Mechanism of Pock Formation by Shope Fibroma Virus on Monolayers of Rabbit Cells

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Received for publication 29 April 1966

ABSTRACT


The mechanism of pock formation by the Shope fibroma virus (SFV) on rabbit cultures in vitro was studied with the use of p-fluorophenylalanine, 5-bromodeoxyuridine, and 5-iododeoxyuridine. The inhibitors were used to inhibit, and to initiate, virus replication at different times after infection. It was shown that pock formation required virus replication to a threshold value of 25 plaque-forming units per pock area, and that this amount of virus can be accumulated during a period about 3 days less than that required for pock formation. Inhibition of virus growth, and of cell multiplication, after this threshold has been reached, did not prevent pock development. A delay in the onset of virus growth required to reach the threshold virus content, caused an about equivalent delay in the time of pock formation. In the absence of inhibitors, pocks were not formed after infection of 84 rabbit embryo clones, or five mixtures of clones containing five to seven clones each. The results indicate that pock formation by SFV in vitro was the result of cell aggregation, and not of cell multiplication, in special types of cells.

The cell-virus interaction of the Shope fibroma virus (SFV) can produce the formation of pocks in vitro at low virus inputs in certain cell cultures. In a previous study on the nature of the pocks produced in rabbit embryo cultures, the mitotic frequency in cultures with pocks was not higher than in the controls, and no pocks were found after infection of 28 single cell rabbit embryo clones (4). These results suggested that pock formation by SFV in rabbit embryo cultures may be the result of cell aggregation in either mixed cell populations or special types of cells, but that it did not seem to be the result of a stimulation of cell multiplication. Infection of rabbit testis cultures with SFV, however, resulted in an increased mitotic frequency in cultures that formed pocks (4). This raised the question of whether the mechanism of pock formation by SFV was the same in the embryo and testis cultures.

The present study was concerned with the further analysis, by the use of inhibitors, of the relationship between cell multiplication, virus growth, and pock formation by SFV in vitro. Data are also presented on the inability of single cell clones and artificial mixtures of such clones to produce pocks.

MATERIALS AND METHODS

Tissue cultures and media. Cells from noninbred rabbit embryo and testes cultures were used. The methods for preparing the cultures and the incubation procedure were as described previously (4). As in previous experiments, the medium used was Eagle's medium with a fourfold concentration of amino acids and vitamins (EM) and 5% calf serum, and infected cultures were incubated at 35 C. All experiments were carried out in 60-mm plastic dishes (Falcon Plastic, Los Angeles, Calif.). Clones of rabbit embryo cells were isolated as in previous experiments (4).

Inhibitors. The inhibitors used were p-fluorophenylalanine (FPA), 5-bromodeoxyuridine (BUdR), and 5-iododeoxyuridine (IUDR), all obtained from Calbiochem. These compounds have previously been found active in inhibiting the growth of vaccinia (1, 3, 8, 9), and some other deoxyribonucleic acid (DNA) animal viruses (2, 6, 7, 10).

The compounds were dissolved in a small volume of saline solution, and, unless otherwise stated, were sterilized by filtration through membrane filters with a pore size of 0.45 μ (Millipore Filter Corp., Bedford, Mass.). Appropriate final dilutions of the inhibitors were prepared in EM prior to use. The concentrations referred to in the following experiments were in all cases based on the initial concentrations, prior to filtration.

Virus titration, pock counting, and inhibitor experi-
ments. Virus was titered on rabbit embryo secondary monolayers by plaque assay as described previously (4). Each sample for titration was a pool of two duplicate plates, from which the total virus was determined.

The number of pocks formed under different experimental conditions was determined in cultures fixed with absolute methanol and stained with May-Grünwald-Giemsa. Pocks were counted under a dissecting microscope at a magnification of 72 times.

Sparse cultures, containing about $3 \times 10^4$ cells, and full monolayers, prepared by seeding $2.4 \times 10^4$ cells per petri dish, were infected at an input multiplicity of 1 to 2 PFU (pock-forming units = plaque forming units) per cell and 200 PFU per dish, respectively. Inhibitors were added either immediately or at various times after virus adsorption. Virus was adsorbed for 1 hr. Titrations of all the samples were made at the end of the experiments.

