Role of Mitochondria in the Production of RNA-Containing Tumor Viruses

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The role of mitochondria in the reproduction of RNA-containing tumor viruses was examined by using ethidium bromide (EB) to induce degenerative effects in mitochondria. The effects of EB in murine and avian cells were monitored by electron microscopy. Chronically infected mouse (JLS-V5) cells, in which extensive mitochondrial changes were induced, continued to produce murine leukemia virus. Also, complete reproductive cycles of Rous sarcoma virus (RSV) occurred in newly infected chicken embryo cells exposed to EB. Morphological transformation characteristic of infection of chicken embryo cells by RSV occurred in cells which contained induced aberrant mitochondria. The results demonstrate that mitochondria play a relatively minor role, if any, in the reproduction of RNA-containing tumor viruses.

A role for mitochondria in the production of RNA-containing tumor viruses has been suggested or implied by several investigators (3-5). In 1969, Gazzolo et al. (5) reported that particles with the morphology of Rous sarcoma virus (RSV) were found in association with mitochondria of hamster cells infected with the Schmidt-Ruppin strain of this virus. There were no stated observations of virions budding from the membranes of these cellular organelles, and no examination of biological activity of virions was recorded. It was suggested by these authors that mitochondria may function in the development of RSV.

Mach and Kara (9) isolated mitochondria from RSV cells by zonal and differential centrifugation procedures. By using electron microscopy and radioactive labeling techniques, they found virions in these mitochondrial isolates. In addition, infectivity of the mitochondrial fraction was demonstrated by obtaining progeny virus from chicken embryo (CE) fibroblasts exposed to this fraction. They concluded that RSV replicates within the mitochondria of RSV cells.

The present study was undertaken to define the role of mitochondria in the reproduction of oncornaviruses using the phenanthridine dye, ethidium bromide (EB).

Several investigations have shown that EB produces degenerative effects on mitochondria populations (6, 10, 13). Soslau and Nass (13)

showed alterations in the mitochondrial cristae accompanied by a loss in cytochromes a plus a_3 and b after exposure of mammalian cells to EB. Apparently, EB can intercalate with mitochondrial DNA, as well as with nuclear DNA, and inhibit DNA synthesis (16). However, selective inhibition of mitochondrial functions is more likely ascribed to an enhanced differential sensitivity of mitochondrial DNA polymerase (11) and mitochondrial RNA synthesis (18) to EB. Therefore, if viruses, or virus components, are synthesized in the mitochondria, EB should inhibit virus production. While this study was in progress, Reichert and Hare reported (12) that production of RSV was not affected by exposure of CE cells to EB.

Degenerative changes can be induced in mitochondria without severely affecting other cellular organelles. Treated cells may continue to grow and divide while exposed to concentrations of EB which have drastic effects on the morphology and physiological function of mitochondria.

An examination of the effects of EB on the mitochondria of mouse and CE cells was made initially to assess the usefulness of this dye in studies of mitochondrial function. After establishing that EB had pronounced effects on the mitochondria of these cells, an investigation was made into the effects of this mitochondrial degeneration on the reproduction of murine leukemia virus (MLV) and of RSV. The results

presented here demonstrate that both of these viruses were produced under conditions which induced degenerative effects in mitochondria.

MATERIALS AND METHODS

Cells. A cell line of mouse spleen and thymus cells (JLS-V5) which had been infected with MLV Rauscher strain and continuously releases infectious virus particles (17) was used. CE cells from 9- to 10-day-old embryos were established as primary cultures. All cells were propagated in Eagle minimal essential medium containing 5 or 10% fetal bovine serum, 10% tryptose phosphate broth, penicillin, and streptomycin. JLS-V5 cells were grown in T-75 plastic flasks (Falcon). CE cells were grown in plastic dishes (10-cm). All cells were maintained in a CO₂ incubator (5%) at 37 C.

