Proflavine Inhibition of Vaccinia Virus Synthesis

H. CURT BUBEL AND DAVID A. WOLFF

Department of Microbiology, University of Cincinnati College of Medicine, Cincinnati, Ohio

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ABSTRACT

BUBEL, H. CURT (University of Cincinnati College of Medicine, Cincinnati, Ohio), AND DAVID A. WOLFF. Proflavine inhibition of vaccinia virus synthesis. J. Bacteriol. 91:977–983. 1965.—The synthesis of vaccinia virus, hemagglutinin, and blocking antigen, as well as the development of cytopathic effects, were inhibited by low concentrations of proflavine. This inhibitor did not exert a selective effect on any particular portion of the virus synthetic cycle. Proflavine added to infected KB cells during the eclipse period or later stages of virus maturation rapidly arrested further production of infectious virus and virus-related products. Suppression of virus synthesis was completely reversible, indicating that permanent damage to the virus synthetic mechanism did not result from a transient exposure to proflavine. Photosensitization of maturing vaccinia virus by subinhibiting concentrations of proflavine suggested an interaction of the inhibitor with viral nucleic acid.

Proflavine inhibition of bacteriophage synthesis has been ascribed to interference with the assembly of virus from subunits of protein and deoxyribonucleic acid (DNA) (DeMars, 1955; Mura-kami, Van Yunakis, and Levine, 1959). Studies with animal viruses have, in general, supported a similar view. For example, the production of virus-specific proteins and infectious ribonucleic acid (RNA), in the absence of complete virus synthesis, occurs in foot-and-mouth disease virus-infected cells inhibited by proflavine (Brown and Stewart, 1959). Other studies (Wilcox and Ginsberg, 1962; Franklin, 1955) suggest that proflavine exerts a differential effect on the synthesis of viral antigens and complete virus.

Proflavine is generally regarded as interfering with the synthesis of cytoplasmic proteins (Ephrussi and Hottingeur, 1950). In the vaccinia virus-infected cell, viral DNA (Joklik, 1962) and protein antigens (Loh and Riggs, 1961) are synthesized in the cytoplasm. It would appear, therefore, that the vaccinia virus-infected cell presents an ideal system for studying the effect of proflavine on the synthesis of virus and virus-related products. This communication will show that proflavine in low concentrations exerts a profound effect on the course of synthesis of vaccinia virus, viral antigens, and hemagglutinin. Experiments are described which provide some insight into the mechanism of proflavine interference with virus synthesis.

1 Present address: Department of Microbiology, Ohio State University, Columbus.

MATERIALS AND METHODS

Virus. The strain of vaccinia virus used in all experiments was obtained from A. W. Moyer of the Lederle Laboratories, Pearl River, N.Y. A stock virus pool was prepared from 48-hr infected KB cells and supernatant culture fluids by treatment for 1 min in a 10-ke Raytheon sonic oscillator, and was stored at −20 C.

Cell culture. KB cells were grown in screw-capped bottles or 50-mm petri dishes in an atmosphere of 5% CO₂ (Bubel et al., 1956). Cell cultures were grown in Eagle’s medium (Eagle, 1955) containing 10% inactivated calf serum.

Infectivity titrations. Samples for virus titration consisted of infected cells and supernatant culture fluids. This material was exposed to sonic vibration for 1 min just prior to virus assay. Appropriate dilutions of virus samples to be titrated were added to confluent KB cell sheets in a volume of 0.3 ml. After a 2-hr adsorption period, the infected monolayers were overlaid with a mixture of 2X Eagle’s medium and 1.8% (v/v) agar. Infected cultures were incubated further for 72 hr, and plaques were counted after being stained with neutral red. Virus titers were expressed as the number of plaque-forming units (PFU) per milliliter of culture fluid.

Hemagglutination titrations. Hemagglutination titrations were performed by adding 0.5 ml of a 0.5% suspension of chicken red blood cells in phosphate-buffered saline (PBS), pH 7.2, to an equal volume of the sample dilution to be assayed. Red blood cells were derived from chickens previously tested for high reactivity with vaccinia virus hemagglutinin. The pattern of sedimented cells was read after 2 hr at room temperature.
and the last tube showing complete hemagglutination was taken as the titration endpoint.

