Herpes-Type Virus Particles in Tissue Culture of Kaposi's Sarcoma From Different Geographic Regions

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SUMMARY—Typical herpes-type virus particles were observed in 5 of 8 selected tissue culture lines derived from different cases of Kaposi's sarcoma from 2 equatorial African regions, Congo and Uganda. Common and different traits of cellular morphology related to viral involvement were found. In one line, morphologic aspects and preliminary immunologic characterization suggested a virus resembling cytomegalovirus. The other 4 cases presented one important trait in common: The appearance of viruses (at an early stage in lines 13, 16, 19) in undifferentiated cells that resembled fibroblastoid or macrophage elements.—J Natl Cancer Inst 49: 1509-1526, 1972.

THE PRESENT REPORT concerns the discovery of herpes-type viruses (HTV) in 5 tissue culture lines derived from tumor biopsies of 5 cases of Kaposi's sarcoma (KS). These cases arose in 2 equatorial African regions, Congo and Uganda.

Although it is premature to suggest that such particles might be causative, contributory, or passenger agents, the presence of HTV in tissue culture lines from KS is noteworthy, since HTV have been associated with human malignancies such as Burkitt's lymphoma, nasopharyngeal carcinoma, cervical carcinoma, and possibly the sarcomatous form of Hodgkin's disease and the poorly differentiated lymphocyte-lymphoblast lymphoma (1). Furthermore, the particular epidemiology of this neoplasm with its high incidence in equatorial African regions, its frequent involvement and association with a second neoplasm, mainly Hodgkin's disease, the clinical course progressive in children while often regressive in adults, and certain histopathologic aspects with marked lymphoplasmacellular infiltration are highly indicative of a possible infectious agent (2, 3).

MATERIALS AND METHODS

Cell cultures.—The establishment or KS and skin cell lines in tissue culture from tumors or draining enlarged lymph nodes and skins biopsies of KS patients is described elsewhere (4). The KS lines studied in detail here were Kap 9, 13, 16, 19, and 22. Of these lines, Kap 13, 19, and 22 were derived from neoplastic skin nodules, and Kap 9 and 16 were obtained from highly enlarged draining lymph nodes. All 5 lines were of African origin.

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Thin sectioning.—Monolayer cultures were fixed in situ with cold 5% glutaraldehyde for 2 hours. The cells were scraped off with a rubber policeman, postfixed with buffered osmium at 1% for 1 hour, and embedded in Araldite. Grids were stained with uranyl acetate and lead citrate. Electron micrographs were made on a Siemens Elmiskop.

RESULTS

From 40 cases of KS, 51 cell lines have been established in tissue culture, some of which have now been continuously propagated for 1½ years: 25 were derived from tumor biopsies, 16 from enlarged draining lymph nodes, and 10 from normal skins. Among 8 selected lines, HTV were observed by electron microscopy in 5. In 4 (Kap 9, 13, 16, and 19) the cells exhibited morphologic changes after 2-3 months in culture and virus particles were seen at the same time. No changes were observed in Kap 22, presently in its 8th month in culture, and HTV were rare during this time. Because there were marked differences, but also many traits in common, the lines will be described separately.

Culture Kap 9

Light microscopy.—The cells of Kap 9 presented a strikingly distinct pleomorphism typical of most other KS lines. The predominant cell type described here as “fibroblastoid” was mainly pyramid shaped, and occasionally had one or more elongated processes and an enlarged globular or bean-shaped nucleus. There seemed to be no growth orientation among the cells. This was best seen in cultures which had reached confluence. Kap 9 cells as well as many others were not contact-inhibited (fig. 1a).

Morphologic changes occurred after 2 months in culture. They were characterized by the development of dense colonies consisting of altered, enlarged, nonrefractile cells, often with bean-shaped nuclei, nuclear and cytoplasmic inclusion bodies, and numerous large, round or oval refractile cells (fig. 1b).

Electron microscopy.—The cells of Kap 9 were a mixture of typical elongated fibroblasts and larger, widely spread, often angular cells (figs. 2-4) with some remarkable aspects: In the cytoplasm, microfibrils were not only developed to an extreme degree but were hypertrophied and sometimes occupied the entire ground substance (fig. 5). Pinocytosis was striking and almost formed, in some places, a lattice of continuous voluminous vesicles easily visible at the surface membrane (fig. 6). Cells with features intermediate between the typical fibroblasts and these could easily be observed (fig. 7).

