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Rhabdovirus Replication in Enucleated Host Cells

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Infection of enucleated TC-7 monkey cells with rabies virus resulted in the synthesis of virus-directed RNA and the production of rabies antigens but not of infectious virus. The yield of infectious vesicular stomatitis virus from enucleated TC-7 cells, on the other hand, was almost as high as that from intact cells. Inhibition of the mitochondrial functions of enucleated cells by treatment with ethidium bromide did not influence the development of rabies antigens or the production of infectious vesicular stomatitis virus.

Simplification of the procedure for enucleating animal cells (7, 17), and hence the availability of the enucleated cells in considerable quantities for exposure to animal viruses, has made it possible to determine the extent of involvment of the nucleus in the replication of virus.

Of the three RNA viruses whose synthesis has been studied in enucleated cells, infectious poliovirus (15) and vesicular stomatitis virus (VSV) (8) replicated in enucleated animal cells but influenza virus did not (8).

This difference between the outcome of influenza and VSV infections was explained by the postulate that, whereas the influenza nucleocapsid is assembled in the nucleus (12), the VSV nucleocapsid does not have these requirements and, therefore, may grow in cells in which nuclear DNA replication and transcription is nonfunctioning (8). Since VSV is a rhabdovirus, it was of interest to extend the information on synthesis of virus in enucleated cells to other members of this group. Of particular interest was the study of the replication of rabies virus since this rhabdovirus, in contrast to VSV (1, 4), does not show activity of virus-bound RNA-dependent RNA polymerase (transcriptase) (20) and its replication is affected by a DNA inhibitor such as cytosine arabinoside (14).

For comparative purposes, we have also studied the replication of VSV in the same cell system as rabies virus and have investigated the effect of ethidium bromide (EB) on viral synthesis in enucleated cells. Since EB, through modification of the tertiary structure of circular DNA (3, 25), significantly inhibits functions mediated by mitochondrial DNA, it seemed useful to determine whether mitochondrial functions are involved in the synthesis of those viruses which replicate in cells in the absence of the nucleus.

MATERIALS AND METHODS

Cells. The TC-7 clone (18) of African green monkey kidney cells (CV-1) (10) was propagated in Eagle minimum essential medium supplemented with 10% fetal calf serum (MEM). Cells were transferred every 4 to 6 days and replated at a density of 3×10^4 cells per cm^2. Trypsin (0.25%)-EDTA (0.5%) in calcium-free and magnesium-free phosphate-buffered saline (PBS) was used for dispersion of cells.

Enucleation procedures. (i) TC-7 cells were grown to confluence (5×10^4 to 6×10^4 cells per cm^2) on 12-mm diameter glass cover slips or on 25-mm diameter plastic Thermanox cover slips (Lux Scientific Corp., Thousand Oaks, Calif.) in 30-mm Falcon plastic petri dishes at 37 C in an atmosphere of 4% CO_2. For enucleation, the medium was replaced with MEM containing 10 μg of cytochalasin B (CB) (6) (Aldrich Chemical Co., Milwaukee, Wis.) and incubated at 37 C for 60 to 90 min. Cover slips were transferred into cellulose nitrate centrifuge tubes (Beckman 1 by 3 in or $^{5}/_{8}$ by $3^{1}/_{2}$ in) containing CB medium with the cells facing the bottom of the tube. The tubes, placed in Beckman SW 27 or SW 41 buckets, were centrifuged for 30 min at 15,000 rpm at 37 C in an L-2 Beckman ultracentrifuge to draw off cell nuclei. After enucleation, cells were washed free of CB with new medium and left for 1 to 2 h in a 37 C CO_2 incubator to fully recover from the action of the drug.

(ii) In some experiments, the method described by Croce and Koprowski (7) was used. Briefly, cells were grown directly on the inner surface of a nitrocellulose ultracentrifuge tube and, after treatment with CB, were enucleated in situ. Enucleated cells were detached from the walls of the tubes by trypsin treatment, seeded on plastic or glass cover slips, and incubated in MEM for several hours (4 to 6 h) to allow the attachment and spreading of cells on the surface of the cover slips.

Enucleation efficiency was checked at the time of the termination of the experiment by staining with Giemsa and counting intact and nucleus-deprived cells. In general, from 95 to 99% of cells were free of nuclei after enucleation by both of the above methods.