Virus. RSV₀ mixed with the nontransforming Rous-associated virus₁, constituted the RSV used in studies involving CE cells. Cells in which infection with RSV had been established were used as the source of virus for infectivity and transformation studies. Virus was assayed by the focus formation method (14), and infectivity is described in focus-forming units. Cells were infected as described by Bader (2). Briefly, CE cells were replated from secondary cultures and, after allowing 6 hr for cells to settle, fluids were replaced with RSV (approximately 3 focus-forming units per cell)-containing medium. Thirty minutes later, unattached virus was removed, and the cells were rinsed four times with warm, minimal essential medium. Growth medium, with or without EB, was then added to the cultures.

Radioactive labeling of virion RNA. Radioactive labeling was done similarly to that reported by Bader (1). Cultures of JLS-V5 and EB-treated JLS-V5 cells were rinsed several times, and uridine-5-³H (300 μ Ci) in 5 ml of growth medium (without tryptose phosphate broth) was added. After 2 hr at 37 C, fluids were removed, the cells were rinsed, and complete growth medium was added. Six hours later, culture fluids were removed, centrifuged (600 $\times$ g, 10 min) to remove large particles, and stored overnight on ice. Virions were pelleted by centrifugation (65,000 $\times$ g for 2 hr), resuspended in buffer (pH 7.6, .01 M tris(hydroxymethyl)aminomethane (Tris), 0.001 M ethylenediaminetetraacetic acid containing Pronase (50 μ g/ml), and the viral RNA was extracted with 1% sodium dodecyl sulfate and phenol. Ethanol-precipitated RNA was resuspended and electrophoresed in polyacrylamide-agarose-mixed gels (1).

Drug. EB was prepared and stored under sterile conditions (made up in 70% ethanol) in stock solutions of 1 mg/ml. The experimental drug dosage used was 1 μ g/ml. Both stock and working solutions were stored in the cold and protected from light. JLS-V5 cells were grown in the presence of EB for a total time period of 37 days. Samples were processed for electron microscopy at 24, 48, and 72 hr and at 7, 8, 12, 21, 26, 33, and 37 days.

CE cells were treated with EB immediately after replating from primary or secondary cultures.

Nontreated cells were fixed for electron microscopy at the same time as drug-treated samples.

Electron microscopy. Cells were washed in Tris buffer, Tris-dextrose, prior to fixation in either 1.5% glutaraldehyde in cacodylate buffer or 3% glutaraldehyde in Sorenson buffer. Fixation in glutaraldehyde was followed by postfixation in Dalton chrome osmium, when cacodylate buffer was used, or in 1% OsO₄ in phosphate buffer when Sorenson was the initial buffer. All fixations were carried out in the cold. After fixation, the cells were scraped from the dishes and pelleted from the fixation or rinsing fluids at approximately 3,400 rev/min (International Clinical Centrifuge model CL) for 5 min. Pellets of cells were further processed as previously described (15).

A slight modification of the procedure was employed with the CE cells. Cells were exposed to fixative for 15 to 20 min, the plates were scraped with a rubber policeman, and then fixation was allowed to continue for 1 hr. Pellets were processed in the cold throughout dehydration. The grids were examined in an AEI-801 electron microscope equipped with a 50- μ m, ultrathin, metal (French) objective aperture and an accelerating voltage of 60 kv.

RESULTS

Effects of EB on cellular morphology and virus production in JLS-V5 cells. The effects of EB on the continuous cell line JLS-V5 were examined. Cells were exposed to EB for 37 days and observed daily by light microscopy. At various time intervals, the cells were fixed and processed for electron microscopy.

Gross morphology. A difference in growth pattern was seen between EB-treated and non-treated JLS-V5 cells as observed with the light microscope. Cells exposed to EB grew slower and appeared more elongated and refractile than did control cells. Cultures treated with EB reached their maximum cell densities before becoming confluent, in contrast to the JLS-V5 controls, which always reached confluency. EB-treated cells typically produced more acid at lower cell concentrations than did controls, as indicated by the color of the phenol red in the medium. The typical growth pattern of EB-treated cells continued with successive transfers until 26 days after treatment when these cells reverted to the normal growth pattern. The cells then became completely confluent.

