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Replication of Vaccinia Virus DNA in Enucleated L-cells

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Enucleated cells and cell fragments, made from L-cells using cytochalasin B, were infected with vaccinia virus. By radioautography it was shown that factories of viral DNA synthesis were set up in enucleated cells or cell fragments. The results prove that the presence of the host cell nucleus is not necessary for uncoating of vaccinia virus and establishment of viral DNA synthesis.

1. Introduction

Uncoating of vaccinia virus DNA occurs by a two-stage process (Dales, 1963; Joklik, 1964*a*,*b*; Dales & Kajioka, 1964): (1) removal of the outer lipoprotein membrane of the virus in phagocytic vacuoles; (2) disruption of the inner or "core" protein coat in the cytoplasm of the cell, resulting in the release of the viral DNA in a deoxyribonuclease-sensitive form.

The second step uncoating is inhibited by various drugs which specifically block either RNA synthesis or protein synthesis (Joklik, 1964*b*; Dales, 1965). Thus it is presumed that the synthesis of a messenger RNA and its translation into a special protein(s) is required before the viral uncoating of stage (2). The true nature of the uncoating protein(s) remains a mystery to date.

Joklik put forward the interesting hypothesis that the vaccinia uncoating function was coded by a cellular gene present in the nucleus of the host, and that this gene was activated in response to an inducer protein brought in by the virus particle (Joklik, 1964*b*). The finding that vaccinia cores can synthesize and release messenger RNA before stage (2) uncoating reduced but did not eliminate the possibility that a host cell gene was involved in vaccinia uncoating (Kates & McAuslan, 1967*a*,*b*).

In the present study we demonstrate that vaccinia virus undergoes DNA replication in enucleated mammalian cells. This proves unambiguously that vaccinia virus uncoating does not depend on the induction of a host-cell nuclear gene.

2. Materials and Methods

Mouse L-929 cells (courtesy of Flow Labs) grown in Ham's F-12 nutrient medium supplemented with 10% fetal calf serum were cultured in 35 mm × 10 mm plastic Petri dishes fitted with 24 mm round glass coverslips. The cultures were treated with cytochalasin B (courtesy of Robert Cahn) for 13 to 16 hr at a final concentration of 10 μg/ml. or 20 μg/ml.

At the end of this time the cytochalasin was removed by washing 3 times with warm (37°C) EBSS (Earle's balanced salt solution; Gibco) and warm (37°C) nutrient medium was then added. In some cases actinomycin D (1 μg/ml.) (> 96% inhibition of [^{3}H]uridine incorporation) was added to cultures 0·5 hr before the addition of cytochalasin and left in the culture during the cytochalasin treatment. All cultures were incubated for 2 hr, at which time they were again washed 3 times with warm (37°C) EBSS. The vaccinia virus (WR strain) suspension was added to the washed cultures in 1 ml. of Ham's F-12 (Gibco) *without* serum at a final concentration of 8×10^6 plaque-forming virus/ml. (plaque-forming units/cell). The cultures were swirled and incubated for 15 min, then the virus was removed by washing 3 times with Ham's F-12 *with* serum. [*methyl*-^{3}H]Thymidine (New England Nuclear, 18 Ci/m-mole) was added to cultures and the cultures were incubated for 5 hr. After this labeling period, the coverslips were washed through EBSS, fixed in ethanol–acetic acid (3 : 1), dehydrated in ethanol and air dried. The coverslips were hydrolyzed in room temperature 1 N-HCl and autoradiographed with NTB-2 (Kodak), exposed for 5 days, developed in D19 (Kodak) and stained with crystal violet.

3. Results

(a) *Effects of cytochalasin B on L-cells*

In the presence of 10 or 20 μg of cytochalasin B/ml. the nucleus becomes segregated into a small pocket of cytoplasm that frequently may separate from the cell but still remain attached by a cytoplasmic stalk. These effects occur within one hour. Within 15 minutes after the removal of cytochalasin B from the medium, the nuclei are drawn into the main body of cytoplasm, and the cells regain their normal form. These effects are about the same as those described by Carter (1967).

Cytochalasin B has been reported to cause complete loss of the nucleus in a small proportion of L-cells (Carter, 1967; Ladda & Estensen, 1970), presumably by the chance severing of the thin cytoplasmic stalks by which nuclei remain attached to the main body of cytoplasm. In our experience with cytochalasin B, self-enucleation of L-cells occurs with a frequency of only one in several thousand cells. Enucleated cells almost as large as nucleated cells are therefore relatively rare. Small pieces of cytoplasm are found more frequently; these apparently form by partial fragmentation of cells, although we have not tried to observe the course of fragment formation. Enucleated cells or cytoplasmic fragments have never been observed in our L-cell cultures that have not been treated with cytochalasin B.