Virus. Stock virus of plaque-purified ERA and HEP strains of rabies (11) and plaque-purified strain of VSV (Indiana serotype) (1) were prepared in monolayer cultures of BHK-21 cells (13) as previously described (19).

Infection of cells. Monolayers on glass or plastic cover slips of enucleated cells and, as controls, of intact cells treated and untreated with CB were infected with rabies virus at an input multiplicity (IM) of 10 PFU per cell, and with VSV at an IM of 1 or 100, or both. After 1 h of adsorption at 37 C, the inoculum was removed and cells were washed twice with MEM and incubated in the same medium at 37 C for different periods of time (see Results). In some experiments, the virus which did not penetrate the membrane was neutralized by corresponding antibodies to remove residual infectivity.

Fluorescent-antibody (FA) staining. The technique for rabies virus antigen was used as described previously (23). Fluorescein-labeled rabbit antirabies globulin was prepared from animals immunized with purified rabies nucleocapsids as described elsewhere (24). The percentage of infected cells was determined after observation of at least 1,000 cells per preparation.

Titration of virus infectivity. The infectivity of rabies virus was determined by the plaque assay technique in agarose suspended BHK-S13 cells (23). The infectivity of VSV was measured in monolayers of BHK-21 cells under a nutrient overlay containing agarose. Infectivity titers were expressed as PFU per milliliter.

Autoradiography procedure. Enucleated and normal TC-7 cells on plastic cover slips were infected with rabies virus and, after 1 h of adsorption and washing, were exposed to 10 μCi of [^{3}H]uridine per ml in MEM. Noninfected cultures of normal and enucleated cells were similarly treated with [^{3}H]uridine. After 48 h of incubation, cells were washed, fixed for 15 min in an ice-cold mixture of acetic acid-ethanol (1:3), washed for 15 min in 70% ethanol, and finally washed for 15 min in ice-cold 5% tricholoracetic acid. Cover slips were then mounted on gelatinized glass slides covered with Kodak AR 10 stripping film and exposed in the dark for 72 h. After routine developing and fixing, the cells were stained with buffered Giemsa stain.

EB. The dye EB, 2,7 diamino-10-ethyl-9-phenylphenanthridium bromide (Calbiochem, Los Angeles), was diluted in PBS at a concentration of 1 mg/ml, sterilized by membrane filtration (0.45 μm pore size; Millipore Corp.), and stored in the dark at 4 C. For cell treatment, EB was diluted at the desired concentration in MEM and used immediately. EB-treated cultures were protected from the light.

RESULTS

Presence of rabies antigen in enucleated cells infected with rabies virus. TC-7 cells were enucleated after growing either in centrifuge tubes (experiment 1, Table 1) or on glass cover slips (experiment 2, Table 1) (see Materials and Methods). After enucleation in centrifuge tubes, the cells were reseeded on glass cover slips. Cells in both experiments were then infected with two strains of rabies virus. The results shown in Table 1 indicate that treatment with CB alone had no effect on the development of FA antigen and yield of infectious virus (assayed only in experiment 1). When cells were enucleated, a smaller fraction of these cells stained for FA antigen 24 h after infection than did intact or CB-treated cells, but the FA-positive fraction of the enucleated cells increased in the next 24 h. Even then, however, the percentage of enucleated cells that stained for FA antigen was lower than that of intact cells, and a smaller number of fluorescing granules per cell was seen in the enucleated cells

TABLE 1. *Replication of two strains of rabies virus (ERA and HEP) in intact and enucleated TC-7 cells*

Expt no.	Virus	Treatment of cells			Intact cells (%)	% FA-positive cells and type of fluorescence[a]		PFU per culture ($\log_{10}$)
		CB	Centrifugation	Trypsin		24 h	48 h	
1	ERA	−	−	+	100	95 B	100 CD	6.6
		+	−	+	100	95 B	100 CD	6.4
		+	+	+	5	20 A	60 B	4.6
	HEP	−	−	+	100	80 A	100 CD	6.5
		+	−	+	100	80 A	100 CD	6.7
		+	+	+	5	40 A	80 B	4.2
2	ERA	−	−	−	100	80 B	100 CD	5.8
		+	−	−	100	80 B	100 CD	NT[b]
		+	+	−	2	60 A	90 B	3.8

[a] A, Faint fluorescence, only few small granules per cell; B, brighter fluorescence, approximately 10 to 20 small and medium granules per cell; C, bright fluorescence, more than 50 medium and large granules per cell; D, bright coalescent fluorescence, granules large when discernible.