Electron microscopy. Sections of control cell lines of JLS-V5 were examined by electron microscopy and were found to contain well-preserved mitochondria. These mitochondria were observed to have electron-dense granular

matrices, specific mitochondrial granules, and well-defined lateral infoldings of the inner membrane (mitochondrial cristae) (Fig. 1 and 2). The majority of the cristae were either thin folds or lamellar in appearance. The cristae were either randomly oriented or in parallel array, with some extending across the organelle (Fig. 1 and 2). Nuclei (Fig. 1) and endoplasmic reticulum (Fig. 1 and 2) were well preserved.

EB-treated cells. JLS-V5 cells exposed to EB for 24 hr were identical to control cells when cell sections were examined by electron microscopy. At 48 and 72 hr some small changes were reflected in a loss of density of the mitochondrial matrix. Also, changes in the orientation of the cristae were noted after 72 hr.

Distinct changes were observed after 7 days of exposure to EB (Fig. 3-6). The mitochondria increased in size and assumed bizarre shapes, with more than 90% of the mitochondria observed in sectioned cells affected. A large decrease in the electron density of the matrix occurred, and the mitochondrial granules observed in control cells disappeared. In many mitochondria, the low-density matrices contained what appeared to be either a granular or filamentous substance, (Fig. 3, 5, and 6). A variety of internal membrane formations were observed, and these changes in the mitochondrial cristae were the most striking effects produced by EB (Fig. 3-6). In many mitochondria, cristae were absent (Fig. 3, 5, and 6). The external membranes of the mitochondria seemed unaffected (Fig. 3, 5, and 6), and no effects of EB on the endoplasmic reticulum

(ER) or nuclei of EB treated cells were observed (Fig. 3-6).

Apparently, degeneration of mitochondria occurred progressively with time and, after 21 days exposure to EB, all the mitochondria observed in any given cell section had morphological aberrations (Fig. 4). Cell sections examined after exposure of cultures to EB for 26 days contained degenerative mitochondria as well as mitochondria which resembled those of control cells. This observation suggested that either mutant mitochondria or mutant cells which are resistant to EB developed. Cells observed 33 days after exposure to EB showed only mitochondria characteristic of control cells.

Reversal of the EB effect. The effect of EB was reversible, and cells exposed to EB for as long as 19 days reverted back to normal after the EB was removed. This reversal was not immediate, and, when the ultrastructure of cells was observed 3 days after removal of EB, the mitochondria looked essentially as they had in the presence of the drug. Eight days after removal, the mitochondrial population resembled control cells.

Virus production. Attention was also given to the continued production of virions by JLS-V5 cells exposed to EB. Virions were found in the extracellular spaces of both control (Fig. 1 and 2) and EB-treated cultures (Fig. 4-6). No difference in the morphology of virions was noted between treated and untreated cultures; virions containing electron-lucent or electron-dense nucleoids were found in both. Depending

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FIG. 1. This figure and Fig. 2-6 represent electron micrographs of nontreated and EB-treated JLS-V5 cells (glutaraldehyde fixation). All sections were doubly stained with alcoholic uranyl acetate and lead citrate. The magnification mark in each figure represents 1 μ m, in insets 0.1 μ m. Mitochondria (M) with electron-dense matrices and well-preserved cristae (small arrow) which are randomly oriented or in parallel array are observed in this field. A portion of the cell nucleus (N) is found in the upper-right-hand corner of the micrograph. Ribosomes are seen attached to membranes or endoplasmic reticulum (ER), or are found lying free in the cytoplasm. A small portion of the Golgi apparatus (G) is present. A virus particle (V) containing a nucleotide with an electron-lucent center is seen in the extracellular space. Magnification, approximately $\times 25,000$.

FIG. 2. Distinct densely staining mitochondrial granules (MG) are seen in some of the mitochondria observed here. A virion budding (VB) from the cell surface of a second cell is apparent. A few extracellular virions (V) are present. Magnification, approximately $\times 24,000$.