Cells in which virus was found had undergone a typical modification: They were more voluminous and rounded, and the large nucleus was lightly stained and convoluted with frequent invaginations. The cytoplasm was also modified; the Golgi area in particular was hypertrophied, but the mitochondria were usually normal (fig. 8).

Virus particles were observed in the nucleus and the cytoplasm but rarely outside the cell. In the nucleus, particles were either free in the nucleoplasm or in contact with the characteristic reticular “inclusion body.” The intranuclear particles were around 100 mμ in diameter and were observed either as empty capsids represented by a shallow ring or as nucleocapsids at diverse stages of maturation with either an electron-lucent or dense nucleoid. The dense cores sometimes appeared to be free in the nucleoplasm and their passage in or out of the particles was readily illustrated (fig. 9).

In the cytoplasm the particles were either immature as in the nucleus and lay free in the ground substance, or they had acquired an outer envelope through budding at the nuclear membrane or at the Golgi vacuoles. Their size varied from 180-200 mμ in diameter, and they were inside Golgi vacuoles and multivesicular bodies. Usually they were clearly associated with round, dense, homogeneous lysosome-like bodies of diverse size (figs. 10, 11).

Culture Kap 13 and Kap 16

These lines were quite similar morphologically to the Kap 19 cells (to be described below) and underwent the same evolution. Typical HTV were seen in the electron micrographs taken 4 months after the onset of the cultures.
**Culture Kap 19**

*Light microscopy.*—The Kap 19 cultures consisted of essentially the same pleomorphic “fibroblastoid” cell types as described for Kap 9. After 3 months, refractile, roundish, mononucleated, attached cells with many pseudopodia were seen. They continued to grow and often reached 2–3 times their original volume. Concurrently small, attached-cell populations with “epithelioid” features appeared (fig. 1c).

During the following 4 weeks, the culture proliferated rapidly and various cell types were continuously released into the supernatant. The cell types were separated and a continuous mixed-suspension line was established and is presently in its 28th passage. It consists of small, round cells some of which exhibit several pseudopodia, and other enlarged, round cells with one or more nuclei. Attempts to select an attaching-cell population are in progress.

*Electron microscopy.*—Cell types typical of KS cultures were the most frequent when examination was made after 3 months in culture: typical fusiform fibroblasts and larger cells, flatly spread, and often angular with hypertrophic fibrils and unusual areas of pinocytosis. Rarely, enlarged rounded and/or elongated cells, some with many pseudopodia, were also present. In the latter type, typical HTV particles were seen in the nucleus and cytoplasm and were much more numerous in the intercellular spaces along the plasma membranes than in the Kap 9 cultures (figs. 12–14).

The few infected cells at this stage differed from the Kap 9 cells described above: No reticular inclusion bodies were in the nuclei, and the chromatin was not regularly dispersed but was extremely dense and usually concentrated along the margin of the nuclear membrane, which itself was modified so as to appear to be doubled in some areas, especially in convoluted portions (figs. 15, 16). In the distinctly nondifferentiated cytoplasm, the sparse ergastoplasm was represented by nondilated, thread-like canals, and the ribosomes were dispersed as in rapidly dividing embryonic or cancerous cells (fig. 14).

The mitochondria of some cells were profoundly modified; the cristae were hypertrophied and fragmented or curved, and occasionally bizarre so-called “beaded mitochondria” were observed (figs. 17, 18). The characteristic association of viruses with lysosomes noted in the Kap 9 cultures was not observed.

In a second sample taken from the suspension-culture line 2 months after separation from the monolayer culture, the predominant cell population consisted of rounded cells, most of which were mononucleated but in some instances were multinucleated and resembled lymphoid or lymphoreticular cells. In those cells where virus was observed, the same modifications occurred. Extracellular particles on cell surfaces were numerous, and penetration by phagocytosis was readily seen.

**Culture Kap 22**

*Light microscopy.*—This culture consisted of the same cell types observed in several KS lines. The predominant type was “epithelioid,” and spindle cells, often refractile, were rare. In the perinuclear area, large amounts of dark granulation were seen which often extended to the cell membrane. Some of these cells exhibited vacuoles. Histochemical analysis revealed strong acid phosphatase positivity, and phagocytosis of vital stain was frequent. The line is now in its 8th month of continuous culture without signs of morphologic changes (fig. 1d).