Antiserum was prepared in rabbits by repeated intravenous injections of fluorocarbon-purified vaccinia virus (Epstein, 1968). 

Assay for blocking antigens. The production of vaccinia virus antigens by infected cells was determined by a modification of the procedure of Oda (1963). Virus samples to be assayed for blocking antigens were first irradiated for 2.5 min with a 15-W germicidal lamp to destroy virus activity. Preliminary experiments with this system showed that the surviving fraction of a suspension of vaccinia virus containing 1.7 × 10⁷ PFU/ml was less than 1 × 10⁻⁶, indicating nearly complete inactivation of virus.

For the test, 0.3-ml samples of irradiated material to be assayed were mixed with an equal volume of antivaccinia serum diluted 1:250. Titrations with active virus had shown that this dilution of serum inactivated approximately 50% of the virus within 2 hr at 37 C. Virus-serum mixtures were then incubated for 2 hr at 37 C, and for 48 hr at 4 C. Next, 0.3-ml portions of "indicator virus," containing a total of 1 × 10⁶ PFU, were added. The mixtures were incubated for 2 hr at 37 C and for 48 hr at 4 C, after which assays for residual virus activity were carried out. Appropriate controls, such as noninfected cell lysates with antiserum and normal rabbit serum with infected cell lysates, were included. Results of blocking tests were expressed as the surviving fraction of indicator virus. This fraction represented residual virus after incubation with antiserum previously exposed to lysates of infected cells maintained in the presence or absence of proflavine. Thus, the surviving virus fraction was directly related to the amount of antibody removed by exposure to antigen-containing cell lysates.

Photoinactivation. Irradiation of virus samples was carried out in an apparatus modeled after that described by Hiatt (1960). Samples were irradiated for 3 min with opposed Westinghouse EBR photoflood lamps. Temperatures during irradiation ranged between 10 and 12 C.

Proflavine experiments. Stock solutions of proflavine hydrochloride were prepared in PBS, sterilized by autoclaving, and stored in the dark. To obviate any photodynamic effects of proflavine on vaccinia virus or virus-infected cells, all incubations were carried out in the dark, and infectivity titrations were performed in subdued light. In all experiments to be reported, a virus input multiplicity of 10 PFU/cell was employed.

RESULTS

Effect of proflavine on cells and vaccinia virus. To determine the toxic effect of proflavine on normal KB cells, selected quantities of the dye (0.5 to 10 µg/ml of cell culture fluid) were added to KB cells, and incubation at 37 C continued for 48 hr. During this period, cultures were examined microscopically for evidence of cytotoxicity. Proflavine toxicity first appeared at 6 hr, and was characterized by elongation and eventual detachment of cells from the surface of the culture vessel. Cytotoxicity became apparent at dye concentrations above 3 µg/ml. Cells removed from an environment of 2.5 µg/ml of proflavine were able to form normal-looking cell sheets upon transfer and further incubation.

Vaccinia virus suspensions were incubated at 37 C in the dark with proflavine concentrations ranging from 0.5 to 10 µg/ml. Virus assays carried out after 48 hr did not reveal a significant reduction in infectivity.

Suppression of virus and hemagglutinin synthesis and cytopathic effects (CPE) by proflavine. Cultures of KB cells were infected with vaccinia virus and freed of unadsorbed virus by several washings with PBS. Growth medium containing the desired amount of proflavine was added to the infected cultures, which were incubated at 37 C for 48 hr. At this time, proflavine-treated cultures were compared with normal infected cultures for CPE by microscopic examination. The cells and fluids were then harvested, disrupted, and examined for virus and hemagglutinin content.

