MORPHOLOGICAL AND BIOLOGICAL STUDIES ON A VIRUS IN CULTURED LYMPHOBLASTS FROM BURKITT'S LYMPHOMA

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Plates 56 to 59

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Burkitt's malignant lymphoma of children in Africa (1, 2) is of special significance because the environmental factors which govern its distribution (3, 4) suggest that causation might depend on an arthropod vector spreading an aetiological agent (2–5). Inoculation experiments with monkeys have recently provided further indications that Burkitt's tumour might contain such a transmissible agent (6), whilst certain viruses have been found in association with a number of patients. Thus, herpes simplex has been isolated from a small proportion of biopsy specimens (7, 8) and a reovirus has been grown from biopsy material in a single instance (9). In addition, in six other cases unidentified filtrable agents have been propagated in tissue culture from various materials including tumour (10).

In the course of tissue culture experiments with samples from Burkitt lymphomas, altered lymphoblasts have been established in continuous culture from both a maxillary tumour (11, 12) and from an ovarian tumour (13, 14). These cells have the unusual ability for members of the lymphocytic series to grow *in vitro* in the absence of other associated cells (12–14); they also show certain fine structural features suggestive of malignancy (14, 15) and have been found, when examined in the electron microscope, to carry a virus (16, 13). So far, only the presence of this virus has been reported in preliminary notes (16, 13); in view of its possible significance in relation to the *in vitro* growth potential of cultured Burkitt tumour lymphoblasts, and perhaps even to the tumour itself, it was considered important to investigate further its structure and biological nature. The present communication gives a full account of the results which have been obtained.

Materials and Methods

Lymphoblast Strains.—All the experiments were done on two strains of cultured human lymphoblasts from Burkitt lymphomas. The cells of the EB1 strain (11), derived from a

maxillary tumour in a 9-year-old girl, were grown either in Eagle's basal medium or in supplemented Eagle's minimal essential medium; the EB2 strain (13), cultured from an ovarian lymphoma in a child of 7, was propagated in Eagle's basal medium. The tissue culture methods used have already been described (12, 14).

The cells of both strains were similar in their mode of growth, floating free without attachment to the glass of culture vessels, in their morphology when stained, and in their fine structural organisation; full details of these cells have been given elsewhere (11–15).

Preparation of Cells for Electron Microscopy.—The free-floating cells were fixed by squirting n suspension into 1 per cent iced glutaraldehyde, were lightly deposited after 30 minutes in the fixative, and were then washed, exposed to phosphate–buffered osmium tetroxide for 15 minutes, dehydrated in graded alcohols, and embedded in epoxy resin. Thin sections were cut on a Porter-Blum microtome and were contrast-stained with uranyl acetate for examination in a Philips EM 200 electron microscope. These techniques have been reported in full (15).

Animals.—(*a*) Newborn golden Syrian hamsters, 1 to 2 days of age, were used for the experiments. They were inoculated intraperitoneally with between 0.05 and 0.1 ml of material.

(*b*) Newborn Swiss albino and C57 black mice were inoculated intraperitoneally at 1 to 2 days of age; the dose used varied between 0.05 and 0.1 ml per animal.

(*c*) Fertile hens' eggs, free from the Rous resistance-inducing factor (RIF) (17), were obtained from Kimber Farms Inc., Fremont, California, and were inoculated after the embryos had been incubated for 8 days. The inoculum in each case measured 0.05 ml and was given by the intraallantoic, amniotic, and chorioallantoic routes to each egg.

Tissue Cultures for Virus Isolation.—Cultures of HeLa cells (S3 line), rabbit kidney cells, primary human embryonic fibroblasts, and L(MCN) cells were prepared in the laboratory. Cultures of rhesus monkey kidney cells and primary human amnion cells were obtained from Microbiological Assoicates Inc., Bethesda, Maryland, and green monkey kidney cells and primary human embryonic kidney cells from Flow Laboratories, Rockville, Maryland; human diploid cells (strain WI-38) were kindly supplied by the Wistar Institute, Philadelphia. All the cultures were set up in 13 × 100 mm soft glass test tubes with rubber stoppers, and materials to be tested were introduced into the tubes in 0.1 ml amounts.

Preparation of Lymphoblasts for Inoculation.—EB1 and EB2 cells were used either directly from culture or after storage in 50 per cent glycerol. Each cell line was inoculated without treatment, as well as after exposure to sonic oscillation or three cycles of freezing and thawing. The glycerinated cells were treated with a sonic oscillator (Raytheon Co., Waltham, Massachusetts) at 9 kc for 10 minutes, while cells derived directly from the culture medium were frozen and thawed three times, using acetone cooled with dry ice. Both treatments resulted in complete destruction of the lymphoblasts, after which cell debris was deposited by centrifugation at 500 *g* for 20 minutes and the supernatant was used as inoculum.