[b] NT, Not tested.

(Fig. 1A and B) than in CB-treated but not enucleated cells (Fig. 1C). Immediately after virus adsorption and the washing of monolayers, FA antigen was not detected in either infected enucleated or intact cells.

Since, in these experiments, the yield of infectious virus from enucleated cells was 100 times lower than the yield from intact or CB-treated cells, an experiment was designed to test whether this fraction of virus represented persistence of the inoculum or actual replication of the virus.

Cover slips of TC-7 cells were enucleated and the cells then exposed to infection with ERA strain of rabies virus. Monolayers of intact cells and cover slips without cells served as a control in this experiment, and the presence of infectious virus in the three preparations was determined immediately after exposure, and again 48 h later. Only small differences were noted between the amount of virus present immediately after exposure (1.6×10^4 PFU per culture) and that recovered from cultures of enucleated cells 48 h later (4×10^4 PFU per culture). In contrast, the amount of virus recovered from cultures of intact cells 48 h after infection was 1,000 times higher (10^7 PFU per culture) than that determined immediately after exposure. In control preparations without cells, the virus titer fell 10-fold during the 48-h incubation period (10^3 PFU per culture).

Effect of antibodies on persistent virus fractions. Since the results of the preceding experiment indicated that infectious rabies virus is recovered from enucleated cells probably because of its persistence and not because of its replication, we devised the following procedure for the removal of residual infectious virus from the exposed cultures. TC-7 cells were enucleated on cover slips and exposed to ERA strain of rabies virus. After 1 h of incubation at 37 C, the cover slips were washed twice with normal medium and transferred to new petri dishes containing 10 IU per ml (2) of rabies immune serum. After the cultures were incubated 30 min at 37 C, the antibody-containing medium was removed and the cover slips were washed twice in normal medium and transferred again into new petri dishes containing normal medium. The cultures were then incubated at 37 C and assayed for the presence of infectious virus. As controls, cover slips containing the same number of intact TC-7 cells as were used for enucleation procedures were also infected with rabies virus and exposed to the action of rabies antibody.

The results of this experiment indicate that, whereas the titer of infectious virus present in cultures immediately after exposure was $10^{4.2}$ PFU per culture, no virus could be recovered from enucleated cell cultures treated with rabies antibody. In contrast, $10^{6.9}$ PFU of virus per culture were recovered from infected intact cell cultures treated with rabies antibody and incubated for 48 h. Provided that enucleated cells produce no infectious virus, the yield of virus from enucleated cells should be proportional to the residual amount of intact cells. Data shown in Table 2 demonstrate, however, that decrease in the yield of virus was much greater than would be expected from the number of intact cells in culture. This discrepancy is probably due to the neutralizing effect of residual gamma globulins drastically decreasing the virus yield in cultures containing only a small number of cells. This of course also applies to cultures of enucleated cells containing a small proportion of intact cells.

Comparative infection of enucleated cells with rabies virus and VSV. TC-7 cells (3×10^5) per plastic cover slip were enucleated. After enucleation, the fraction of residual nucleated cells was determined by counting the nuclei (see Materials and Methods) and found to be not higher than 1,000 per cover slip, whereas the number of "ghost" cells deprived of nucleus was 100,000 to 150,000 as compared to 300,000 cells seeded. Enucleated cells and cultures not subjected to enucleation were infected either with rabies virus or with VSV at multiplicity levels of 1 or 100. At the same time, plastic cover slips previously seeded with decreasing numbers of TC-7 cells (Table 2) and not subjected to the enucleation procedures were also infected.

Cultures infected with VSV and treated with the corresponding antibody virus neutralizing titer 1:3,000 (kindly supplied by H F. Clark, The Wistar Institute), showed, 17 h after infection, marked cytopathic effect characterized by cell rounding, regardless of whether or not the cells contained nuclei. Cultures infected with rabies virus showed, during the 48-h observation period, no morphological changes regardless of whether they consisted of nucleated or enucleated cells. VSV in culture medium was collected for assay 17 h after exposure, and rabies virus, 48 h after exposure.

