Yaba Tumor Poxvirus Synthesis In Vitro

I. Cytopathological, Histochemical, and Immunofluorescent Studies

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ABSTRACT

YOHN, DAVID S. (Roswell Park Memorial Institute, Buffalo, N.Y.), VICTORIA A. HAENDIGES, AND JAMES T. GRACE, JR. Yaba tumor poxvirus synthesis in vitro. I. Cytopathological, histochemical, and immunofluorescent studies. J. Bacteriol. 91:1977-1985. 1966.—Yaba tumor poxvirus synthesis in BSC-1 cell culture was followed sequentially with light microscopy, immunofluorescent microscopy, and various histochemical stains. The first evidence of infection was detected at 24 hr when nucleoli became hypertrophic, reflecting enhanced ribonucleic acid (RNA) synthesis. At 36 hr, deoxyribonucleic acid synthesis was detected in the cytoplasm. This was immediately followed by or associated with antigen synthesis at paranuclear sites and enhanced RNA synthesis in the cytoplasm. Cytoplasmic inclusions were readily apparent at 4 days in initially infected cells. Contiguous spread of virus was judged to have occurred around the third day of infection. The time required to complete the synthetic cycle from time of infection to production of infectious progeny was estimated to be of the order of 60 hr. This cycle is 6 to 10 times longer than for vaccinia virus. By light microscopy, cytopathic effects (CPE) were detectable in 5 days in heavily infected cultures. With 100 units or less of infectious virus, CPE was not readily detected until the 10th to 14th day. At this time, focal areas of infection classified either as microtumors or microplaques were present. Secondary foci appeared during the 4th week of incubation.

Yaba tumor poxvirus was isolated by Bearcroft and Jamieson (2) in 1957 from an outbreak of subcutaneous tumors which occurred among a colony of rhesus monkeys housed in Yaba, Nigeria. Propagation of the virus in primary rhesus monkey kidney cultures was reported by Andrewes et al. (1). These workers obtained only limited serial passage of the virus in cell culture. Recently, we (15) described briefly the propagation, serial passage, and assay of Yaba virus in a continuous line of cercopithecus kidney, BSC-1, developed by Hoppes et al. (3). Levinthal and Shein (4) described serial passage of Yaba virus in primary human and simian kidney cells by monitoring the cultures with fluorescent antibody.

The present report is the first of a series of detailed studies on Yaba virus replication in BSC-1 cells. Included are a description of the cytological changes induced by Yaba virus in BSC-1 cells as observed by light microscopy, histochemical, and immunofluorescent staining techniques. A preliminary report has appeared (Yohn et al., Bacteriol. Proc., p. 132, 1964).

MATERIALS AND METHODS

Virus. A 3-year-old rhesus monkey (Macaca mulatta), no. 10, and a 5-year-old pigtail macaque monkey (Macacus nemestrinus), no. 36, were injected at multiple subcutaneous sites with 10^6 tumor-inducing doses of virus. Tumors were surgically removed 2 to 3 weeks later and homogenized as a 10% suspension in equal volumes of chilled 0.015 M phosphate-buffered saline (PBS) and Genetron (fluorocarbon) with a Servall Omni-mixer at maximal speed for 2 min, and then were centrifuged at 2,000 rev/min for 20 min in head no. 284 of a PR-2 International centrifuge. One-half the aqueous phase was removed, and an equal volume of chilled PBS was added. The above extraction procedure was employed two or three times. The final residue of tumor-Genetron gel was mixed with an equal volume of PBS, frozen, thawed, and centrifuged as above. The freezing procedure released the Genetron from the tumor residue; the aqueous phase represented the final extraction. Extracts were assayed for complement-fixing antigens and for infectious virus in BSC-1 cells and in rhesus monkeys. Antigen and infectious virus titers of these preparations are shown in Table 1. Virus was titrated as described by Yohn et al. (17). The complement-fixation procedure was
TABLE 1. Complement-fixing (CF) antigen and infectivity titers of Yaba tumor genetron extracts