FIG. 3. This figure and Fig. 4-6 represent electron micrographs of ultrasonic changes occurring in mitochondria of EB-treated cells (1 μ g/ml) and also virus production in these cells. Cell sections representative of structural changes occurring at different time periods are shown. This figure shows mitochondria after 7 days exposure to EB. The density of the mitochondrial matrix has lessened and the cristae have become rearranged. Filamentous material (F) is observed within the mitochondrial matrix. The densely staining mitochondrial granules are absent. Mitochondria have changed their shapes and increased in size. Mitochondrial cristae (C) no longer have the orientation observed in control cells and have formed irregular patterns. External membranes of the mitochondria are intact (arrow). The endoplasmic reticulum (ER) has not been altered. Magnification, approximately $\times 24,000$.

FIG. 4. Extracellular virions (V) are observed in this section where aberrant mitochondria (M) are seen after 21 days of exposure to EB. Magnification, approximately $\times 24,000$.

FIG. 5. This figure and Fig. 6 show virus particles budding from cells after 7 days of exposure to EB. This figure shows a portion of cell showing virus particle (V) budding from cell surface (upper left). A higher magnification of the particle is seen in inset. The "intermediate membrane" of the particle is clearly defined. Degenerative mitochondria with filamentous material (F) and very few cristae (C) are seen. The mitochondrial matrix is of a very low density. The outer membranes (OM) of the mitochondria are seen to be unaltered. Magnification, approximately $\times 25,000$; inset, $\times 50,000$.

upon the sections being screened, there appeared to be fewer or equivalent numbers of extracellular particles in EB cultures when compared with controls. Whether the differences observed were attributable to the effect of EB or to random selection of thin sections being screened could not be determined. Nevertheless, large numbers of virions were

found associated with cells in EB-treated cultures (Fig. 4 and 6) in which greater than 90% of the mitochondria in sectioned cells showed major degenerative effects. These observations indicated that virions were being produced by cells containing defective mitochondria. Virions were found budding from the cellular membranes of cells taken from cultures ex-

posed to EB for short time periods as well as from those exposed for 7 to 21 days. These cell sections showed virus formation at the surfaces of cells which contained aberrant mitochondria (Fig. 5 and 6).

Incorporation of ^3H -uridine into virion

RNA. Cells were exposed to ^3H -uridine for 2 hr, and culture fluids were collected. Virions were sedimented by high-speed centrifugation and then were extracted with sodium dodecyl sulfate and phenol, and the ethanol-precipitated nucleic acid was analyzed by electrophoresis in

FIG. 6. Portion of a cell section showing several degenerative mitochondria (M) and virus particles budding (VB) at two different sites of the plasma membrane. Insets of each of the particles at higher magnifications are shown (bottom left and right). Other cellular organelles are unaffected. Extracellular virions with nucleoids containing electron-dense centers as well as electron-lucent centers are seen. Approximately $\times 25,000$. Insets are approximately $\times 56,000$.

polyacrylamide-agarose gels. Less than 10% of the radioactivity was found in cellular nucleic acids. The total radioactivity in viral RNA was substantial in EB-treated as well as in non-treated culture fluids (Table 1). Although fewer counts were found in EB-treated cultures, differences could be attributed partially to differences in cell number. When the amount of radioactivity in viral RNA was related to the radioactivity incorporated into the total cellular nucleic acids (Table 1), a measure of both cell number and cellular physiological activity, it was estimated that EB-treated cultures produced one-third to one-half the virions of a comparable control culture.

Cultures exposed to EB only during the labeling period contained radioactivity in virion RNA similar to nontreated cultures. Cycloheximide (10 μ g/ml), which prevents incorporation of radioactive uridine into virion RNA (1), was added to some cultures simultaneously with ^{3} H-uridine. No peak of radioactivity was found in the region of viral RNA, although substantial radioactivity was incorporated into cellular nucleic acids (Table 1).