Haemadsorption and Haemagglutination Tests.—Haemadsorption tests were performed with chicken, guinea pig, and human group O Rh-negative erythrocytes, by the method of Vogel and Shelokov (18). Haemagglutinations with human, chicken, guinea pig and monkey erythrocytes were carried out by the pattern test as described by Salk (19).

Test for Mycoplasma.—After omission of penicillin and streptomycin from the medium for 2 weeks the EB1 and EB2 cells were tested for the presence of mycoplasma in broth and on agar, through the courtesy of Dr. Leonard Hayflick of The Wistar Institute.

EXPERIMENTAL PROCEDURE

In a first set of experiments cultured EB1 and EB2 lymphoblasts were prepared for electron microscopy and examined for the presence of morphologically recognisable virus particles.

In further experiments the lymphoblasts were studied in the electron microscope in order to determine both the morphology of any associated virus, and its relationship to the cells.

In a final group of experiments attempts were made to isolate the virus by inoculating cultured lymphoblasts, either as whole cells or as cells disrupted by sonic vibration or freezing and thawing, into animals and test tissue culture systems.

OBSERVATIONS AND RESULTS

Electron Microscopy.—

Recognition of virus particles: As already reported, particles with the characteristic morphology of a virus (Figs. 1 to 5, and 8) were found when EB1 cultures were first examined by electron microscopy after 75 days *in vitro* (16); during more than 11 months of culture the particles were subsequently seen in samples taken on the 82nd, 119th, 168th, 216th, 267th, 287th, and 334th days.

The EB2 cultures were first examined in the electron microscope and found to carry a morphologically identical virus (Figs. 6 and 7) on the 32nd day of culture (13). The virus was thereafter observed in this cell strain on the 59th, 97th, 117th, 147th, and 181st days of culture.

In both EB1 and EB2 cultures virus particles have only been found in association with cells; about 1 to 2 per cent of the lymphoblasts in a culture usually carried the virus.

Structure of virus particles: The particles present in EB1 and EB2 cells were structurally indistinguishable (Figs. 1 to 8) and occurred in two forms.

Firstly, there were particles, measuring about 75 mμ across, which often appeared hexagonal in profile and which were limited by a single membrane (Figs. 1, 2, and 4 to 8). These particles were either empty (Figs. 1, 4, and 6 to 8) or contained an inner body in the form of a ring (Figs. 1, 5, 6, and 7), a round central nucleoid (Figs. 1, 2, 4, and 6) or an elongated eccentric nucleoid frequently touching the limiting membrane (Figs. 1, 2, and 7). Such particles, which seemed to represent immature forms with nucleoids in various stages of development, were generally profuse and were located in both the nucleus and cytoplasm of affected cells (Figs. 1, 2, and 4 to 8).

Secondly, there were less numerous larger particles with a diameter of between 110 and 115 mμ which were surrounded by two membranes and always had a dense nucleoid measuring about 45 to 50 mμ across (Figs. 2, 3, 7, and 8). These large, presumably mature, virus particles were invariably cytoplasmic (Figs. 2, 3, 7, and 8).

Relationship of virus to cells: In both strains of lymphoblasts the particles occurred in cells which were either morphologically altered but intact (Figs. 2 and 4 to 6) or more frequently, degenerated and dead (Figs. 1, 7, and 8). The intact virus-containing cells always showed two or more of the following fine structural changes.

(*a*) Decreased electron opacity of the nucleoplasm in general but with concentration of the nucleoli and other dense material into small peripheral clumps (Figs. 4 and 6).

(*b*) Fragmentation of the nuclear envelope giving nucleo-cytoplasmic continuity (Fig. 6) between scattered segments of nuclear membrane which often showed the multi-layered structure found in the unique nuclear projections characteristic of EB1 and EB2 cells (15, 13) (Figs. 4 and 6).

(*c*) Altered mitochondria in which the cristae and matrix were replaced by beaded electron-opaque material (Figs. 2 and 4).

(*d*) In addition, EB1 cells with virus contained sheaves of unusual cytoplasmic structures (Figs. 2, 4, and 5) which could be seen in cross-section to have a hollow construction (Fig. 5).

In the intact cells the immature particles lay free in the nucleoplasm or cytoplasmic matrix (Figs. 2 and 4 to 6) whilst the mature particles were always surrounded by a fine membrane within the cytoplasm (Figs. 2 and 3). Evidence suggesting that immature particles matured by budding out through these membranes was sometimes found (Fig. 2).

In the dead cells with virus it was often difficult to recognise the exact site of the particles; however, immature particles usually appeared to be in both nuclear and cytoplasmic debris (Figs. 1, 7, and 8), whereas mature particles seemed to be present only within membrane-bounded vacuoles (Fig. 7) or attached to the remnants of the cell surface (Fig. 8).