<table>
<thead>
<tr>
<th>Tumor source</th>
<th>Ex-</th>
<th>CF</th>
<th>Infectivity/</th>
<th>FFU</th>
</tr>
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<tbody>
<tr>
<td>Macaca mu-</td>
<td></td>
<td></td>
<td>ml (log range</td>
<td>in vivo)*</td>
</tr>
<tr>
<td>latta no. 10</td>
<td>1</td>
<td>3,200</td>
<td>10^4 to 10^6</td>
<td>7.9 X 10^4</td>
</tr>
<tr>
<td>2</td>
<td>3,200</td>
<td>10^4 to 10^6</td>
<td>1.6 X 10^6</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1,600</td>
<td>10^4 to 10^6</td>
<td>1.6 X 10^6</td>
<td></td>
</tr>
<tr>
<td>Macaca nem-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>strina no. 36</td>
<td>1</td>
<td>3,200</td>
<td>10^4</td>
<td>5.3 X 10^6</td>
</tr>
<tr>
<td>2</td>
<td>6,400</td>
<td>10^4 to 10^6</td>
<td>1.2 X 10^6</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1,600</td>
<td>10^4 to 10^6</td>
<td>1.5 X 10^6</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>200</td>
<td>10^4 to 10^6</td>
<td>7.9 X 10^6</td>
<td></td>
</tr>
</tbody>
</table>

* Results of titrations in two to six rhesus monkeys per extract.
† Focus-forming units.

deoxyribonuclease were employed to confirm the specificity of the acridine orange staining. The preparation of these enzymes and the digestion procedures were as described by Mayor et al. (7). Other staining procedures included routine hematoxylin-eosin, methyl green thionin for nucleic acids according to the method of Roque et al. (10), and Sudan IV for free lipids.

RESULTS

Gross cytopathology. The time of appearance of virus-induced cytopathic effects (CPE) was dose-related. With high virus input, infection was evident by day 5, whereas with lower virus dose, 100 focus-forming units (FFU) per tube or less, CPE was rarely evident before 10 days. At this time, discrete focal involvement of three to five or occasionally more cells, containing large eosinophilic cytoplasmic inclusions, was present. During the next 10 days, the size of the primary foci increased as did the number visible. Comparison of the rate at which foci appeared in tubes inoculated with 100 FFU or less with the total number of foci expected revealed that secondary foci rarely appeared before day 24 and generally not before day 28 (Table 2).

The morphology of the foci was essentially of two types. In areas of low cell density, the cytoplasm of the infected cells contracted about the inclusion body and nucleus, thereby detaching the cell from its neighbors and the glass at all but one or two sites. The relatively slow progressive CPE resulted in a microplaque as shown in Fig. 1 and 2. The contiguous spread of infection in areas of high cell density resulted in decreased contact inhibition and the cells piled up, forming dense microtumors (Fig. 3 and 4). After approximately 18 to 20 days of incubation, cells on the periphery of the microtumors detached from the glass, and eventually all but a small portion of the microtumor sloughed.

Sequential immunofluorescent and histochemical studies of Yaba virus infection in BSC-1. Immunofluorescence was employed to establish the interval between infection and appearance of viral antigen. Approximately 100 FFU of Yaba virus from monkey no. 10 pooled tumor extracts were added to BSC-1 cells on cover slips. The tubes were incubated at 35 C. Cover slips were removed at 12- to 24-hr intervals and reacted with fluorescein isothiocyanate-conjugated hyperimmune monkey γ globulin. No fluorescence was obtained with the 12- and 24-hr postinfection specimens. Between 36 and 48 hr, small areas of pinpoint fluorescence appeared at perinuclear sites (Fig. 5). At 60 and 72 hr, additional fluorescent sites appeared that occasionally radiated into the cytoplasm, while initial sites enlarged or

modified according to Mayer (6) from the method of Metzgar et al. (8).

Cell culture. BSC-1 cells were grown in either medium 199 or Eagle’s basal medium (EBM) supplemented with 10% fetal calf serum and maintained in either medium 199 or EBM diluted 1:1 with bovine amniotic fluid (BAF) at pH 7.6. For preparation of assay tubes, stock cultures were treated for 10 min with prewarmed trypsin prepared according to the method of Wallis et al. (14). Approximately 1.5 X 10^4 cells contained in 2 ml of growth media were added to 80-mm Leighton tubes and incubated at 37 C.

Immunofluorescence. Two adult rhesus monkeys whose Yaba virus-induced tumors had regressed were hyperimmunized with repeated subcutaneous injections of 10^4 tumor-inducing doses of Yaba virus. Weekly 50-ml bleedings were obtained from each animal, and the sera were assayed for complement-fixing antibodies. The highest titered sera were pooled, and γ globulin fractions were precipitated by half saturation with (NH_4)_2SO_4. The globulins were washed and conjugated with fluorescein isothiocyanate according to the method of Riggs et al. (9). All conjugated γ globulin preparations and unlabelled reagents were adsorbed three times with monkey liver sediment prepared according to the method of Metzgar et al. (8). Rhodamine-labeled bovine albumin was added as a counterstain at a final concentration not greater than 1:6.