RESULTS

Inhibition of virus growth by FPA, IUDR, and BUDR. To choose the appropriate inhibitor and concentration for further experiments, different concentrations of FPA, IUDR, and BUDR were tested for their inhibitory effect on SFV growth in rabbit embryo secondary cultures. The inhibitors were incorporated into the medium added to the cultures after virus adsorption, and virus growth in the presence of the inhibitors was determined during the next 7 days (Table 1).

It can be seen from Table 1 that FPA gave a marked inhibition at $0.8 \times 10^{-3}$ M and complete inhibition at $1.6 \times 10^{-3}$ M. At $0.8 \times 10^{-3}$ M, there was a rise in virus titer after 3 days, whereas $1.6 \times 10^{-3}$ M gave complete inhibition for the 7 days of the experiment. With IUDR, there was complete inhibition with $5 \times 10^{-4}$ M for 48 hr, and with $10^{-4}$ M IUDR, for 3 days, but in both cases there was a rise in virus titer after this period of inhibition. BUDR gave complete inhibition for the 7-day period at $5 \times 10^{-4}$ M. In the experiment with $10^{-4}$ M BUDR (Table 1), there was a 2% yield of the control at 3 days, whereas in another experiment with $10^{-4}$ M BUDR (not shown in Table 1), in which the inhibitor solution was not filtered before use, $10^{-4}$ M BUDR gave complete inhibition. This indicates that there was presumably some decrease in inhibitor concentration during the filtration process.

The possibility that the increase in virus after a period of inhibition may have been due to inhibitor inactivation was tested in an experiment with $0.8 \times 10^{-3}$ M FPA. In this experiment, the effect on virus yield of a single addition of inhibitor was compared with daily addition of inhibitor. No significant difference was found between the two groups of culture, and in both cases there was a rise in virus titer after an initial inhibition.

Correlation between pock formation and virus replication. SFV pocks produced on rabbit cultures generally appear on the 5th day after infec-

TABLE 1. Inhibition of SFV multiplication by FPA, IUDR, and BUDR*

| Expt. | Inhibitor | Inhibitor concn | Virus titer 48 hr postinfection (PFU/plate) | Virus titer 3 days postinfection (PFU/plate) | Remarks
|-------|-----------|-----------------|------------------------------------------|--------------------------------------------|----------
|       |           |                 | Control | Inhibited | Per cent inhibition | Control | Inhibited | Per cent inhibition |                       |
| MT 16 | FPA       | $0.8 \times 10^{-4}$ | $1.6 \times 10^4$ | 0 | 0 | 100 | $8.8 \times 10^4$ | 0 | 99.32 | Complete inhibition until end of experiment |
|       |           | $1.6 \times 10^{-4}$ | $1.6 \times 10^4$ | 0 | 0 | 100 | $8.8 \times 10^4$ | 0 | 99.32 |                       |
| MT 24 | IUDR      | $5 \times 10^{-4}$ | $2.2 \times 10^4$ | 0 | 0 | 100 | $3.4 \times 10^4$ | 8.0 | 100 | 99.77 | Rise in titer in inhibited cultures at the 4th to 5th day |
|       |           | $1 \times 10^{-4}$ | $2.2 \times 10^4$ | 0 | 0 | 100 | $3.4 \times 10^4$ | 0 | 100 |                       |
| MT 34 | BUDR      | $5 \times 10^{-4}$ | $2.2 \times 10^4$ | 0 | 0 | 100 | $3.4 \times 10^4$ | 0 | 100 | Complete inhibition until end of experiment |
|       |           | $1 \times 10^{-4}$ | $2.2 \times 10^4$ | 0 | 0 | 100 | $3.4 \times 10^4$ | 0 | 100 |                       |
|       |           | $5 \times 10^{-5}$ | $2.2 \times 10^4$ | NT | NT | 100 | $3.4 \times 10^4$ | 0 | 100 |                       |

* Monolayer cultures were infected with 200 PFU per plate.

All experiments were terminated at 7 days after virus infection.

Not tested.
tion, and continue to enlarge during subsequent days. The inhibitors were used to study the relationship between virus replication and pock formation. Two series of experiments were carried out. In the first virus growth was inhibited at different times after infection, and in the second virus growth was initiated at different times after infection.