The cultures pretreated with EB contained cells in which most of the mitochondria were degenerative, as observed by electron microscopy, demonstrating that synthesis of viral RNA and its incorporation into virions is not dependent upon intact mitochondria.

Effects of EB on CE cells and RSV. The role of mitochondria on oncornavirus reproduction was examined further by using CE cells and RSV. In this system, complete cycles of virus reproduction occurred, and possible ef-

fects of EB on both early and late stages of the reproductive cycle could be examined.

CE cells-gross morphology. CE cells treated with EB for brief intervals were not easily distinguished from control cells at the light microscopy level. There was no difference in the growth rate and plating properties of control CE cells and those treated with EB for up to 7 days, but CE cells exposed to EB could not be propagated beyond this time. An increase in acidity occurred in EB-treated cells, which was indicated by a more rapid change in color of the phenol red in the medium.

CE cells-electron microscopy. Examination of CE cells by electron microscopy revealed a general morphology similar to JLS-V5 cells. As in the JLS-V5 cells, the mitochondria were the only cellular organelles affected by EB at the dosage levels used in these experiments (Fig. 7-9), but the mitochondrial changes occurred earlier in treated CE cells. In contrast to the JLS-V5 cells, detectable changes in the rearrangement of the mitochondrial membranes were evident as early as 48 and 72 hr after exposure to EB (Fig. 8). Exposure of CE cells to EB for 3 or 4 days (Fig. 8 and 9) produced mitochondrial populations that resembled those after 7- or 8-day exposures in JLS-V5 cells (Fig. 3-6). Perhaps the higher sensitivity of CE cells to EB can be related to the recent embryonic origin of the CE cells compared with the long period in culture of JLS-V5 cells.

CE cells-virus reproduction. Cultures of CE cells with and without EB were infected with high multiplicities of RSV per cell, allowing both virus production and morphological

TABLE 1. Effect of ethidium bromide on incorporation of ^{3} H-uridine into virion RNA^a

Treatment		^{3} H in viral RNA (counts/min $\times 10^{-3}$)	Counts per min in viral RNA/counts per min in cellular RNA ($\times 10^6$)
Before labeling	During labeling		
None	None	776	52
None	None	754	60
EB ^b (7 days)	EB	403	26
EB ^b (7 days)	EB	145	20
None	EB	541	47
None	Cycloheximide	(22) ^c	(3.4)

^a JLS-V5 cells and JLS-V5 cells treated with EB for 7 days were exposed to uridine-5- ^{3} H (0.3 mCi per flask) for 2 hr. Fluids were removed, and the cultures were rinsed before replacing nonradioactive growth medium. Six hours later, culture fluids from similar cultures were pooled, and virions were concentrated by pelleting in the centrifuge. Viral RNA was extracted and separated from possible cellular contaminants by electrophoresis in polyacrylamide-agarose gels, and radioactivity was determined. The radioactivity in viral RNA was related to the total incorporation of uridine-5- ^{3} H into acid-precipitable cellular RNA.

^b EB-treated cultures plated at different concentrations of cells.

^c Counts in parentheses were distributed evenly through the gel track rather than as a peak in the region of viral RNA.

transformation to be observed in the same system. Cultures treated with EB for 2 days prior to and for 2 days after infection contained cells with degenerative mitochondria as determined by electron microscopy (Fig. 9). Likewise, cells treated only for the 48 hr prior to or immediately following infection also contained aberrant mitochondria.

Fluids from these cultures collected 24 and 48 hr after infection were assayed for infectious RSV. Full cycles of virus reproduction occurred in EB-treated cultures as is indicated by the amount of infectious virus found. Slightly less virus was found in the EB-treated cultures after the first 24 hr as compared with controls, but this difference was no longer apparent at 48 hr (Table 2). It is possible that this lag in virus production at the earlier times is caused by the effect of EB on RNA-dependent DNA polymerases (7). Quantitative estimation of cell sections which contained degenerative mitochondria was performed to insure that the majority of the mitochondria were degenerative. Approximately 75% of the cell sections counted showed major degenerative effects in three-fourths or more of their mitochondria after 4 days of exposure to EB.