The results of this experiment (shown in Table 2) indicate that the yield of VSV from cultures of 100,000 enucleated cells was similar to that from cultures of 100,000 nucleated cells and six logs higher than from a culture of 1,000 nucleated cells, a fraction representing those cells which may have retained the nucleus after

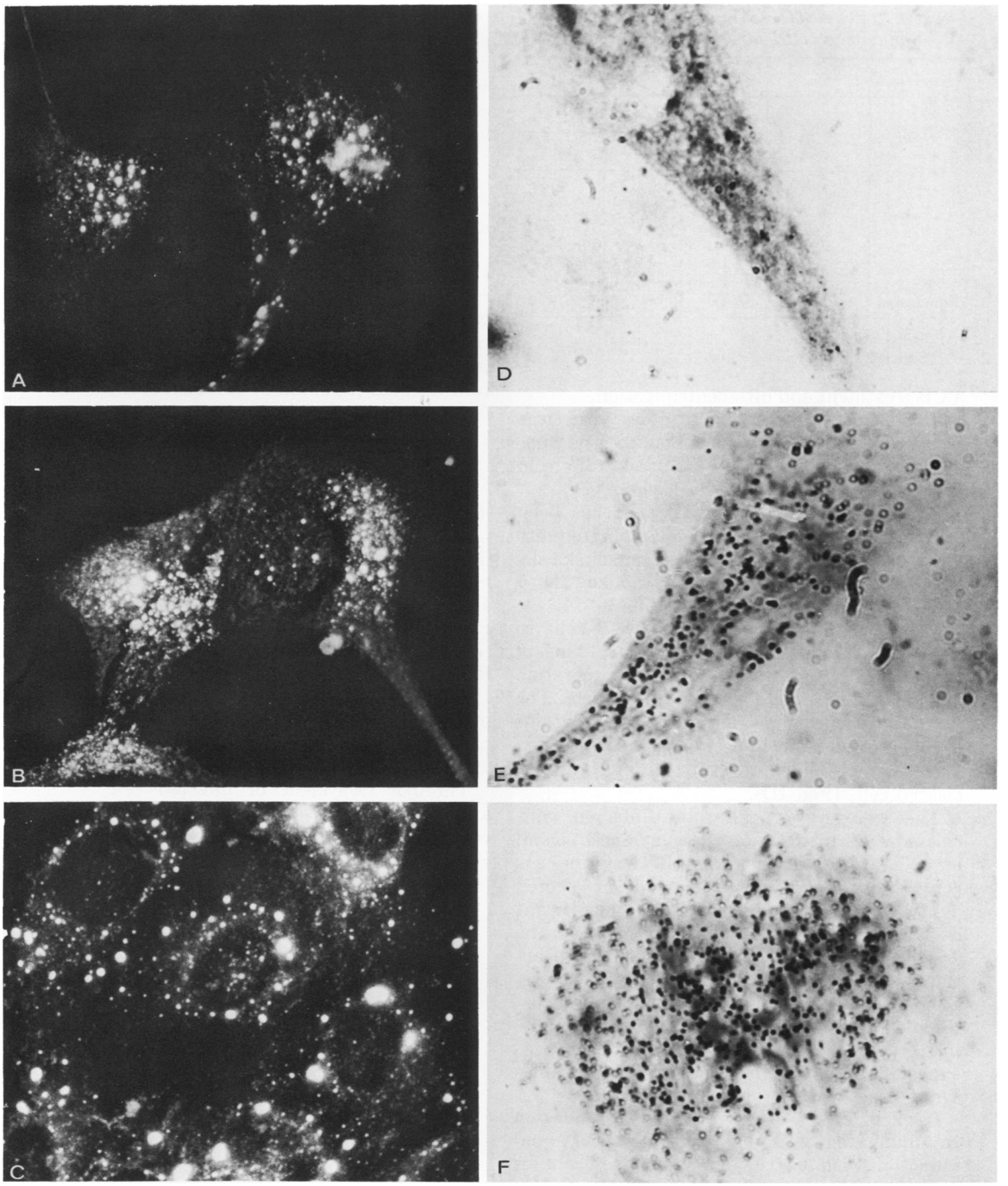

FIG. 1. *Immunofluorescent staining of TC-7 cells infected with ERA strain of rabies virus. A and B, enucleated cells; C, intact cells (CB-treated but not enucleated), 48 h after infection. Incorporation of [^3H]uridine into RNA of enucleated TC-7 cells. D, Uninfected cells; E, cells infected with ERA strain of rabies virus; F, ERA-infected cells treated with actinomycin D, 48 h after infection.*