Inhibition of virus growth at different times after infection. If the formation of pocks on the 5th day after infection is the result of a continuous process of virus growth during all the preceding days, inhibition of virus growth at any time during this period should prevent pock formation. On the other hand, if virus growth is required only in the early days for pocks to be formed on the 5th day, pock formation should not be prevented by inhibiting virus growth on the later days.

To differentiate between these two possibilities, experiments were first carried out in which 1.6 < 10^{-3} \text{ M} \text{ FPA} was added to rabbit embryo secondary cultures at different times after virus adsorption (Table 2). When the FPA was added at 24, 37, or 48 hr after virus adsorption, new infectious virus was produced for 1 to 2 days, although at a rate lower than in the controls. This virus was presumably the result of synthesis that had occurred before the application of the inhibitor, and that had reached a stage of insensitivity to FPA. After this period of 1 to 2 days, new virus growth stopped, and this was followed by a steep reduction in virus titer presumably due to thermal inactivation. The half-life of SFV in E A at 35 C was found to be about 5 hr.

In all experimental groups where the treatment allowed pock formation, pocks appeared on the 5th day after infection. Addition of FPA at 0, 5, and 11 hr after virus adsorption completely inhibited virus growth and pock formation. When added at 24 hr, there was some virus growth, which ceased on the 3rd day, but no pock formation. However, in this case some flat foci appeared, which presumably could have developed to pocks in the absence of the inhibitor. The virus content of such foci was 7 PFU per focus. The virus content per focus, or per pock, was calculated on the assumption that all the virus in these cultures was present in the focus or pock areas, taking into account the fact that only cells in the pock or focus area contained inclusion bodies, thus suggesting the synthesis of virus in such areas only. The addition of FPA at times later than 24 hr after virus adsorption allowed the formation of pocks, and the virus titer per pock was 25 PFU when FPA was added at 37 hr and 2.3 × 10^{9} PFU when added at 48 hr.

The lower amount of virus per pock, compared with the control, when FPA was added at 37 and 48 hr (i.e., 25 PFU and 2.3 × 10^{9} PFU, respectively, compared to 6.1 × 10^{9} PFU, was also reflected by a smaller percentage of cells with inclusion bodies (10 to 20% and 30 to 50%, in comparison with ~80%), and by an initially smaller pock size (Table 2). However, in the inhibited cultures, pocks continued to grow after the 5th day postinfection, i.e., after the time of their appearance, without any increase in virus titer. This was particularly pronounced in the group in which the inhibitor was added at 48 hr.

### Table 2. Pock formation in cultures inhibited by 1.6 × 10^{-3} M FPA added at different times after infection

<table>
<thead>
<tr>
<th>Addition of inhibitor (hr postadsorption)</th>
<th>Titer at time of inhibitor addition (PFU/plate)ᵃ</th>
<th>Maximal virus titerᵇ (PFU/plate)</th>
<th>No. of pocksᵇ</th>
<th>Titer/pock (PFU/pock)</th>
<th>Shape of pocks</th>
<th>Percentage of cells with inclusion bodies in pock</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>2.2 × 10^{4} (5)ᶜ</td>
<td>328</td>
<td>6.1 × 10^{3}</td>
<td>Flat foci, no pocks</td>
<td>~80</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>4.0 × 10^{3}</td>
<td>(88)ᶜ</td>
<td>(7)ᵈ</td>
<td>Flat foci, no pocks</td>
<td>10-20</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>7.6 × 10^{3}</td>
<td>4.4 × 10^{3} (3)</td>
<td>25</td>
<td>Well developed but very small</td>
<td>30-50</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4.2 × 10^{3}</td>
<td>5.5 × 10^{4} (3)</td>
<td>237</td>
<td>Well developed but smaller on time of appearance than in the control</td>
<td></td>
</tr>
</tbody>
</table>

ᵃ Determined by titration of two pooled plates for each time.
ᵇ Counted on 7th day postinfection. Each number is an average of two to three plates. There was no increase in pock number in the inhibited cultures after 7 days.
ᶜ Numbers in parentheses indicate day at which titer reached its maximal value.
ᵈ Numbers in brackets indicate flat foci that did not develop into pocks.
In this group, after several days of growth, the pocks reached the same size or even exceeded the size of pocks in the controls.

These data thus indicate that pock formation required a minimal accumulation of 25 PFU per pock, and that this amount of virus can be accumulated during a period shorter than that required for pock development.

