained in EBM containing 50% BAF. All media contained 100 units of penicillin and 20 μ g of streptomycin per ml.

Electron microscopy. Yaba virus-infected BSC-1 cells were gently scraped from glass surfaces with a rubber policeman and centrifuged at $800 \times g$ for 3 min. The supernatant fluid was decanted, and the cell pellet was fixed for 10 min at 0 C with glutaraldehyde diluted to 1% in 0.1 M phosphate buffer (pH 7.5). The glutaraldehyde was removed by rinsing the pellet three times with buffer containing 0.33 M sucrose, and the pellet was fixed for 1 hr at 0 C in buffered 2% OsO₄. The pellet was dehydrated through absolute ethyl alcohol and embedded in Epon (16). Sections were examined with a Siemens Elmiskop I electron microscope, with the use of the double condenser system, 80-kv accelerating voltage, and 50- μ molybdenum objective apertures. Ilford N.60 "photo-mechanical" plates were employed.

RESULTS

Time course of Yaba virus synthesis in BSC-1 monolayers. Three replicate studies of Yaba virus synthesis were performed. Yaba virus was added at a multiplicity of 2.0 to fully sheeted BSC-1 monolayers grown in 3-oz prescription bottles and in 80-mm Leighton tubes. Adsorption was permitted for 18 hr at 25 C, and the cultures were rinsed five times with 5.0 ml of phosphate-buffered saline (PBS), pH 7.2, per rinse. Maintenance medium was added, and the cultures were incubated at 35 C. Virus titers of the pooled rinses, of the supernatant fluid prior to rinsing, and of the cell-associated virus prior to and 6 hr after rinsing were determined. These determinations revealed that approximately 50% of the original inoculum was recovered in the supernatant fluid and rinses after 18 hr at 25 C (Fig. 1). An additional 24 to 32% of the inoculum, originally cell-associated at 18 hr, appeared in the supernatant fluid after 6 hr at 35 C. Thus, the amount of cell-associated titratable virus at this time, i.e., after the first 24-hr period, varied from 14 to 20% of the original inoculum. The amount of original inoculum which was not accounted for ranged from 4 to 8%, and represented the sum of inactivated virus, error, and of eclipsed virus. The expected percentage of surviving virus was calculated from the results obtained from preceding inactivation studies (25).

At 24-hr intervals thereafter, duplicate determinations were made of titratable cell-associated and free virus. The average value of the two samples for each replicate is plotted in Fig. 1. Lines in Fig. 1 connect either the average values of the three replicates, as for total infectious virus, or occasionally represent the general trend, as with infectious virus in the medium.

Titration at 48 hr after virus inoculation revealed that titratable virus decreased an average of 90% during the second 24-hr interval. Since this decrease represented the sum of inactivated and eclipsed virus, subtraction of the amount of virus estimated to be inactivated during this interval indicated that at least 20%, probably more, of this decrease represented eclipsed virus.

At 72 hr after virus inoculation, two of the three replicates contained twice as much titratable cell-associated virus as the 48-hr samples, whereas the third contained only one-half as much. The amount of infectious virus in the medium decreased to one-half, one-third, and one-fourth in the three replicates. These results indicate that a slight amount of infectious virus synthesis was detected between 48 and 72 hr in one, or possibly two, of the replicates.

FIG. 1. Time course of Yaba virus replication in BSC-1 monolayers.

By 96 hr (day 4), infectious virus synthesis was apparent in all replicates, as reflected by one- to fourfold increases in infectious cell-associated virus. These comparatively small increments were followed by logarithmic increases between day 4 and 5. Increment constants, k values, calculated from the monomolecular formula $T_t = T_0 e^{kt}$, for this 24-hr interval ranged from 2.428 to 3.733, and averaged 3.256. Peak titers were noted on days 6 through 8 and decreased thereafter. Consistently very little virus was detected in the supernatant fluid during the period of maximal synthesis and maturation (day 5 through day 10). The amount of cell-associated virus during this interval ranged from 99.75 to 99.96% of the total assayed. Viral release remained at low levels throughout the observation period of 15 days. (Viral release is probably minimal at all times since cell lysis does not occur readily.)