Yaba virus-infected cultures of BSC-1 grown on cover slips were fixed for 30 min in 1% phosphate-buffered formalin (pH 7.2), fixed in cold acetone for 10 min, and washed three times. Cover slips were floated with reagents and incubated at 37 C for 60 min, rinsed three times, mounted in buffered glycerin, and examined with Reichert-Lux or Leitz ultraviolet equipment.

Histochemistry. Acridine orange staining of Yaba virus-infected BSC-1 cells on cover slips was performed according to the method of Mayor et al. (7). Enzymatic treatments with pepsin, ribonuclease, and
coalesced, or both (Fig. 6). After 96 hr, fluorescent inclusions were prominent in the primary infected cells, and contiguous cells contained pinpoint areas of fluorescence (Fig. 7). At 8 days, large fluorescent inclusions were seen in many adjacent cells (Fig. 8). Yaba virus antigen was not detected in the nucleus and nucleolus at any time.

Evidence of viral nucleic acid synthesis was not readily detected by acridine orange staining until discrete cytoplasmic inclusions formed at 96 hr. These inclusions fluoresced green, indicative of deoxyribonucleic acid (DNA) content. The staining reaction was not removed by digestion with pepsin, ribonuclease, deoxyribonuclease, or pepsin followed by ribonuclease. Sequential digestion with pepsin and deoxyribonuclease abolished the staining. Inclusions developed to their maximal size in approximately 8 days. At this time, the cytoplasm surrounding the inclusion stained an intense flame-red with acridine orange, and the inclusion took on a yellow cast. Ribonuclease treatment removed the flame-red color, and the inclusion again stained green.

With hematoxylin-eosin staining, little evidence of cytoplasmic pathology was discernible before the 4th day. However, as the inclusions increased in size and age, the intensity of their eosinophilic staining increased. Lipids were noted in association with the mature inclusions.

With methyl green-thionin staining, synthesis of DNA in the cytoplasm was detected at 36 hr. Minute blue-green granules were observed. From 48 to 72 hr, the number and size of these granules increased. At 96 hr, the major portion of the green staining reaction corresponded with that of the inclusions seen by previous techniques.

However, occasionally minute blue-green granules were observed outside the inclusion toward the periphery of the cells. The cytoplasm surrounding the inclusions stained more intensely with thionin than did the cytoplasm of uninfected cells.

Hypertrophy of the nucleolus was a prominent and consistent feature of Yaba virus infection in BSC-1. Nuclear enlargement was quite apparent in 36 to 48 hr and occasionally was discernible in 24 hr, apparently preceding cytoplasmic pathology. Although nucleolar hypertrophy was detectable in all stained preparations, acridine orange and methyl green-thionin staining were the most sensitive means of demonstration. The nucleoli of infected cells when stained with acridine orange fluoresced with an intense orange in contrast to the paler yellow of uninfected cells. Examples of hypertrophied nucleoli are seen in Fig. 2, 5, 6, and 7.

**DISCUSSION**

The sequence of cytological changes observed in BSC-1 cells infected with Yaba virus is quite similar to that described for other poxviruses in various cell cultures. The most distinguishing feature of the present virus-host cell interaction is the relatively protracted period required for the sequence of events to occur as compared, for instance, with vaccinia virus in HeLa cells (5). The earliest evidence of cytological change was nucleolar hypertrophy, which occurred 24 to 36 hr after infection. This observation indicates that one of the early events associated with Yaba virus synthesis involves ribonucleic acid (RNA) synthesis. Similar events have been described for vaccinia virus. Tamm et al. (13) noted that RNA synthesis is required for vaccinia virus production.