TABLE 2. *Replication of rabies virus and VSV in intact and enucleated TC-7 cell cultures*

Cells		PFU per culture (log_{10})		
Type	No. of cells	Rabies IM 10	VSV	
			IM 100	IM 1
Intact	300,000	5.9	9.1	9.0
	100,000	6.1	8.7	8.7
	30,000	3.9	7.6	7.7
	10,000	3.3	5.3	5.0
	3,000	1.8	4.2	3.8
	1,000	1.5	2.3	1.8
Enucleated	100,000	<1.0[a]	8.3[b]	7.8

[a] Enucleation efficiency 99%.
[b] Enucleation efficiency 97%.

a CB treatment and ultracentrifugation.

In contrast, no infectious rabies virus was recovered from enucleated cell cultures as compared to the yield of $10^{6.1}$ PFU per culture from cultures containing 100,000 nucleated cells.

Rabies virus RNA synthesis. It has been shown that [^{3}H]uridine is not incorporated into an acid-insoluble form in enucleated chick cells (16). Although the enucleated monkey TC-7 cells do not incorporate [^{3}H]uridine into RNA, the exposure of the enucleated TC-7 cells to rabies virus, followed by labeling with 10 μCi of [^{3}H]uridine per ml, results in the incorporation of [^{3}H]uridine into an acid-insoluble form. The autoradiographs show numerous silver grains over the rabies-infected enucleated cells (Fig. 1E), but only a few over the uninfected enucleated cells (Fig. 1D).

The exposure of the rabies-infected enucleated cells to 10 μg of actinomycin D per ml for 48 h (at this concentration, RNA synthesis is completely shut off in nucleated, uninfected TC-7 cells) does not result in any significant decrease in the number of silver grains over the enucleated infected cells not treated with actinomycin D (Fig. 1F).

These results seem to indicate that virus-directed RNA synthesis occurs in rabies virus-infected enucleated monkey cells and that, as expected, actinomycin D does not inhibit rabies virus-directed RNA synthesis.

Lack of effect of EB on virus replication in enucleated cells. That mitochondrial apparatus remained functional in enucleated cells for at least 24 h after enucleation was indicated by the unchanged level of mitochondrial protein synthesis during this period (J. England and G. Attardi, personal communication). To determine if mitochondrial function in the enucleated cell is involved in the synthesis of VSV or rabies virus antigens, enucleated TC-7 cells were exposed to EB at concentrations known to inhibit mitochondrial functions in intact cells (3, 4) (Table 3), 1 h after infection of cells with either rabies virus or VSV and treatment with antibody. The EB was kept in the medium during the entire observation period.

The results of the experiment shown in Table 3 indicate that treatment with EB had no effect either on the development of rabies FA antigen or on the production of infectious VSV.

When enucleated TC-7 cells were pretreated with EB at 40 μg/ml concentration 1 h before infection with rabies virus, a slight decrease (from 80 to 60%) in the fraction of cells staining for FA antigen was observed in comparison to untreated enucleated cells. This difference, however, was too small to be considered significant.

DISCUSSION

The results of the experiments summarized in this paper indicate that infectious rabies virus cannot be produced in enucleated cells but that the virus-directed RNA synthesis and production of rabies antigens take place in these cells. This presents a unique situation as far as the growth of an RNA virus in enucleated cells is concerned. In the case of two other enveloped RNA viruses, the yield of infectious VSV from enucleated cells (8) was almost as high as from intact cells, whereas no virus-directed components could be detected in enucleated cells exposed to influenza or fowl-plague virus (8).