The results of this study, although failing to establish the precise time sequences of Yaba virus adsorption, eclipse, synthesis, and maturation in BSC-1 monolayers, nevertheless provided reasonable estimation of the time required for certain

steps. Viral eclipse did not occur during the 18-hr period at 25 C. A small proportion of virus may have been eclipsed during the first 6 hr at 35 C; however, the maximal rate of viral eclipse occurred during the next 24 hr at 35 C. At least another 24 hr at 35 C was required before any evidence of infectious viral synthesis was obtained. Maximal viral maturation occurred after the 4th day, i.e., 18 hr at 25 C plus 78 hr at 35 C.

Electron microscopic observations on the development of Yaba virus in BSC-1. Detailed descriptions of the fine structure of Yaba poxvirus as determined from these studies and others carried out on thin sections of Yaba tumors have been reported elsewhere (8). A summary of the in vitro results is included here. Yaba virus was added to T-30 flask cultures of BSC-1 at a multiplicity of 5, and the cultures were placed at 35 C without preadsorption at 25 C. Cultures were removed for examination at 20, 44, 68, 92, and 116 hr after infection. No evidence of virus assembly was detected until the 68th hr sample. At this time, immature virus particles were observed. At 92 hr, the number of immature particles had increased markedly. Although a few particles of increased density (Fig. 2 and 3), indicative of increased maturation and designated as transitional forms (8), were present at 92 hr, fully mature particles with typical biconcave disc shape and dumbbell-shaped core were not seen until 116 hr (Fig. 3).

DISCUSSION

These studies were undertaken to establish the sequential time relationships of Yaba tumor poxvirus synthesis in BSC-1 monolayers at 35 C. Certain other information obtained by histochemical and immunofluorescence studies described previously (24) will be discussed in conjunction with the present results.

The initiating event in virus infection, irreversible adsorption and penetration, presumably requires active cellular phagocytosis as with other poxviruses (6), and was, for reasons as yet not clear, relatively inefficient in monolayers of BSC-1 at temperatures normally employed for virus adsorption, 25 to 37 C (25). Because of the comparatively long interval required for Yaba

virus adsorption by BSC-1 monolayers, the adsorption portion of the growth curve study was carried out at 25 C for 18 hr. Analysis of the amounts of extracellular and cell-associated virus recoverable at the end of this period indicated that viral eclipse (uncoating) had not occurred during this period. During the next 6 hr, now at 35 C, a maximum of 4 to 8% of the original inoculum may have entered the eclipse phase.

During the next 24-hr period, day 2, eclipse was completed and synthetic processes were initiated. Nucleoli became hypertrophic, and pinpoint sites of DNA synthesis were detected in the cytoplasm; however, viral antigen (protein?) synthesis was not demonstrated until the end of this interval, between the 36th and 48th hr (24). At this point, the growth curve analysis indicated that no infectious virus had been produced. In addition, no evidence of viral assembly was seen by electron microscopy.

During the third 24-hr interval, day 3, analysis of growth curve results indicated that infectious virus had been synthesized in one, or possibly two, of the three replicates. By electron microscopy, only immature particles were discernible at 68 hr. The synthesis of Yaba virus has been described to consist of six stages (8), the first two of which are immature forms; the next two are transitional forms, and the final two stages are morphologically mature with differences only in the number of exterior coats. Whether a very few transitional or even mature forms were present at this time and were not located by electron microscopy is not known. Additional evidence that infectious virus is synthesized by 72 hr was obtained from immunofluorescence studies (24). After addition of virus to BSC-1 monolayers, approximately 48 hr elapses before viral antigen appears. Contiguous cells contain viral antigen 3 days later. Thus, the infection of the contiguous cells must have occurred at least 48 hr earlier, i.e., during the 3rd day after infection of the primary cell. The physical nature of the infectious material which passes from cell to cell is not known at this time. It is tempting to speculate that contiguous spread may not require morphologically mature particles.

The onset of logarithmic maturation occurred

FIG. 2. Portion of the cytoplasm of a BSC-1 cell 5 days after infection with Yaba virus. A group of immature particles is seen corresponding to the stages 1, 2, and 3 described elsewhere (8). A group of free ribosomes (arrow) is seen in the vicinity of this "factory area." $\times 32,500$.