### Table 2. Appearance of Yaba virus foci in BSC-1 over a 40-day period

<table>
<thead>
<tr>
<th>Titration* no.</th>
<th>No. of primary FFU expected</th>
<th>Virus dilution</th>
<th>Observation day</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>1</td>
<td>64</td>
<td>$10^{-4}$</td>
<td>13.5‡</td>
</tr>
<tr>
<td>2</td>
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</tr>
<tr>
<td>3</td>
<td>64</td>
<td>$10^{-4}$</td>
<td>9.5</td>
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<td>1</td>
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<td>$10^{-2}$</td>
<td>2.3</td>
</tr>
<tr>
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<td>6.4</td>
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<td>$10^{-2}$</td>
<td>1.3</td>
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<tr>
<td>3</td>
<td>0.64</td>
<td>$10^{-4}$</td>
<td>0.50</td>
</tr>
</tbody>
</table>

* Three separate titrations of extract no. 2 from monkey no. 10.
† Focus-forming units.
‡ Each figure represents the average of counts from four tubes; 0.4 ml of diluted virus added per tube. TMTC = too many to count.
FIG. 1. Hematoxylin and eosin stain of Yaba poxvirus-infected BSC-1 cells 20 days after infection, showing a microplaque. X 200.

FIG. 2. Higher magnification of center area of microplaque shown in Fig. 1. Note hypertrophied nucleoli and dense cytoplasm (inclusions) in cells immediately peripheral to hole in the cell monolayer. X 450.
FIG. 3. Hematoxylin and eosin stain of a middle stage (14 days) microtumor. A few inclusion bodies are discernible in the central cells. \( \times 450 \).

FIG. 4. Hematoxylin and eosin stain of later stage (20 days) microtumor. \( \times 450 \).
FIG. 5. Immunofluorescent staining of a single BSC-1 cell 48 hr after infection with Yaba poxvirus. Note paranuclear pinpoint areas of fluorescence. X 950.

FIG. 6. Immunofluorescent stain of a single BSC-1 cell 72 hr after infection with Yaba poxvirus. Note progression of immunofluorescent staining as compared with Fig. 5. X 800.
Fig. 7. Immunofluorescent stain of an early Yaba poxvirus focus in BSC-1 at 5 days after infection. Note inclusion in one cell and widely distributed diffuse staining in adjacent cells. X 800.

Fig. 8. Immunofluorescent stain of a Yaba poxvirus focus in BSC-1 at 8 days after infection. Note intensive fluorescence of inclusions in four cells. X 800.
Sheek and Magee (12) felt that the initial stimulation of RNA synthesis after vaccinia infection occurred in the nucleus and proceeded to the cytoplasm. Salzman et al. (11) described the synthesis of a vaccinia DNA-like RNA in the cytoplasm of vaccinia-infected HeLa cells. Loh and Riggs (5) noted increased RNA synthesis in both the nucleolus and cytoplasm. Our observations were similar; increased cytoplasmic RNA synthesis, as determined by both acridine orange and methyl green-thionin staining, followed nucleolar stimulation, and this hyperactivity apparently persisted during maturation of the inclusion. The amount of RNA surrounding the mature cytoplasmic inclusion was sufficient to require ribonuclease digestion to obtain a true green color with acridine orange, which is indicative of DNA content in the cytoplasmic inclusion.

Cytoplasmic DNA synthesis was first detected histochemically at 36 hr with methyl green-thionin stain but not with acridine orange staining. The insensitivity of the latter technique may have been due to a masking effect of cytoplasmic RNA or protein. The methyl green and thionin stains are believed not to react with protein, as does acridine orange (10).

Viral antigen synthesis, as revealed by immunofluorescence staining, was detected between 36 and 48 hr. The chemical nature of the antigen(s) and its relationship to the virus, i.e., whether viral structural or viral associated, have yet to be determined. Antigenic analysis of Yaba virus has not been reported. The fluorescein-labeled antibody employed was obtained from monkeys hyperimmunized with crude virus preparations (tumor extracts), and undoubtedly contained antibody to the presumed multiple virus components.

The immunofluorescence studies provided evidence that the time required to complete synthesis of infectious virus is of the order of 60 hr. Since approximately 48 hr were required before antigen appeared in the initially infected cell and antigen synthesis in contiguous cells was first observed between 96 and 120 hr, the minimal average difference, 60 hr, represents the time at which infectious material passed from the initially infected cell to its neighbors. Although this represents a crude approximation of the cycle length, growth curve analyses have indicated that the minimal cycle requires 50 hr (16). The length of this cycle is six to eight times longer than the infectious cycle of vaccinia virus in HeLa cell monolayers (5). Whether the comparative protracted cycle will lend itself to accurate kinetic and biosynthetic analysis remains to be seen. Studies along these lines are in progress.

Acknowledgments

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Literature Cited