Virus production in cells in which infection had been established was also investigated by doing simultaneous experiments with EB and actinomycin D. As seen in the graph (Fig. 10), production of virus decreased within a few hours in the actinomycin-treated cultures, whereas EB had no effect on virus production during this time.

The effect of EB on transformation of CE cells by RSV was also examined. In these experiments, using a high multiplicity of RSV per cell, morphological changes were detectable in all infected cultures within 24 hr, and by 48 hr transformed cells were predominant in both control and EB-treated cultures. No difference was seen between control and EB-treated cells prepared from one embryo, but a second embryo showed less transformation in treated cultures (Table 2). The extent of transformation occurring over this brief interval using high multiplicities of RSV was not quantitated.

When cells were infected with a low multiplicity of RSV and cultures were overlaid with nutrient agar, the development of foci was inhibited. This result is similar to that reported by Reichert and Hare (12). Additional studies of these preliminary findings of the effect of EB on transformation are in progress.

Electron microscopy of virus-productive

cells. Other workers (5, 9) have reported the association of virions or virion components with mitochondria, as observed by electron microscopy. In the experiments described herein, both JLS-V5 cells producing MLV and CE cells producing RSV have been examined for the presence of virions or subvirion particles in association with their mitochondria. No differences were observed between the mitochondria of virus-infected and noninfected cells, and no virions or virion components suggestive of virus synthesis within or on the mitochondria were found. Likewise, in the course of other reported (15) and unreported studies on oncornavirus-infected cells, no particles suggestive of virions were observed in association with mitochondria.

DISCUSSION

EB-induced changes in the mitochondria of mammalian and avian cells and structural rearrangements of mitochondrial membranes were observed at the ultramicroscopic level.

The production of neither MLV nor avian sarcoma virus was inhibited in cells which had drastically altered mitochondria. The disruption in mitochondrial integrity observed here probably was concomitant with EB-induced biochemical changes in mitochondria noted by other investigators (13). Therefore, it is unlikely that mitochondrial function plays any direct role in the production of RNA containing tumor viruses.

The observation of budding particles in the JLS-V5 cultures during the course of exposure of cells to EB indicates that virions were being produced by mitochondria-defective cells and that the large numbers of particles observed extracellularly were not merely residual virions produced at an earlier time. Also, because extracellular virions rapidly change from particles with electron-lucent to electron-dense nucleoids, the presence of particles containing nucleoids with electron-lucent centers among the extracellular virions is indicative of newly synthesized particles.

The interpretation of the electron microscopy data on the JLS-V5 cells was supported by the incorporation of ^3H -uridine into virions and in the avian cell system by infectivity studies. Incorporation of ^3H -uridine into the viral RNA of expressed virions demonstrated that neither viral RNA synthesis nor other virion constituents were significantly suppressed under conditions in which mitochondria were severely altered.

Deliberate infection of EB-treated CE cells

TABLE 2. Effects of ethidium bromide on reproduction of RSV^a

Treatment with EB		Focus-forming units/culture ($\times 10^{-5}$)		Extent of transformation ^b
Before infection	After infection	24 hr	48 hr	(Embryo)
None	None	240	1,190	3+, 3+
None	EB	100	1,950	3+, 2+
EB	None	200	1,090	3+, 3+
EB	EB	85	1,200	3+, 2+

^a CE secondaries were prepared in either growth medium or growth medium plus EB (1 $\mu\text{g}/\text{ml}$). After 48 hr, the cells were trypsinized and plated in 60-mm petri dishes. Cells were allowed to settle (5 hr) and then were infected with freshly harvested RSV (ca. 3 focus-forming units per cell). Cells were exposed to the virus for 30 min at 41 C, and then plates were washed 4 $\times$ with warm minimal essential medium to remove residual virus. Fresh growth medium or growth medium with EB was then added to the cells. Fluids were collected after 24 and 48 hr and assayed for infectivity.