Attempts at Virus Isolation—

Animals: Seventy-two chick embryos, inoculated with whole glycerinated lymphoblasts or with supernatant from sonicated or frozen and thawed lymphoblasts, were incubated for 8 to 10 days after inoculation, at which time their fluids and membranes were harvested; each type of inoculum of each lymphoblast line (EB1 and EB2) was tested in a separate group of 12 embryos. None of the chorioallantoic membranes showed evidence of pock development and the extraembryonic fluids were without haemagglutinating activity for erythrocytes of human, chicken, guinea-pig, or monkey origin.

The glycerinated lymphoblasts and supernatants from sonicated and frozen and thawed lymphoblasts of both EB1 and EB2 strains likewise failed to cause any ill effects following inoculation in newborn hamsters, newborn Swiss mice, and newborn C57 black mice. Two litters of each kind of animal, containing 5 to 7 newborns, were used for each preparation and were observed over a period of 5 weeks following inoculation.

Tissue cultures: Both cell extracts obtained from EB1 and EB2 lymphoblasts by sonic vibration or freezing and thawing, and the samples of untreated glycerinated lymphoblasts, failed to cause detectable changes in any of the 9 types of test cultures used for virus isolation. Each preparation was inoculated into a separate group of 4 tubes of each type of culture and these were then observed for 2 to 3 weeks after

which the cells and medium were passaged into fresh cultures. A total of 8 such blind passages was attempted in each case. None of the cultures in this series of attempted passages showed haemadsorption of chicken or guinea pig erythrocytes.

Where live EB1 and EB2 lymphoblasts were inoculated into the various test tissue cultures, they multiplied there readily. Despite this active growth of the lymphoblasts, no cytopathic effect was evident in any of the test cells during an observation time of 2 months.

Test for mycoplasma: No mycoplasma was isolated from the EB1 or EB2 cultures.

DISCUSSION

By all morphological criteria the virus in the EB1 cells (Figs. 1 to 5, and 8) is indistinguishable from that in the EB2 cells (Figs. 6 and 7), and the fact that each has given the same negative results when subjected to a large number of isolation procedures provides supporting biological evidence that they are two examples of the same agent.

In general appearance this virus somewhat resembles herpes simplex virus (20) and it is on the basis of what is known of the development of the latter (20) that the various particles described here have been identified as immature (Figs. 1, 2, and 4 to 8) and mature (Figs. 2, 3, and 8). Findings suggesting that maturation of the present agent may occur by budding through cellular membranes (Fig. 2) increase the resemblance to herpes, which is known to mature in this way (21, 22).

However, the mature form of the virus in the lymphoblasts was consistently smaller than epoxy-resin–embedded herpes virus (22–24) and the failure to isolate and identify it by biological methods suitable for herpes further emphasises its distinctness. This virus is also different from salivary gland viruses and from equine abortion virus, despite morphological similarities (25, 26), since the former are pathogenic for hamsters and the latter can be cultured in human fibroblasts. On the other hand, the virus described here is very similar in appearance to that associated with the Lucké frog carcinoma (27) and further resembles it in causing the production of unusual intracytoplasmic sheaves of tubules (Figs. 2, 4, and 5) (27). These structures were described in the case of the Lucké carcinoma cells as "bundles of coarse, dense filaments," their actual construction not being evident in the preparations available at that time (27). Nevertheless, it is clear that the structures found in the EB1 cells carrying virus (Figs. 2, 4, and 5) are identical in appearance with those in the frog carcinoma cells, are hollow (Fig. 5), and consist of unusual groupings of altered spindle tubules. The fact that similar altered tubules were not present in EB2 cells with virus is probably to be explained by functional differences between the two cell strains such as are evidenced by their slightly different morphologies (15, 13) and nutritional requirements (12, 14). Apart from the

sheaves of tubules, the changes observed in intact virus-containing cells were not unequivocally specific and could be regarded merely as reflecting the onset of cellular degeneration.

The negative results obtained with a wide range of procedures for virus isolation indicate that the present agent is not any ordinary known virus. It has already been pointed out above that it differs from herpes virus; it cannot therefore be equated with the herpes simplex viruses which have been isolated from certain Burkitt tumours (7, 8) and whose presence there is not surprising in view of the many cases with involvement of the nasal or oral cavities (2). Similarly, it is clearly not a reovirus such as that reported from a single biopsy sample (9), and whatever the nature of the filtrable agents described by Dalldorf and Bergamini (10) these, too, differ from it by their ability to replicate in tissue culture systems. In addition, the fact that a morphologically similar virus has been found quite recently in a new strain of cultured lymphoblasts isolated from a Burkitt tumour in a different laboratory (28) makes it unlikely that the unusual, unknown virus in EB1 and EB2 lymphoblasts has entered the cells as a laboratory contaminant.