The production of virus and hemagglutinin was markedly inhibited by proflavine concentrations of 2.5 µg/ml and above, as was the development of a CPE characteristic of vaccinia virus infection (Table 1). Proflavine concentrations of less than 2.5 µg/ml exerted a progressively diminishing effect on the virus infection. The small quantity

<table>
<thead>
<tr>
<th>Proflavine conc µg/ml</th>
<th>Virus conc (PFU/ml)²</th>
<th>Hemagglutinin titer</th>
<th>CPE:</th>
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<tr>
<td>0</td>
<td>8.0 × 10⁷</td>
<td>2,500</td>
<td>4+</td>
</tr>
<tr>
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<td>&lt;5</td>
<td>0</td>
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<td>3.0</td>
<td>2.7 × 10⁷</td>
<td>&lt;5</td>
<td>2+</td>
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</tr>
<tr>
<td>10.0</td>
<td>2.6 × 10⁷</td>
<td>&lt;5</td>
<td>4+</td>
</tr>
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</table>

* Plaque-forming units per milliliter of culture fluid, 48 hr after proflavine addition.
† Hemagglutinin titers are expressed as the reciprocal of the end-point dilution.
‡ The degrees of CPE rank the severity of the cellular response from rounding of cells (1+ to 2+), to aggregation of cells (3+), and detachment from the surface of the culture vessel (4+).
of virus (approximately 2.5 × 10⁷ PFU/ml) which was routinely detectable in infected cultures receiving more than 2.5 µg/ml most likely represented virus firmly bound to cells, but which did not participate in the infectious process beyond the initial adsorptive phase. Thoroughly washed, normally-infected cultures disrupted during the first 5 hr of the infectious cycle characteristically yielded virus titers of this magnitude.

Infected cultures in which virus synthesis was completely suppressed showed no evidence of a CPE characteristic for vaccinia virus (rounding, aggregation, and detachment of cells). Cytopathic responses of proflavine-inhibited infected cultures which did not produce virus were attributed to a direct cytotoxic effect of the dye. On the basis of data from these and the previous experiments, 2.5 µg/ml of proflavine was selected as an appropriate concentration for use in subsequent experiments.

**Suppression of viral antigen synthesis.** The foregoing experiments showed that proflavine profoundly altered the course of infectious virus and hemagglutinin synthesis. It was also of interest to investigate the effect of proflavine on the synthesis of viral antigens. Proflavine selected at various concentrations was added to infected KB cells, and incubation at 37°C continued for 48 hr. At this time, the cells and culture fluids were harvested, disrupted, and examined for the presence of blocking antigen. Fig. 1 demonstrates that proflavine arrested the synthesis of viral antigen in proportion to the amount added to the virus-synthesizing system. Low concentrations of the inhibitor (0.5 to 1.0 µg/ml) allowed some synthesis of viral antigen, as indicated by relatively high survival fractions of indicator virus used in the blocking antigen test (see Materials and Methods). Proflavine at higher concentrations completely suppressed viral antigen synthesis, as indicated by the consistently low surviving fractions (less than 10%) represented by the asymptotic portion of the curve.

**Effect of proflavine added during the virus growth cycle.** The foregoing experiments revealed that proflavine acted on the virus-cell complex rather than on the mature virus. To determine whether proflavine exerts a selective effect on any particular phase of the virus growth cycle, the inhibitor was added to infected cells at selected intervals after infection. At the time of proflavine addition to an infected culture, a replicate infected culture was frozen and stored at −20°C for future virus assay. Cultures receiving proflavine were incubated at 37°C until 48 hr after infection.

The results from such an experiment (Fig. 2) indicated that proflavine did not exert a selective effect on any specific phase of the virus growth...
cycle. Proflavine added during the early stages of virus synthesis (0 to 5 hr) or later (14 to 22 hr) rapidly arrested virus synthesis. Infected control cultures killed at the time of proflavine addition to replicate cultures always displayed lower virus titers than did inhibited cultures incubated until termination of the experiment. Comparison of virus titers from such proflavine-inhibited with normal cultures revealed that proflavine inhibition became maximal within 1 hr. These results are in agreement with the concept that proflavine blocked an early event in the infectious cycle, as well as the later assembly of virus from precursor materials.