^b Morphological transformation (1+ = first detectable, 2+ = <25% of cells transformed, 3+ = >25% of cells transformed (but not all), 4+ = fully transformed control RSV. Cell cultures established from two different embryos were used.

with RSV resulted in complete cycles of reproduction equivalent to the reproduction of RSV found in control cells. The slight inhibition in the amount of RSV produced during the first 24 hr could be attributed to an effect of EB on the viral RNA-dependent DNA polymerase. Hirschman (7) reported that EB, at a similar concentration used in this study, inhibited the *in vitro* activity of the reverse transcriptase of MLV. Any slight inhibition in virus production noted herein easily could be attributed to other physiological effects of EB on cells.

A selective action of EB on the synthesis of mitochondrial RNA compared with nuclear RNA was shown by Zylber et al. (18). Because virus production continues in cells with altered

FIG. 10. Effects of EB and actinomycin D on production of RSV. Chicken embryo cells were infected with RSV and transferred twice during the next 9 days. Virus-producing cells were exposed to EB (1 $\mu\text{g}/\text{ml}$) or actinomycin D (1 $\mu\text{g}/\text{ml}$). Drug-containing medium was collected and replaced at hourly intervals, and infectious RSV in the culture fluids was assayed and compared with fluids from nontreated cultures collected at the same times.

mitochondria, it is unlikely that RSV is transcribed on a mitochondrial template.

The data presented here, and the failure to detect any electron-microscopic evidence of mitochondrial participation in virus production, argue against the conclusions of Kara et al. (8) and Mach and Kara (9) that RSV is synthesized within the mitochondria of infected cells. Probably, the virions found in their

FIG. 7. This figure and Fig. 8 and 9 show nontreated and EB-treated chicken embryo cells. EB-treated chicken embryo cells infected with RSV (glutaraldehyde fixation-Sorenson phosphate buffer). Marker in each figure represents 1 μm ; insets 0.1 μm . This figure shows a cell section representative of nontreated chicken embryo cells showing mitochondria (M) with lamellar-like cristae (C), electron-dense matrices, and mitochondrial granules. Nuclei (N), Golgi (G), and endoplasmic reticulum (ER) are shown to be well preserved in this cell. Some vesicles of the Golgi complex are seen in this field (G). Approximately $\times 15,000$.

FIG. 8. Chicken embryo cell after 72 hr of exposure to EB. Degenerative mitochondria (M) are apparent. Nucleus (N) and endoplasmic reticulum (ER) are not altered. Approximately $\times 15,000$.

FIG. 9. Cell sections from CE cultures which have been in the presence of EB prior to and after infection with Rous sarcoma virus. Degenerative mitochondria (M) and a virus particle (VB) being produced at one of the cell surfaces are observed. Virus (V) particles are found in the extracellular spaces. A higher magnification of the budding virus particle is seen in the inset. Approximately $\times 20,000$; inset, approximately $\times 87,500$.

mitochondrial fractions were adventitiously associated with the mitochondria. One would not expect to find infectious viruses in situ associated with mitochondria because virions of oncornaviruses are completed by budding from the cell surface.

In studies using high multiplicities of RSV per cell, transformation of cells during the next 2 days was not inhibited by EB. However, by using low multiplicities of infection and nutrient agar overlays, development of transformed foci was inhibited. Clarification of the effect of EB on transformation requires further study, which is currently in progress.

Because the EB-induced changes are reversible and JLS-V5 cells eventually become resistant to continued exposures to EB, the possibility that EB-resistant cells are the ones producing virus must be considered. The incorporation of ³H-uridine into the 70S RNA of virions from cultures in which more than 90% of the mitochondrial population had degenerated indicates that cells with defective mitochondria are replicating and producing virus. Also, virions are found in JLS-V5 cell sections in which all the mitochondria are degenerative. The large yield of RSV, as determined by the infectivity tests, from CE cells with EB-induced degenerative mitochondria is inconsistent with a mitochondrial function in virus production.

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