FIG. 3. Higher magnification of another cell from the same sample as Fig. 2. The viral membrane has a typical unit membrane structure and is covered by short subunits. The arrow points to a more mature particle corresponding to stage 5 in the previously quoted reference. This form is characterized by the appearance within the dense matrix of additional membranes which are going to constitute the inner dumbbell-shaped core of the fully mature virions. $\times 67,500$.

FIG. 2-3.
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during the 4th day and reached its maximal rate during the 5th day. With electron microscopy, these intervals were characterized by increased numbers of immature and transitional forms at 4 days and the appearance of large numbers of mature particles at 5 days. Cytopathology was now discernible with routine bright-field examination. In all stained preparations, cytoplasmic inclusion bodies were well formed and approached the size of the nucleus. Thereafter, the inclusions enlarged, equaling or exceeding the size of the nucleus. This latter process coincided with the interval when maximal virus yields were extracted from the cultures.

The minimal latent period, defined as the interval between irreversible adsorption and initial appearance of infectious progeny, appears to be of the order of 50 hr. In the growth curve analysis, this interval encompassed 54 hr. The previously discussed immunofluorescence results suggested a minimal period of 48 to 60 hr. The electron microscopic study placed the interval at more than 44 hr but less than 68 hr including adsorption time. The minimal latent period required for vaccinia virus synthesis in suspended L cells (5, 13) or in HeLa cell suspensions (12, 19, 20) or monolayers (15) is 6 to 10 hr. The time required for maximal yields of the two viruses under the above different conditions is 18 to 24 hr for vaccinia virus and 100 to 120 hr for Yaba virus. These differences can be attributed to the combination of many factors: (i) the 2 C temperature differential; (ii) the use of cell culture-adapted vaccinia virus as opposed to our use of virus extracted from tumor; (iii) the use of high multiplicity of infection with vaccinia virus under optimal adsorption conditions; (iv) the inherent biochemical differences between the cell hosts; and (v) the inherent differences between the synthetic rate directing capacities of the two viruses. At present, it appears that a direct comparison can only be made at 35 C in BSC-1, since some process of Yaba virus synthesis in BSC-1 at 37 C appears thermal-sensitive and virus yields are markedly lower than at 35 C (Marmol and Yohn, *unpublished data*). Furthermore, Yaba virus is not infectious for HeLa (1) or L cells (Marmol and Yohn, *unpublished data*). Initial studies of vaccinia virus replication in BSC-1 cells at 35 C indicate that the difference in kinetics between Yaba and vaccinia virus synthesis is largely viral genome-controlled. The vaccinia infectious cycle is four to six times more rapid.

Means to obtain maximal adsorption of Yaba virus in short-term suspension cultures of BSC-1 cells in a minimal time interval, i.e., less than 1 hr, are presently being developed. Once this

technique is available, it should be possible to compare under identical *in vitro* conditions the biochemistry of an oncogenic poxvirus with that of a normally nononcogenic poxvirus.

The protracted cycle of Yaba poxvirus synthesis in BSC-1, although unlike vaccinia, rabbit pox, and Shope fibroma virus in this respect, appears to be similar in length to the synthetic cycles of fowlpox in chick chorioallantoic membrane (4) and of molluscum contagiosum (9) in HeLa cells. The antigenic relationship of Yaba virus to these and other poxviruses has been examined somewhat limitedly. Niven et al. (17) found no cross-protection in monkeys between Yaba, vaccinia, and orf viruses. Similar results (1) have been obtained with the monkey poxvirus strains of von Magnus et al. (21) and of Prier and Sauer (18). However, Woodroffe and Fenner (22) obtained a weak precipitin reaction with vaccinia and myxoma nucleoprotein antigens reacted with Yaba antisera. Cross-reactions did not occur in complement-fixation tests, by immunofluorescence tests, or in precipitin tests with myxoma and vaccinia soluble antigens. Thus, it appears that Yaba tumor poxvirus may differ from the well-known primate poxviruses both biochemically and antigenically. Future studies along both these lines should define these differences more precisely.

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