Reversal of proflavine inhibition. The reversibility of proflavine inhibition in virus-infected cells was examined as follows. Virus-infected cells were exposed to proflavine, and, at selected intervals after infection, growth medium containing proflavine was removed, the cell sheets were washed twice, and fresh medium was added. Incubation was continued for 48 hr. It is manifest that infected KB cells maintained in the presence of proflavine for 14 hr were capable of producing maximal virus yields (Table 2). These data indicated that transient exposure to proflavine did not result in permanent damage to the virus-synthetic mechanism.

To study the kinetics of virus synthesis after reversal of proflavine inhibition, cell cultures were infected in the presence of proflavine. After virus adsorption, the cell sheets were washed twice with PBS containing proflavine. Growth fluid containing proflavine was replaced, and incubation was continued for 20 hr. The cultures were then washed free of inhibitor, and fresh growth medium was added. At this time replicate cultures were infected with virus and maintained in the absence of proflavine. At selected intervals, cultures from the proflavine-inhibited group and the control group were frozen and stored for future virus assay. It is apparent (Fig. 3) that proflavine did not block virus adsorption or penetration, since cells infected in the presence of the dye were capable of synthesizing virus after removal of proflavine. In such cultures, however, the appearance of newly synthesized virus could not be detected until several hours after the inhibitor had been removed. It is significant that an eclipse period, characteristic for vaccinia virus, followed reversal of proflavine inhibition. This finding again suggests that proflavine may block an early stage in the infectious cycle, e.g., interference with the viral uncoating process or the synthesis of enzymes associated with viral DNA replication (Magee, 1962; Green and Pina, 1962).

Evidence for the incorporation of proflavine into developing virus. Photosensitization of poliovirus through incorporation of acridine dyes into the maturing virus particle has been reported (Wilson and Cooper, 1962; Schaffer, 1962; Crowther and Melnick, 1961). This effect was attributed to the binding of dye to viral RNA, and the subsequent absorption of light energy by the dye-RNA complex. Consequently, photosensitization of virus by acridine dyes, such as proflavine, may be considered indicative of nucleic acid-dye interaction.

To gain some insight into the mechanisms by which proflavine interferes with vaccinia virus synthesis, KB cells were infected with virus and at hourly intervals thereafter were "pulsed" with 0.5 μg/ml of proflavine. This concentration was

<table>
<thead>
<tr>
<th>Exposure time to proflavine*</th>
<th>Final virus titer†</th>
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<tbody>
<tr>
<td>hr</td>
<td>PPV/ml</td>
</tr>
<tr>
<td>2</td>
<td>$2.1 \times 10^7$</td>
</tr>
<tr>
<td>4</td>
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<tr>
<td>0</td>
<td>$2.4 \times 10^7$</td>
</tr>
</tbody>
</table>

* Proflavine, 2.5 μg/ml, was added to cell cultures after virus adsorption, and allowed to remain in contact with infected cells for the amount of time indicated.
† Virus titers of cultures 48 hr after virus addition.

Fig. 3. Kinetics of virus synthesis following inhibition by proflavine (2.5 μg/ml). KB cells were exposed to proflavine for 2 hr prior to, and for an additional 20 hr after, infection (O). At the time of removal of proflavine from infected cultures, a replicate group (●) was infected with virus. At selected intervals, cultures from the proflavine and control group were killed and examined for virus content.
selected since it did not materially depress virus synthesis (Table 1). Proflavine was allowed to remain in contact with infected cells for 1 hr, after which the cultures were washed free of the inhibitor, and fresh medium was added. The cells were disrupted 48 hr after virus infection and the released virus was irradiated, as described in Materials and Methods. Preliminary experiments had demonstrated that vaccinia virus particles exposed to 0.5 μg/ml of proflavine for 1 hr at 37°C were not rendered photosensitive.

The selective photosensitization of vaccinia virus during the early stages of virus synthesis (Fig. 4) suggested that proflavine interacted with vaccinia DNA while this material existed in an naked form or in loose complex with other virus precursors. Proflavine-DNA interaction coincided with the period shown by Salzman (1960) to include the synthesis of the major portion of vaccinia DNA. The inability of proflavine to photosensitize during the first 2 hr of infection may be attributed to the inaccessibility of viral DNA to proflavine. This period of resistance to photosensitization is equivalent to that necessary for uncoating of vaccinia virus (Joklik, 1964). By the 8th hr, resistance to photosensitization again became manifest, most likely because the incorporation of DNA into maturing virus particles prevented further DNA-proflavine interaction.