*Electron microscopy.*—The cells of a Kap 22 sample (passage 15, 8-month culture period) were similar to those of the KS cultures described above. Most were typical fibroblasts, others were flatly spread with numerous fibrils and noteworthy pinocytotic vesicles. However, in these cells abundant lysosome-like structures, usually heterogeneous with frequent myelin figures, were present (fig. 19). These structures were quite different, however, from those of the virus-associated cells of Kap 9, which were dense, completely homogeneous, and regular in contour. Typical herpes-like virus particles were observed here in only a few cells that exhibited the same nondifferentiated cytoplasm as those of Kap 13, 16, and 19 (fig. 20).

**Skin Control Cultures**

Skin cultures from KS patients (biopsies selected from apparently uninvolved areas) served as controls. In 3 cultures (Kap 8C, 19C, 33C), no
morphologic changes occurred for 6 months. Repeated searches for viruses at various passages were unsuccessful. Light-microscope studies of continuously propagated cultures revealed a typical parallel orientation of fibroblasts and a few pyramid-shaped cells. Electron micrographs of skin cells from the 3 cultures showed mainly normal, elongated fibroblasts or the larger cell type with unusual development of fibrils in the ground cytoplasm.

**DISCUSSION**

In all 8 KS lines a distinct pleomorphism was evident. In 6 cases the cell population consisted predominantly of pyramid-shaped, fibroblastoid cells. The cells of the remaining 2 showed mainly an epithelioid morphology. Electron-microscope examination of both types of cultures showed flatly spread, often angular, cells with hypertrophic fibrils and remarkable areas of pinocytosis in addition to elongated fibroblasts in various proportions. Some cells of this flatly spread type were also found in normal skins. It is impossible at this point to determine whether they are of endothelial (5-7) or mononuclear-phagocytic origin. However, in 3 cases (Kap 13, 16, and 19) after 2-3 months in culture other elements developed and became the predominant types. They were rounded cells of various sizes, some with multiple pseudopodia. Many went into suspension, and gave rise to continuously propagated sublines.

Under the electron microscope these cells were sometimes elongated but larger than mature fibroblasts; usually they were distinctly globular. They were poorly differentiated with a light cytoplasm containing few organelles and dispersed ribosomes; centrioles indicative of active multiplication were frequently observed, and mitosis was seen. Some cells of this category, occasionally greatly enlarged, developed conspicuous pseudopodia and displayed intense phagocytic activity. The mononuclear-phagocytic and lympho-reticular nature of such cells is suggested by these traits.

In relation to viral involvement it should be noted that in Kap 9, where the cell population was predominantly fibroblastoid, the modifications and lesions induced by the virus were different from those observed in the other cultures. While the infected cells became extremely hypertrophic and rounded, they retained the basic fibroblastic characters and dedifferentiation was not observed especially as far as the Golgi apparatus was concerned. Virus development was indicated by the presence of a typical inclusion body in the nucleus and the association of particles with lysosomes in the cytoplasm. These observations together with preliminary immunological characterization suggest the involvement of a virus resembling cytomegalovirus (CMV) (8, 9).

In the other cultures, Kap 13, 16, and 19, after a 3-month culture period the cell population was more heterogeneous. The cells that were susceptible to, and modified by, the virus were generally undifferentiated with fibroblastoid or macrophage features, but some in a later stage resembled cells of the lymphoreticular system. The main lesions induced by the viruses were the condensation of chromatin at the margin of the nucleus, thickening and duplication of the cell membranes, especially the nuclear membrane, increased production of fibrils in the cytoplasm and alteration of the morphology of mitochondria. This picture resembles in some aspects that described for other HTV-associated human malignant diseases such as Burkitt's lymphoma (10) and nasopharyngeal carcinoma (11).

It is important to mention culture Kap 22 separately. Under light microscopy the cells were predominantly epithelioid with high phagocytic activity and strong acid phosphatase positivity. No morphologic changes were observed during the 8-month culture period, in contrast to the other cultures. When typical HTV were found, on extremely rare occasions, the infected cells presented the same nondifferentiated cytoplasm as was observed in Kap 13, 16, and 19, which suggests that most cells in this culture were of mononuclear-phagocytic nature.

In conclusion, in all culture lines in which viruses were found, similarities as well as differences in morphology were observed. In Kap 9, morphologic aspects and preliminary immunologic characterization suggest a virus resembling CMV. The other 4 lines present the common, important trait of having viruses (at an early stage in cultures of Kap 13, 16, and 19) in undifferentiated cells.
HERPESVIRUSES IN KAPOSI'S SARCOMA CULTURES

resembling fibroblastoid cells or macrophages. Although there are common ultrastructural traits in 3 cultures reminiscent of Epstein-Barr virus infection, it has never been demonstrated that this virus could replicate in cells other than those of lymphoreticular origin (1).