(b) *Viral infection of nucleated cells*

Under the conditions described in Materials and Methods, 70 to 90% of the nucleated L-cells treated with cytochalasin (with or without actinomycin D) and then with the virus, showed (by autoradiography) cytoplasmic foci of viral DNA synthesis (Plate I(1)). A few cells showed a single cytoplasmic focus of DNA synthesis, but most cells had several.

(c) *Viral infection of enucleated cells or cytoplasmic fragments*

In cultures treated with cytochalasin but not with actinomycin D, 41 cells were clearly identified as enucleated and 34 of these showed foci of heavy incorporation of [^{3}H]thymidine (Plate I(2a) and (2b)). Of these enucleates, 31 had a single focus of DNA synthesis and three had two foci (Plate I(3)). In cultures treated with cytochalasin and actinomycin D, 22 out of 29 enucleates showed similar foci of heavy

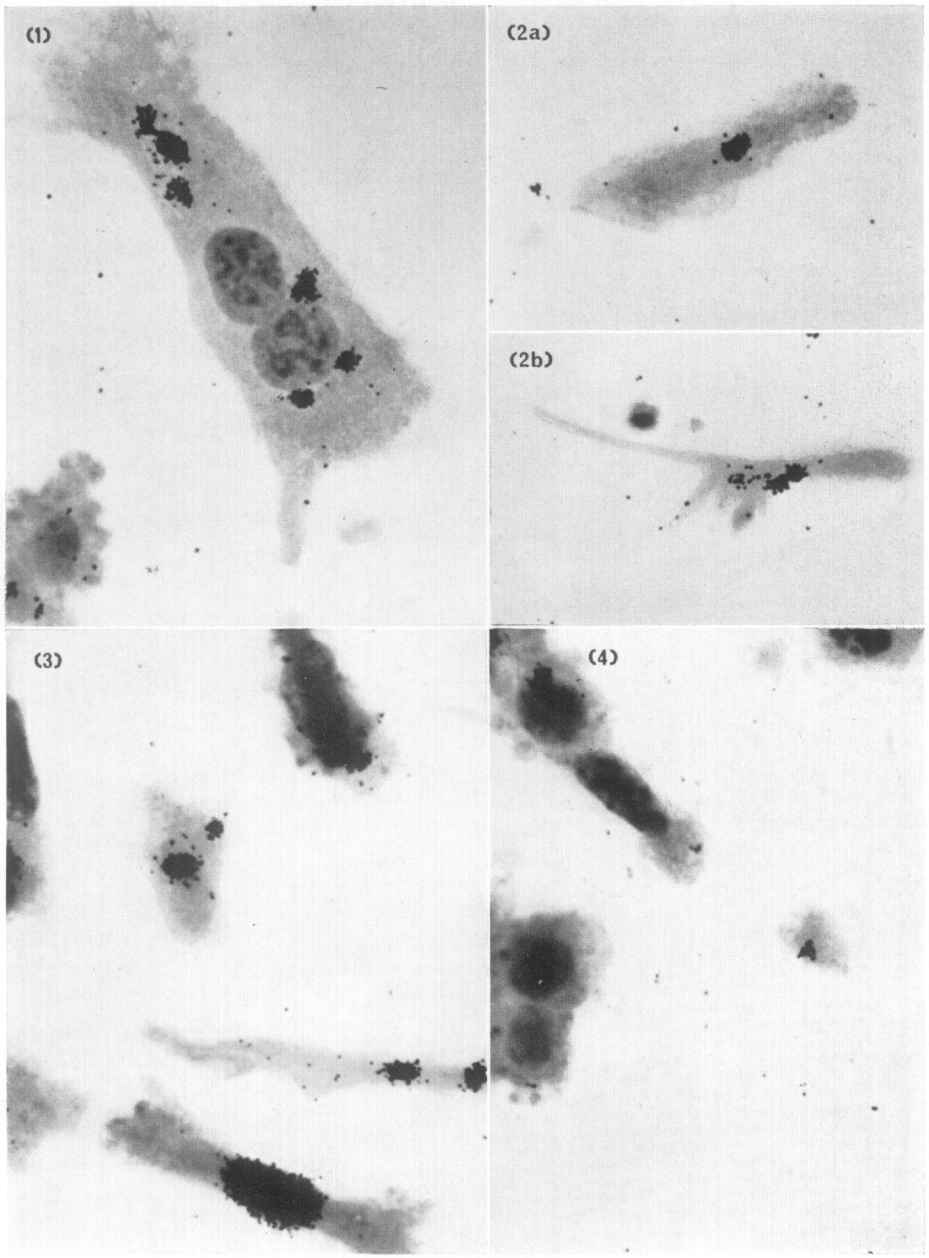

PLATE I. (1) An L-cell treated with cytochalasin B for 16 hr. The cell underwent mitosis during the treatment and has become binucleated. Cytochalasin inhibits cytokinesis. The cytochalasin was removed, the cell infected with vaccinia virus, and incubated for 5 hr with [^{3}H]thymidine in the medium. Six viral DNA factories are present in the cytoplasm as shown by the radioautograph.

(2a) and (2b) Autoradiographs of enucleated L-cells containing single foci of viral DNA synthesis.

(3) An autoradiograph of an enucleated L-cell containing two viral DNA factories.

(4) An autoradiograph of a small cytoplasm fragment of an L-cell with one viral DNA factory.

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incorporation of [^{3}H]thymidine. The use of actinomycin D to block RNA synthesis during cytochalasin treatment obviates the possibility that cytochalasin itself derepresses a host gene for uncoating.

Foci of DNA synthesis in the more numerous, small cytoplasmic fragments were infrequent. The smallest such fragment with a focus of [^{3}H]thymidine incorporation is shown in Plate I(4). The low frequency of success with fragments might be due to the small amount of surface available for contact with viral particles, or to the inability of small fragments to engulf viral particles, or to metabolic inadequacy of small fragments for support of viral DNA synthesis.

4. Discussion

At the time that Joklik proposed the cellular gene hypothesis for vaccinia uncoating, it seemed unlikely that the highly incapsidated viral DNA in the core could act as a template for RNA synthesis, particularly in view of the fact that as small a protein such as pancreatic DNase did not seem to penetrate the protein coat of the core (Joklik, 1964*a*). The activation of a host gene by a viral product had a precedent in the case of interferon induction (Magee & Miller, 1968).