**DISCUSSION**

The data presented demonstrate that proflavine in low concentrations profoundly alters the course of synthesis of vaccinia virus and virus-related products, thus reinforcing the findings with other animal viruses (Wilcox and Ginsberg, 1962; Franklin, 1958). Ledinko (1958) found that although infectious poliovirus synthesis was suppressed by proflavine, large quantities of noninfectious virus could be detected by electronmicrographic and serological methods. These data cannot be interpreted in the light of the present findings, since no precautions were taken to exclude a photodynamic effect. The noninfectious poliovirus produced in the presence of proflavine may have been inactivated by exposure to light subsequent to its maturation in the presence of proflavine. The present study revealed that appropriate concentrations of proflavine effectively suppressed the synthesis of virus, antigen, and hemagglutinin. Such inhibition occurred in the absence of an observable CPE, as is the case when vaccinia virus synthesis is suppressed with actinomycin D (Reich et al., 1962). Preliminary experiments, not included in this report, failed to yield electronmicrographic evidence of vaccinia-like particles in cells exposed to proflavine concentrations which did not permit virus synthesis.

Adsorption and penetration of vaccinia virus occurred in the presence of proflavine, since cells exposed to virus and maintained in the presence of the inhibitor were capable of synthesizing normal quantities of virus when placed in a proflavine-free environment. This would suggest that the proflavine acts on an early critical event of the virus synthetic cycle. Additional evidence of this action is implied by the occurrence of an eclipse period of normal time proportions, after the removal of inhibitor from infected cells (Fig. 3). An early change occurring in vaccinia-infected cells is the stimulation of DNA-synthesizing enzymes (Green and Pina, 1962; Magee, 1962), and in this regard it is significant that proflavine inhibits the enzymatic synthesis of DNA and RNA (Hurwitz et al., 1962).

That interference with virus synthesis may be reversed was indicated by data which showed that proflavine suppression was relieved if the inhibitor was withdrawn. With regard to the reversibility of proflavine inhibition, it is noteworthy that proflavine can undergo reversible binding with native DNA, and that DNA which has reacted reversibly with acridine dyes retains its biological activity (Lerman, 1961).

Proflavine in subinhibitory concentrations photosensitized maturing vaccinia virus. It is conceivable that, with low proflavine concentrations, enough of the dye was bound to viral DNA to render it light-sensitive, yet not enough to interfere with biological functions assigned to viral DNA. The indirect evidence presented here points to viral nucleic acid as one site of proflavine action, and finds support in work with other viruses. Tricyclic compounds containing amino groups
in specified positions (Crowther and Melnick, 1961) have a strong affinity for nucleic acids, and are capable of photosensitizing a variety of viruses. Mayor and Diwan (1961) demonstrated direct interaction of proflavine and tobacco mosaic virus by fluorescence techniques, and Wilson and Cooper (1962) suggested that neutral red exerts its photodynamic effect by binding to poliovirus RNA. A similar conclusion was drawn by Schaffer (1962) who studied poliovirus-proflavine binding.

Whereas the action of proflavine on vaccinia virus synthesis may be attributed to early interaction of the compound with vaccinia nucleic acid or the viral DNA-synthesizing system, this may not be the only mechanism of action. Such a conclusion seems reasonable from the fact that proflavine inhibited virus maturation at a late stage in the infectious process. It may be assumed that access of proflavine to viral DNA already incorporated into developing particles is blocked; hence, the possibility that proflavine interferes with the virus assembly process must also be considered. This is consistent with the literature dealing with the action of proflavine on phage infections (DeMars, 1955; Kellenberger and Kellenberger, 1957) and such a mechanism of action has been suggested as an explanation for the accumulation of virus precursors in adenovirus-infected cells treated with proflavine (Wilcox and Ginsberg, 1962).

ACKNOWLEDGMENT

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