The actual role of the virus in these cultured cells from Burkitt lymphomas is a more difficult question. Biological studies on one such cell line (EB2) have provided evidence that the agent is present as a persistent infection in a high proportion of the cells, which are therefore insusceptible to challenge infection with other known viruses (29) and which can be shown to produce a protective principle which complies with present criteria for an interferon (29). The virus would therefore appear to be replicating slowly in most or all of the cells in the cultures, but with visible infectious particles only being finally assembled in that small proportion of cells in which it was found with the electron microscope. This assembly of the virus seems to lead to eventual cell death, since the virus particles were both commoner and more profuse in debris than in intact cells.

The fact that this unknown virus has now been observed in each of three lines (Figs. 1 to 8) (28) of cultured Burkitt tumour lymphoblasts examined by electron microscopy is perhaps no more than a coincidence; this type of cell could be picking up as a passenger a particular unknown non-pathogenic agent harboured by patients with Burkitt's lymphoma. Alternatively, the cultured cells may have grown *in vitro* just because they are persistently infected with this agent, which might be responsible for their growth potential. In view of this it seems particularly important to determine whether or not the fourth existing strain of cultured Burkitt lymphoblasts, from West Africa (30), also carries a similar virus.[1]

[1] Since this paper was submitted for publication, a fifth tissue culture strain of lymphoblast (EB3) from a Burkitt lymphoma has been established and found to carry a structurally similar virus to that reported here. Epstein, M. A., Barr, Y. M. and Achong, B. G., Wistar Institute Symposia, 1965, in press.

It has not so far been demonstrated that the virus in fact induces cell proliferation, but two pieces of evidence at least hint that this could be the case and that the virus might even be of significance in the aetiology of the tumour. Firstly, the unknown virus in cultured lymphoblasts is in several ways like that of the Lucké carcinoma (27), whilst secondly, and much more important, it has been shown recently that the virus-infected EB1 and EB2 cells (Figs. 1 to 8) (29) carry an antigen immunologically closely related to a known animal tumour virus (31).

SUMMARY

Lymphoblasts of two tissue culture strains (EB1 and EB2) from different biopsy specimens of Burkitt's lymphoma have been examined in thin sections by electron microscopy, and have each been found to carry a morphologically identical virus.

The virus was observed in samples taken over many months, being present in about 1 to 2 per cent of the cells in two forms: Immature particles about 75 mμ in diameter which were seen in both the nucleus and cytoplasm; and larger mature particles with a diameter of 110 to 115 mμ, which were either within membrane-bounded cytoplasmic spaces or at the cell surface. There was some indication that the particles matured by budding through the cytoplasmic membranes.

Both types of particle occurred in dead degenerating cells or, less frequently, in intact altered cells. The characteristic alterations of the latter included margination of the chromatin, fragmentation of the nuclear envelope, beaded opaque material in the mitochondria, and, with one of the cell strains (EB1), sheaves of altered spindle tubules.

All attempts to isolate and identify the virus carried by the two strains of lymphoblasts failed. No pathological effects were caused in 8-day chick embryos inoculated either with whole lymphoblasts or extracts of disrupted lymphoblasts, using the intraallantoic, amniotic, and chorioallantoic routes, and the extraembryonic fluids of such chicks were without haemagglutinating activity for human, chicken, guinea pig, or monkey erythrocytes. Whole lymphoblasts or lymphoblast extracts were likewise without effect when inoculated intraperitoneally into newborn hamsters or two strains of newborn mice. Similar lymphoblast inocula did not cause detectable changes in 9 different test tissue culture systems even after 8 blind passages.

The nature of the unknown, unidentified virus in the cultured lymphoblasts from Burkitt's lymphomas is considered and its possible relationship to the cells discussed.

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EXPLANATION OF PLATES

All the figures are electron micrographs of thin sections cut through human lymphoblasts from two *in vitro* strains (EB1 and EB2) derived from Burkitt lymphomas.

PLATE 56

FIG. 1. Part of a degenerating EB1 lymphoblast with numerous immature particles scattered in condensed nuclear debris. The particles measure about 75 mμ in diameter, are bounded by a single membrane, and are mostly hexagonal in profile. They are either empty or contain a central structure in the shape of a ring or a dense round or elongated nucleoid. × 76,500.

FIG. 2. Portion of cytoplasm from an intact but altered EB1 lymphoblast. A mature virus particle about 110 mμ in diameter, and with two limiting membranes and a dense nucleoid, lies within a membrane-bounded cytoplasmic space (long arrow). Another particle appears to be maturing by budding into a similar space (at *x*) while two immature particles (short arrows) lie free in the cytoplasmic matrix; one of these has a dense central nucleoid and the other an elongated nucleoid touching the limiting membrane. The cytoplasm contains altered mitochondria (*m*) with cristae and matrix replaced by beaded material, and sheaves of spindle tubules (*s*) cut longitudinally and obliquely. × 42,000.