In an experiment with rabbit testes cultures, 1.6 × 10^{-3} M FPA, added on the 3rd or 4th day after virus adsorption, also did not prevent the formation of pocks on the 5th day and their subsequent growth.

Initiation of virus growth at different times after infection. The second series of experiments were carried out to determine whether the length of time between virus infection and pock formation was determined by the initiation of virus infection, or by the initiation of virus replication. Virus growth was initiated at different times after infection by use of IUDR at concentrations which completely inhibited SFV growth for 1 to 2 days, after which time the inhibitory effect was overcome and virus growth was initiated. Another experiment was carried out with BUDR, at a concentration that did not completely inhibit the initiation but reduced the rate of virus growth. The results of these experiments are shown in Fig. 1.

In the experiments with IUDR, 5 × 10^{-4} and 1 × 10^{-5} M IUDR were added to rabbit embryo secondary cultures after virus adsorption (infection was 150 PFU per plate). The concentrations of IUDR caused a complete inhibition of virus growth for 1 and 2 days, respectively, followed by a rate of virus synthesis equal to the initial rate of synthesis in the controls. The delay in initiation of virus synthesis was correlated with about an equivalent delay in the time of pock appearance. These results thus indicate that the 5-day period required for pock production is initiated at the time of the beginning of virus replication.

In the experiment with 10^{-3} M BUDR, there was no delay in the initiation of virus synthesis, or if there was it was shorter than the 24-hr period used for sampling in this experiment. Although the initial rate of virus synthesis was slower than in the controls, pocks appeared on the 5th day, as in the controls. The pocks were, however, less developed than in the controls on the day of their appearance, although they continued to develop. The initial smaller pock size in the BUDR-treated cultures was correlated with a smaller virus content. On the 3rd day after infection, the virus titer was 6.8 × 10^{4} PFU per culture in the inhibited cultures, in comparison with 3.4 × 10^{6} PFU in the controls, and on the 5th day 86 pocks appeared in the inhibited cultures and 225 pocks appeared in the controls. The virus content on the 5th day was thus 8 PFU per potential pock in the BUDR-treated cultures in comparison with 1.5 × 10^{6} PFU per potential pock in the control. This value of 8 PFU was enough for the formation of flat foci, as was shown above; however, the process of virus synthesis continued, enabling further accumulation of virus in the focus area, a fact which could explain the appearance of pocks in time, although they were smaller than the control. Thus, this experiment again shows the relationship between virus growth and pock formation.

Effect of FPA, IUDR, and BUDR on cell multiplication. To determine the correlation between pock formation and cell multiplication, the concentrations of FPA, IUDR, and BUDR used in the two sets of experiments described above were tested for their effect on the multiplication of rabbit embryo cells. FPA was also tested on rabbit testes cells. Secondary cultures were seeded at 2 × 10^{4} cells per plate, and the medium was changed at a time taken as zero time of the experiments. In each group, inhibitor was added to half the plates, and half the plates served as controls. Cell counts were made at zero time, and at 1, 2, and 3 days.

The results (Table 3) indicate that 1.6 × 10^{-3} M FPA completely inhibited cell multiplication immediately or after one cell division. BUDR at 10^{-3} M did not inhibit cell multiplication, whereas 5 × 10^{-4} M and 1 × 10^{-5} M IUDR inhibited mul-
TABLE 3. Multiplication of rabbit embryo and testes cells in the presence of FPA, IUDR, and BUDR

<table>
<thead>
<tr>
<th>Type of cells</th>
<th>Inhibitor</th>
<th>Concen</th>
<th>No. of cells X 10⁶ on day³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0³</td>
</tr>
<tr>
<td>Embryo</td>
<td>Control</td>
<td></td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>FPA 1.6 X 10⁻³</td>
<td></td>
<td>3.9</td>
</tr>
<tr>
<td>Testes</td>
<td>Control</td>
<td></td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>FPA 1.6 X 10⁻³</td>
<td></td>
<td>1.2</td>
</tr>
<tr>
<td>Embryo</td>
<td>Control</td>
<td></td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>IUDR 5 X 10⁻⁶</td>
<td></td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>1 X 10⁻⁵</td>
<td></td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>1 X 10⁻⁵</td>
<td></td>
<td>4.5</td>
</tr>
</tbody>
</table>

* Each number is an average of three to five plates.

sup| Inhibitor added at time zero.