REFERENCES

FIGURE 1.—Light micrographs of KS in tissue culture. a) Kap 9 A (passage 15); predominant cell type is fibroblastoid with striking disoriented growth pattern. b) Kap 9 B (passage 2); colony of altered round, oval, refractile and “epithelioid” cell, often with bean-shaped nuclei and inclusion bodies. c) Kap 19 (passage 3); note development of individual, highly enlarged rounded refractile cells, often with many pseudopodia and small round cells. d) Kap 22 (passage 15); the predominant cell type considered as epithelioid; note spindle-shaped elements in center. Phase contrast. X 360
Figures 2, 3.—Low-power view of tissue culture cells from Kap 9 to illustrate 2 main types of cells. Figure 2: typical elongated fibroblasts; figure 3: flat angular type of cell. Note also a typical fibroblast. Figure 2: × 4500; figure 3: × 2100
Figures 4, 5.—Higher magnification of 2 cell types typical of Kap 9 and most KS cultures. Figure 4: note typical canalar ergastoplasm; figure 5: note angular shape of cells and abundance of fibrils. Figure 4: $\times 25,500$; figure 5: $\times 5500$
Figure 6.—Details of cytoplasm of angular cell type. Numerous fibrils and remarkable development of pinocytotic vesicles are clearly visible. Small segment of cell surface is at top right. $\times$ 85,200
Figure 7.—Low-power view of cell with features intermediate between the 2 types already illustrated. Typical fibroblastic cytoplasm (horizontal part of cell) with hyperfibril development and pinocytotic vesicles (vertical part of cell). \( \times \) 3650
FIGURE 8—General view of a Kap 9 cell infected with HTV. Note its large size and rounded shape. Typical reticulate inclusion body is in nucleus. In cytoplasm viruses are associated with lysosome-like bodies. Note also the advanced differentiation of the cell and hypertrophy of the Golgi region (G). × 11,200
FIGURE 9.—Highly magnified segment of nuclear inclusion body showing fine granular structure and topographic association with immature virus particles. These are typical of herpes and occur either as empty capsids or nucleocapsids at diverse stages of maturation with an electron-lucent or dense nucleoid. Core material may be observed free in nucleoplasm (arrow) and/or penetrating or erupting from capsid (double arrows). Note immature nucleocapsid in cytoplasm. Nucleus (N), cytoplasm (Cy), nuclear membrane (NM). X 94,000
Figures 10, 11.—Details of cytoplasm of infected cells illustrate remarkable relationship between viruses and lysosomes. Note both structures together inside vacuoles of Golgi area. A typical Golgi (G) is in figure 10. Figure 10: $\times$ 36,000; figure 11: $\times$ 43,200
Figure 12.—Low-power view of Kap 16 cells illustrates fibroblastic cell type and the round or elongated type with light undifferentiated cytoplasm. × 25,650
FIGURES 13, 14.—Two examples of light undifferentiated cell types from Kap 13, 16, and 19. Figure 13 (Kap 13): another view of nondifferentiated types showing unusual phagocytic activity illustrated by formation of pseudopodia. *Note* virus particles on cell surface at right. Figure 14 (Kap 19): Higher magnification illustrates poorly differentiated cytoplasm; ground cytoplasm is represented mainly by dispersed ribosomes. HTV-induced lesions at right are margination of nuclear chromatin and modifications of mitochondria. Figure 13: × 14,000; figure 14: × 11,000

GIRALDO, BETH, AND HAGUENAU
Figures 15, 16.—Typical nuclear membrane modification of infected Kap 19 cells. Figure 15: general view illustrates margination of chromatin, figure 16: higher magnification shows margination of chromatin and thickening and duplication of nuclear membrane (arrows). Figure 15: $\times 36,000$; figure 16: $\times 44,000$
Figures 17, 18.—Typical modifications of mitochondria (fig. 17), so-called "beaded" mitochondria. Polymorphic changes are seen in figure 18. Figure 17: × 36,000; figure 18: × 36,000
Figure 19.—Fibroblastic cells with macrophagic inclusions typical of Kap 22. × 5600

Figure 20.—Note undifferentiated aspect of cytoplasm of infected cell. Two viruses are present, one in nucleus, another in cytoplasm. × 8000. Inset: higher magnification of HTV particle embedded in cytoplasm. × 80,000