FIG. 3. Detail of a mature virus particle within a cytoplasmic space whose limiting membrane lies on the right of the field. The particle measures about 115 mμ in diameter, is surrounded by two electron-opaque membranes, and contains a dense central nucleoid 45 mμ across. × 213,500.

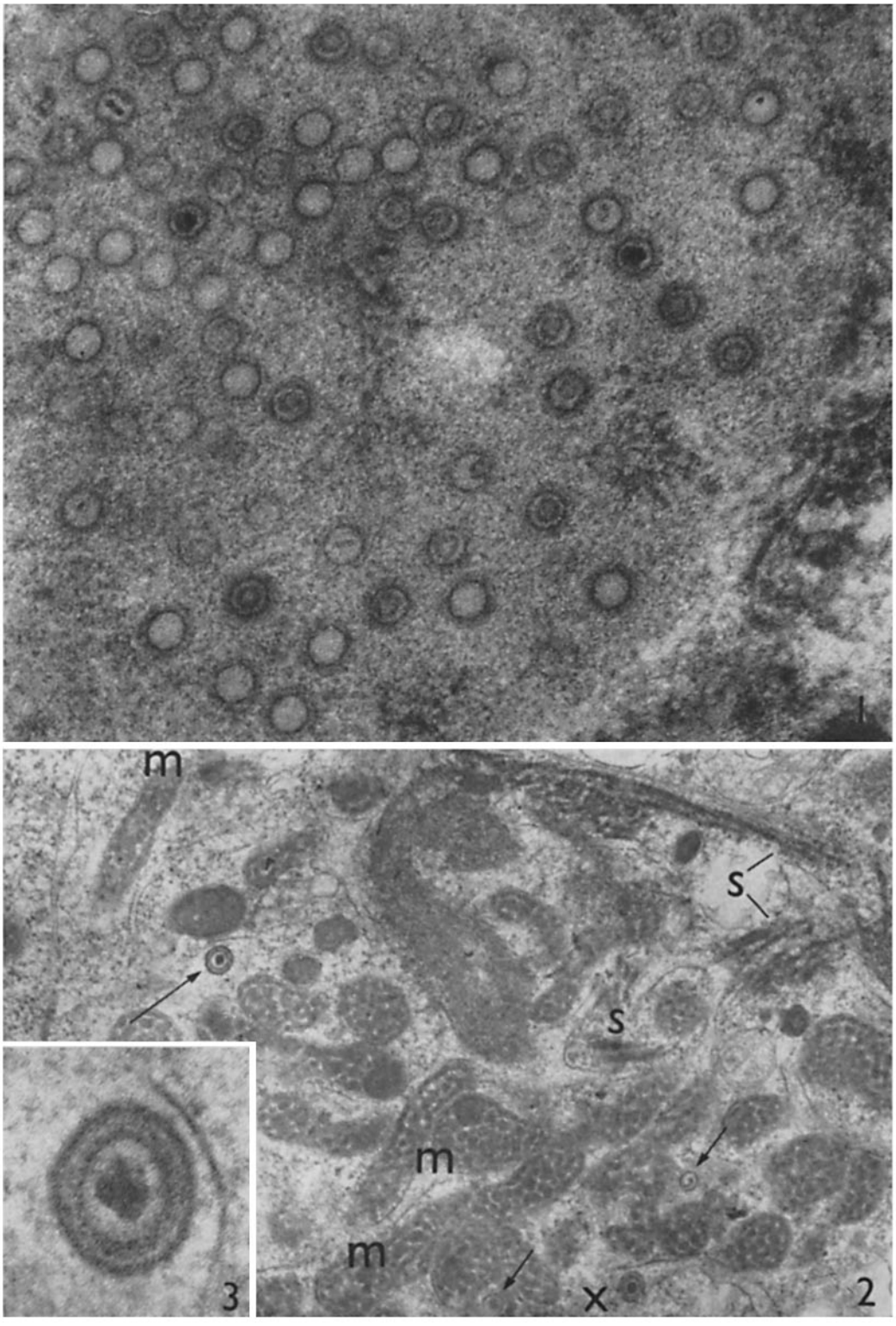

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PLATE 57

FIG. 4. Part of the nucleus (above) and cytoplasm of an intact EB1 lymphoblast with the cell surface just visible in the bottom right-hand corner of the field. The nucleoplasm is of low electron opacity apart from clumps of dense material lying against the nuclear membrane; the latter appears to be in the process of fragmentation and shows layering at two points (*l*). Immature virus particles can be seen in the nucleus at the top of the field, as at *v*, and also lying free in the cytoplasm (arrows). The cytoplasm contains altered mitochondria (*m*) with beaded contents, sheaves of spindle tubules (*s*) sectioned obliquely, Golgi elements (*g*), and masses of free ribonucleoprotein particles. × 30,000.