Through treatment of infected cells with antibody in the present study, it was possible to completely eliminate the "background" infec-

TABLE 3. *Lack of effect of EB on development of rabies FA antigen or production of VSV*

Concn of EB (μg/ml)	Effect on			
	Rabies		VSV	
	% FA positive cells and type of fluorescing granules		PFU per culture (log_{10})	
	Intact cells	Enucleated cells	300,000 Intact cells	100,000 Enucleated cells
None	100 BC[a]	60 B[b]	8.5	7.8
0.5	NT[c]	NT	8.3	7.5
2.0	NT	NT	8.5	7.5
4.0	100 BC	60 B	NT	NT
8.0	NT	NT	8.6	7.9
40.0	80 C[b]	60 B	NT	NT

[a] Treated with cytochalasin only.
[b] For type of granules, see legend to Table 1.
[c] NT, Not tested.

tivity of parental virus (8) and show the striking differences in the behavior of the two rhabdoviruses, VSV and rabies virus, in enucleated cells.

The fact that the fraction of enucleated cells which contained rabies-specific antigens was lower than that of intact cells is not surprising since loss of a part of the plasma membrane and some cellular disorganization after enucleation may account for this fact. It is interesting that findings similar to those observed in enucleated cells were reported for the synthesis of rabies-specific FA antigen in intact cells after treatment with cytosine arabinoside (5).

The ability of rabies virus-infected cells to maintain production of viral antigens in the cytoplasm without a concomitant synthesis of infectious virus is not surprising since the same situation was observed in chronically infected cultures (22), e.g., in infected human diploid cultures maintained for a long period of time without cell transfer (T. J. Wiktor and H F. Clark, unpublished data, and in cultures of BHK cells infected with a high IM and subjected to frequent changes of medium (9). Thus, synthesis of viral antigen in enucleated cells indicates that this process does not seem to depend on the presence of the nucleus in the host cell, whereas the assembly of the infectious virus seems to depend on its presence.

Rabies virus does not seem to contain a virus-bound RNA-dependent RNA polymerase (unpublished observations cited in 20) when this enzyme was assayed under conditions which permitted its detection in VSV (1). Thus, the contribution of host cell functions to the replication of rabies virus must be greater than in the case of VSV. Rabies virus must either carry the mRNA or the transcriptase, or, if the "undetected" virion-bound RNA polymerase is present in rabies virus in the form of a proenzyme, the host cell must provide for an "activator" of the proenzyme. Synthesis of rabies virus-specific RNA probably does take place in enucleated cells, since in the autoradiography experiment we have demonstrated clearly a significant increase in the incorporation of [^{3}H]uridine in cells infected with rabies virus over such incorporation in noninfected cells. Moreover, production of rabies FA antigen which is first observed 5 to 6 h after infection occurs concurrently with the appearance of nucleocapsids in the cytoplasm (9) and is not significantly affected by the absence of a nucleus. If viral-directed RNA synthesis occurs in enucleated cells, it cannot be excluded that the synthesized RNA may be defective in its capability to be translated into all viral proteins which are needed for late viral functions including infectivity. On the other hand, absence of some viral proteins or suppression of their function in enucleated cells may be related to other sequential steps in the assembly of the infectious virus not related to the involvement of defective RNA. Alternatively, the viral RNA may replicate in the nucleus and thus, in enucleated cells, not be available for virus assembly. Obviously, the study of the replication of other viruses in enucleated cells and the characterization of products of rabies infection in the enucleated cells by biochemical and biophysical means may throw light on this problem.

Up to now, the role of mitochondria in the intracellular synthesis of viruses has been difficult to study because of problems in establishing a concentration of inhibitors of mitochondrial function, such as EB, that, in cells containing nuclei, would act selectively only on mitochondria without affecting nuclear function. For instance, the dosage of EB necessary to inhibit replication of herpes and vaccina viruses in intact cells was considerably higher than that necessary to inhibit function of mitochondria (21) and the action of EB was probably not mediated by its effect on mitochondrial DNA. This difficulty has now been overcome by the use of enucleated TC-7 cells in which the mitochondrial function is inhibited by EB known to be inhibitory in intact cells (3, 4). This, and even higher concentrations of the drug, has not had any effect on either the development of rabies FA antigen or on the production of VSV in TC-7 cells.

These results do not rule out the possibility of finding viruses the synthesis of which may depend on functioning mitochondria. Whatever the outcome of these and other experiments, it is now clear that enucleation of cells is a valid adjunct to the study of the mechanisms of growth of viruses in their host cells.

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