Indirect evidence against Joklik's hypothesis has recently been obtained. Aside from the fact, mentioned above, that cores can synthesize RNA, it was shown that vaccinia uncoating could occur in cells whose RNA synthesis had been blocked with actinomycin D and which had been infected after washing out the drug. Such cells allowed the synthesis of viral RNA but not of cellular RNA for at least a few hours (Magee & Miller, 1968).

In another study (Magee, Levine, Miller & Hamilton, 1968) it was demonstrated that pretreatment of cells with interferon inhibited vaccinia uncoating. Interferon is quite specific in causing inhibition of viral, but not host, protein synthesis (Marcus & Salb, 1968). Thus, interferon-induced inhibition of vaccinia uncoating strongly suggests that a *viral* protein must be synthesized before the uncoating event.

Our study, in addition to showing that vaccinia uncoating occurs in cells without a nucleus, also demonstrates that vaccinia DNA replication and all of the enzyme and protein synthesis required for DNA synthesis occurs in enucleates. This suggests that vaccinia does not utilize any host enzymes that are exclusively present in the host nucleus before infection, in order to replicate the viral DNA. Since enucleates obtained by cytochalasin plus actinomycin D treatment are able to uncoat vaccinia and proceed to viral DNA synthesis, the possibility that the activity of the enucleates stems from derepression of a host uncoating gene by the cytochalasin is eliminated.

The replication of ornithosis agent (a member of the psittacosis–lymphogranuloma group recently classified as *Chlamydiae*—not true viruses; in fact, they are bacteria lacking means of energy production) has been observed in enucleated mammalian cells (Crocker & Eastwood, 1963). More relevant to our studies is the demonstration by Marcus & Freeman (1966) that poliovirus is able to infect anucleated pieces of HeLa cell cytoplasm and to initiate viral RNA synthesis.

The present demonstration of at least partial replication of a DNA virus in enucleated cytoplasm suggests an interesting possibility for future investigations in animal virology. For example, it is not known whether the replication cycle of some viruses occurs entirely in the cytoplasm or requires entry into the nucleus at some stage. Studies with enucleates could indicate nuclear involvement and pinpoint the

stage in the viral growth cycle during which the host nucleus is required. Such questions as involvement of the cell nucleus in the process of interferon-induced protection from viral infection could be determined with enucleates. Also of interest is the fate of nuclear viruses in the absence of a nucleus and in the presence of cytoplasmic viruses that produce enzymes capable of transcribing or replicating the nucleic acid of the nuclear virus.

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