FIG. 5. Detail of cytoplasm in an intact EB1 lymphoblast, showing spindle tubules on the right of the field. Where these have been cut transversely, as at *t*, their tubular construction can be seen. The cytoplasm also contains free ribonucleoprotein particles, some smooth elements of the endoplasmic reticulum, and, on the extreme left, immature virus particles with a ring-shaped structure in the centre. An altered mitochondrion (*m*) is also present. × 80,500.

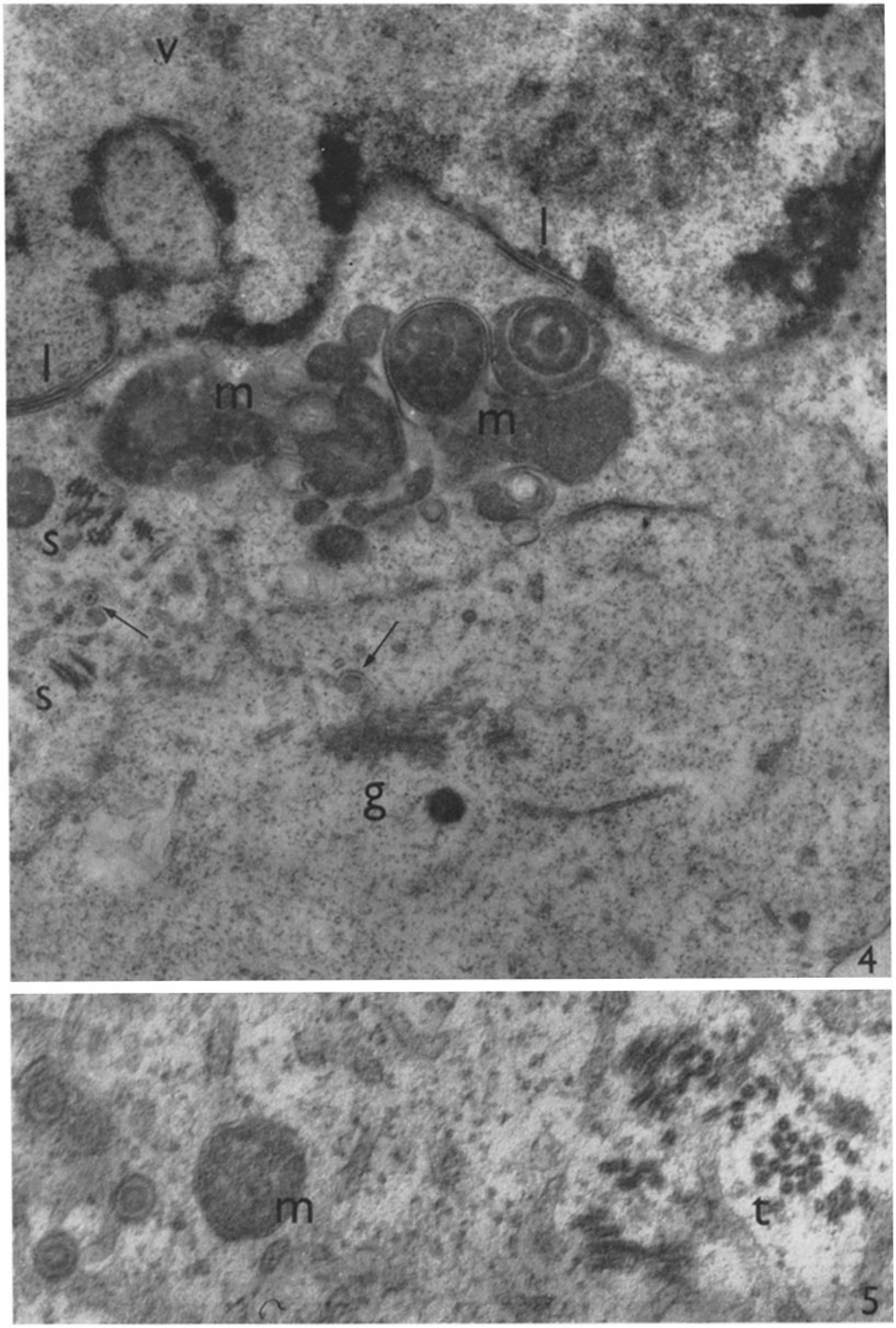

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PLATE 58

FIG. 6. Part of an intact EB2 lymphoblast. The nucleus (above) with marginated chromatin, contains immature particles both empty and with ring-shaped central structures. The remains of the nuclear membrane cross the centre of the field from side to side and show layering and interruption on the left. Three immature particles with central rings lie in the cytoplasm in the bottom right-hand corner of the field and others with dense central nucleoids are also present (arrows). Many free cytoplasmic ribonucleoprotein particles can be seen. × 62,500.