Duplication after allowing one cell division. The results with 1.6 X 10⁻³ M FPA show that pocks produced after reaching an appropriate virus concentration in cultures treated with this concentration of FPA could not have been formed by cell multiplication. Cells incubated with this concentration of FPA were partially damaged after incubation for 4 to 5 days, but this did not prevent pock formation.

Absence of pock formation in rabbit embryo clones. It has previously been reported that SFV did not form pocks after infection of 28 clones made from rabbit embryo cultures. The number of clones examined has now been extended to 84. The clones were tested about 4 weeks after they were first isolated. They showed various degrees of sensitivity to the cytopathic effect of SFV, but none formed pocks after inoculation of monolayers with 100 to 200 PFU.

Ten of these clones were examined for virus yield per cell. Cultures containing 0.8 X 10⁶ to 2.0 X 10⁷ cells were infected at input multiplicities of 2 to 10 PFU per cell, and total virus yield per culture and number of cells with inclusion bodies was determined at 24 hr after infection. The virus yields per cell in these clones were 0.8 (1 clone), 2 (1 clone), 3 (4 clones), 7 (1 clone), 9 (1 clone), 11 (1 clone) and 25 PFU (1 clone). The control value for rabbit embryo secondary cultures in these experiments was 1 PFU per cell, and the other values are within the range previously observed for rabbit embryo secondary cultures (4). The absence of pock formation by the clones thus does not seem to be correlated with any difference from the mixed embryo secondary cultures regarding virus yield per cell.

To determine the behavior of artificial mixtures of the clones, five mixed cultures derived from mixtures of five to seven clones were inoculated with SFV. These cultures were tested at different seeding levels ranging from 2.0 X 10⁶ to 2.4 X 10⁶ cells per plate. All produced a cytopathic effect, but none of these mixed clones formed pocks. As mentioned previously, pocks were not found on rabbit embryo secondary monolayers derived from a low seeding of 2 X 10³ to 3 X 10³ cells per plate, i.e., monolayers that can also be considered as a mosaic of clones.

**Discussion**

Three conclusions regarding the mechanism of pock formation by SFV on rabbit monolayers can be derived from the present experiments. (i) Virus growth to a threshold value of 25 PFU per pock was required for pock formation. This value was calculated on the assumption that the whole virus content of a culture is concentrated in the pocks or foci areas. Since inclusion bodies were found only in such areas, and since it was shown (5) that virus synthesis takes place in the inclusion bodies only, the above assumption is very likely. The threshold amount of virus could be accumulated after 2 days of virus synthesis, although pocks appeared 5 days after infection, and a delay in the initiation of virus growth to reach this threshold value caused an approximately equivalent delay in the time of pock appearance. (ii) After the threshold value had been reached, complete inhibition of cell division did not prevent the formation of pocks. (iii) Pocks were not formed after infection of 84 cell clones or five mixtures of clones containing 5 to 7 clones each, although the virus yield per cell in 10 clones that were studied was of the same order of magnitude as that found after infection of secondary monolayers that allowed the formation of pocks.
It can thus be concluded that pock formation by SFV on rabbit monolayers is due to cell aggregation, and not cell multiplication, in special types of cells that were presumably not present in the clones. Cells in the pocks were piled up on one another; thus, the cellular change that results in pock formation seems to be associated with a decrease in contact inhibition.

In the previous study, an increased mitotic frequency in cultures that formed pocks had been observed in rabbit testis cultures. The present experiments indicate that with these cells the complete inhibition of cell division, after allowing sufficient time for accumulation of the threshold amount of virus, did not prevent the formation and growth of pocks. This suggests that in the rabbit testis cultures the increased mitotic frequency found in cultures with pocks was not essential for pock formation.

These results, however, do not necessarily exclude a relationship between the processes leading to the formation of either a tumor or pocks, in view of the fact that the conditions used in vitro differ greatly from the situation in vivo. The in vitro conditions could, no doubt, eliminate certain essential steps in the cell-virus interactions, leaving only the less complicated ones, like the formation of pocks.

ACKNOWLEDGMENT
I am indebted to L. Sachs, Section of Genetics, The Weizmann Institute of Science, Rehovoth, Israel, for help in preparing this manuscript.

LITERATURE CITED