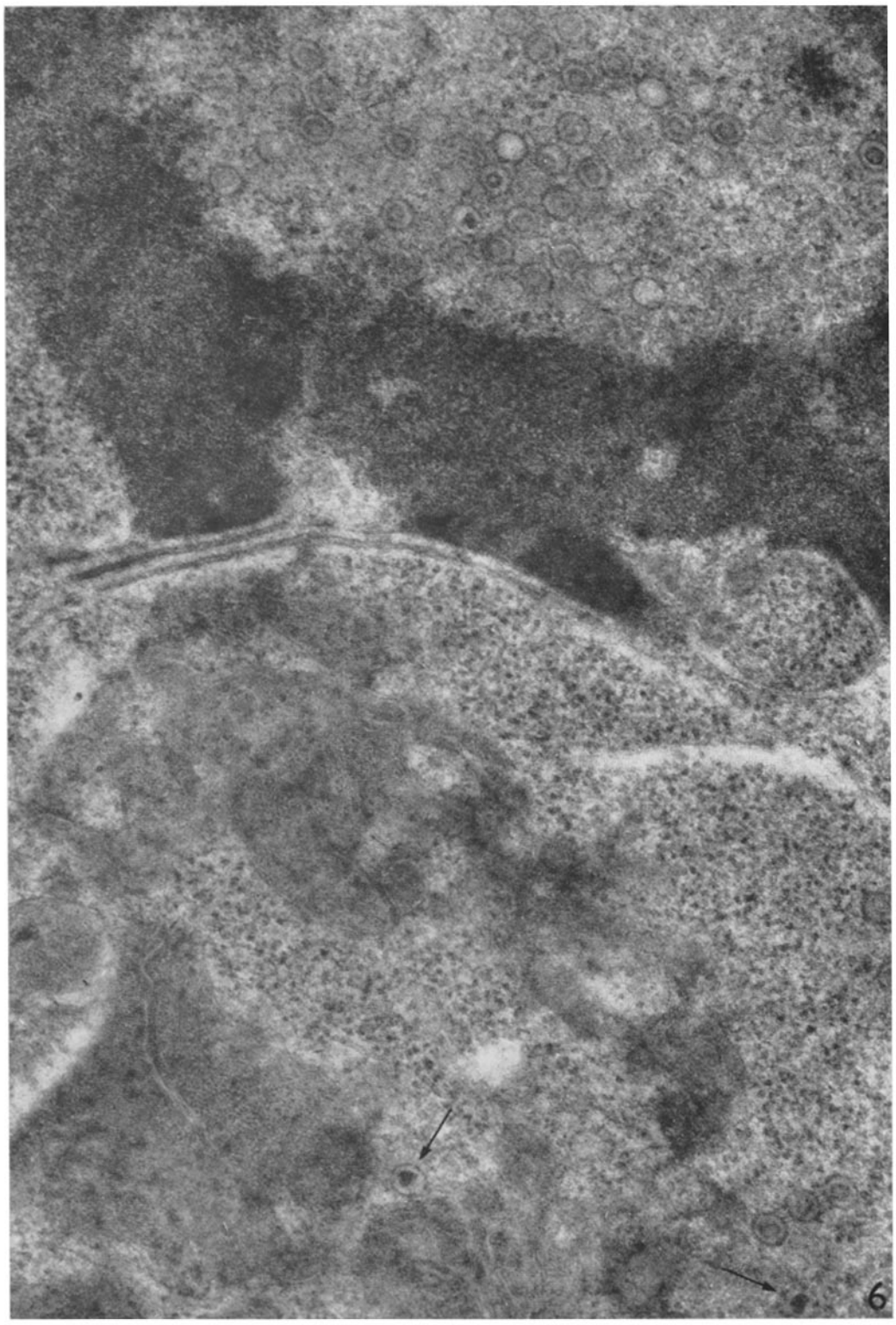

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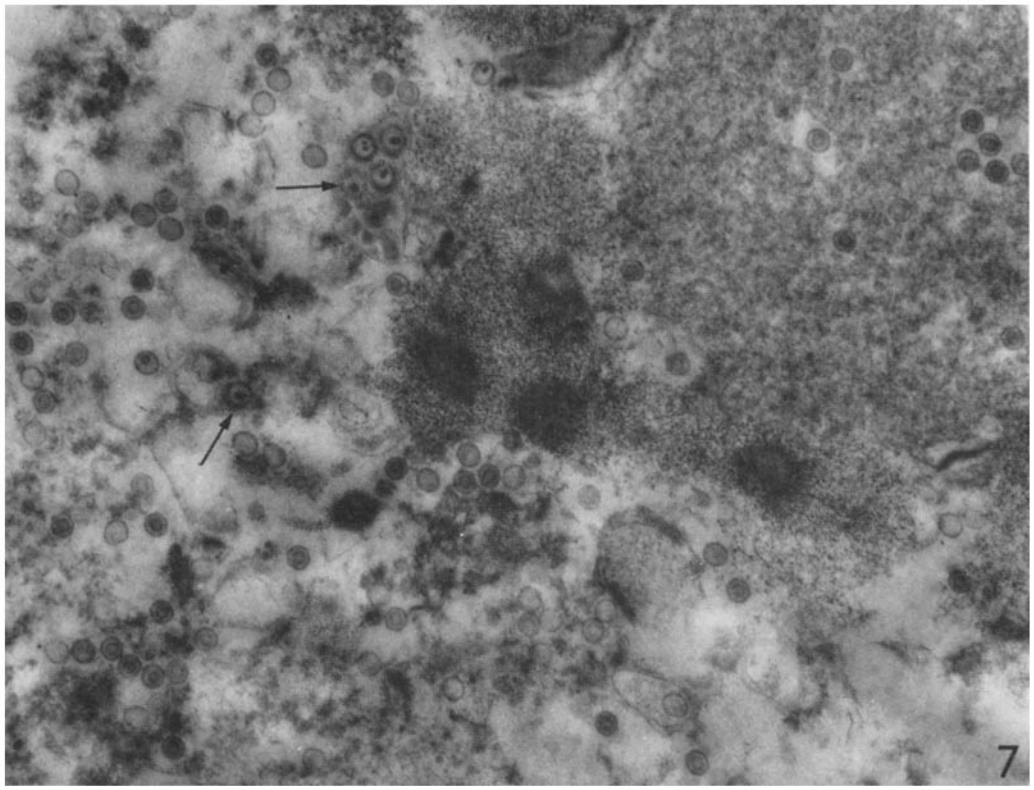
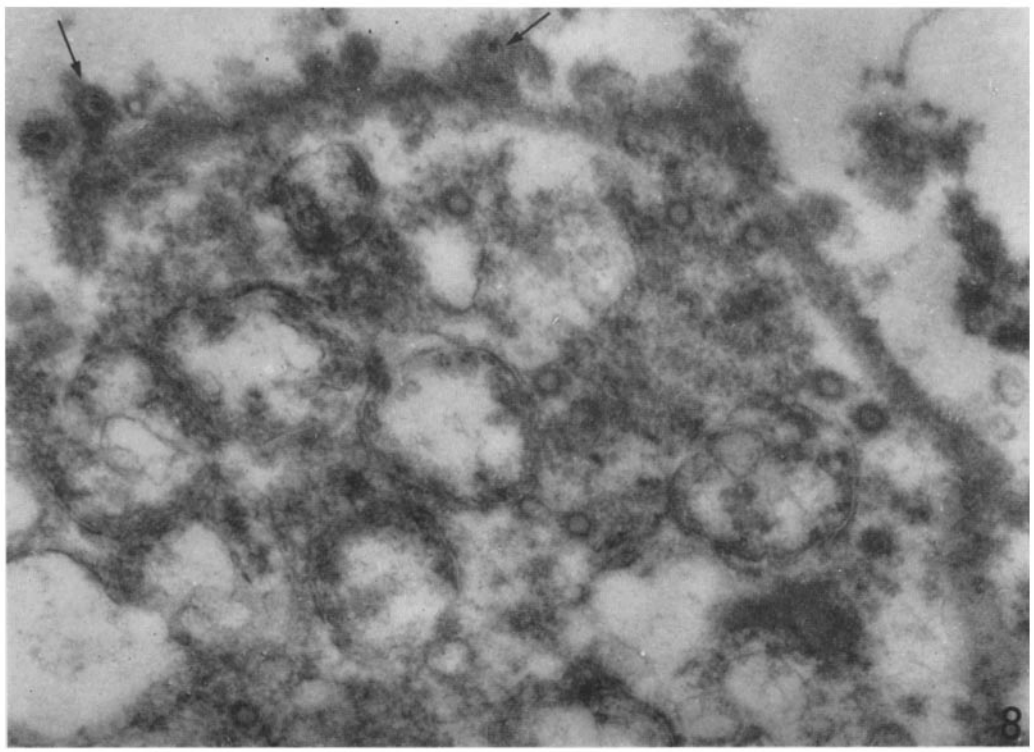
PLATE 59

FIG. 7. Nuclear and cytoplasmic debris in a degenerating EB2 lymphoblast. Immature particles with nucleoids in various stages of development are scattered throughout the field. Mature particles lie surrounded by fine membranes (arrows) in what appears to have been the cytoplasm. × 37,500.

FIG. 8. Part of a degenerated EB1 lymphoblast. Immature particles lie in the cytoplasmic debris with mature particles (arrows) attached to the cell surface. × 31,000.





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