ELECTRON MICROSCOPE STUDIES INTO THE MORPHOLOGY AND LOCALIZATION OF OMSK HEMORRHAGIC FEVER VIRUS IN INFECTED TISSUE CULTURE CELLS

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ELECTRON MICROSCOPE STUDIES INTO THE MORPHOLOGY AND LOCALIZATION OF OMSK HEMORRHAGIC FEVER VIRUS IN INFECTED TISSUE CULTURE CELLS

[Following is the translation of an article by N. M. Shestopalova, V. N. Reyngold, I.N. Gavrilovskaya, A.P. Belyayeva and M.P. Chumakov, Institute of Poliomyelitis and Viral Encephaliteses, USSR Academy of Medical Sciences, Moscow, published in the Russian-language periodical Voprosy Virusologii (Problems of Virology) No 4, 1965, pages 425--430, It was submitted on 11 Jul 1964. v/0 Translation performed by Sp/7 Charles T. Ostertag, Jr.]

The Omsk hemorrhagic fever (OHF) virus was isolated by M.P. Chumakov, A.P. Belyayeva, A. V. Gagarina and coworkers in 1947--1948 from the blood of a patient during a febrile condition and also from Dermacentor pictus and D. marginatus ticks -- carriers of this disease /5/. The isolated virus possesses a wide spectrum of pathogenicity. In infected animals it accumulates in large concentrations in the brain, blood, spleen and other internal organs, and is discharged with the urine /1/. During cultivation outside the organism it multiplies in epithelial normal and tumorous cells, in cultures of fibroblasts, HeLa cells /6/, Sots, in transplanted cultures of human angiosarcoma /3/, and in the primary and transplanted culture of swine embryo kidney /2/.

In antigenic properties the OHF virus is closely related to other viruses of the Ixodes tick group of tick-borne encephaliteses. The virus filters through the smallest pores. In the first investigations of imprint-preparations from chick embryo membranes, in which the OHF virus multiplies, stained by the Romanovskiy-Giemsa method and investigated with a light microscope, no specific changes and elementary bodies were detected /1/.

The mission of this work is a study of submicroscopic changes in tissue culture cells after infection with the OHF virus, uncovering the localization of the virus, investigating its morphology and clearing up the bond between virus multiplication and cellular organoids. Investigation with a light microscope, making it possible to expose specific changes in the structure of infected cells, was carried out preliminarily with the aim of a specialized selection of cells, infected with the virus, for study in the electron microscope by the method of ultrathin sections. A detailed cytological and cytochemical investigation using the fluorescent antibody method was performed by V. Ya. Karmysheva, I.N. Gavrilovskaya and M.P. Chumakov.

Materials and Methods

In the work we used the transplanted tissue culture of a pig embryo kidney, in which the clear cytopathic action of the OHF virus is manifested. The cells were suspended in a medium consisting of 0.5% enzymatic lactalbumin hydrolyzate, 10% bovine serum and Erle balanced solution. For sustaining

the culture we used medium No 199 with 2% bovine serum. The culture was incubated on narrow microscope slides inserted in test tubes. After formation of a monolayer on the slides a suspension of OHF virus (Goloshubin strain), preliminarily passaged 15 times on mice, was placed in the test tube. Infection was performed on the basis of 3 LD_{50} per cell. A noninfected culture served as the control. The material was fixed 2 hours after infection and then daily for 5 days. For fixation we used: 1) 1% solution of osmic acid, buffered according to Kolfild; 2) Buen fluid; 3) a buffered solution of neutral formalin. After fixation in Buen fluid preparations of control and infected cultures were stained with hemotoxylin and eosin; the fixed preparations were treated in formalin by the Felgen method. After fixation in osmic acid and drenching in methacrylate the material intended for electron microscope study was investigated in a phase contrast microscope. Cells with specific changes were found, they were labeled and separated for the preparation of ultrathin sections. For electron microscope investigation the cell monolayer, incubated on glass, was fixed and dehydrated by the generally accepted method. Subsequently we used the drenching technique by way of plane-parallel bi[illegible]lastic plates in the form of microscope slides for light microscopy /4/. The ultrathin sections were prepared on a LKB 4800 A ultratome, contrasted with uranyl acetate /8/ and lead salts /7/, and studied with the help of an IEM-5Y electron microscope at magnifications of 4800, 15,700 and 47,600. The work regimen of the microscope was specified by the following parameters: Accelerating voltage 80 kilovolts, intensity of current of electron beam 30--35 microamperes, diameter of aperture diaphragm 30--40 microns, diameter of condensor diaphragm 180--200 mg.

Results

The results of the preliminary light microscope study of preparations of the control and infection culture showed that infection with OHF virus is accompanied with the appearance in the cells of distinct changes in the fine structure of the nucleus and the cytoplasm with the formation of oxyphilic cytoplasmic inclusions. For electron microscope investigation we used the culture from 2 days after infection, since by this time there were all the types of cell affections which were observed during the 5 days observation; the only change was in the quantitative ratio of cells with this or that degree of infection depending on the length of time after inoculation of the virus. In the control culture, corresponding to 2 days after infection, we observed epithelioid cells forming the main mass of the cellular layer (figure 1, A; see inset between pages 424--425). These cells have round or oval nuclei, somewhat differing in dimensions, with finely pulverized chromatin and several nucleoli. The form of the cellular body is round or oval, sometimes polygonal. The cytoplasm has a delicate netlike structure. The cells lie close to each other, forming a layer of the membrane type. Sometimes between the epithelioid cells there are cells of a spindle-shaped form with more dense nuclei and a more dense cytoplasm. These are apparently cells of connective tissue origin.

By this time among the cells of normal structure in the preparations of the infected culture there are foci of affected cells (see figure 1, B). There is a noticeably expressed increase in the dimensions of the nuclei and the cellular body and a sharp change in the nature of the relative disposition of the cells. Inclusions are exposed in the cytoplasm of the paranuclear area. They have the appearance of oxyphilic zones of a roguhened fibrous structure. In preparations stained with hemotoxylin and eosin they are stained with eosin in a saturated orange-red color and are noticeably different from the color of the remaining cytoplasm. In preparations treated by the method of Felgen with light green dyeing they stain an intensive green color. The degree of intensity of staining and the density of these sectors vary in preparations from the same period. Cells are exposed with a delicate network located near the nucleus on one side or surrounding the nucleus. We encountered cells with compact oxyphilic masses with an irregular form and outgoing thinning clearly outlined appendages. The dense inclusions are surrounded by a light corona. The nuclei of infected cells are enlarged, many of them have lobes contracting into the cytoplasm. Cases are observed of the destruction of the nuclear membrane and the appearance in the cytoplasm of a Felgen positive substance in the form of accumulations of fine granules, surrounded by a membrane or dispersed freely. The nucleolus swells and changes its form.

By using the phase contrast microscope to study preparations fixed with a buffered solution of osmic acid and flooded in methacrylate, it was possible to detect in the infected culture cells with cytoplasmic dense masses in the paranuclear area. Based on the nature of distribution and external appearance they corresponded to the previously described inclusions.

By electron microscope study of ultrathin sections of infected cells it was cleared up that the sectors of the cytoplasm with the netlike structure corresponded to the inclusions revealed with the help of the light microscope (figure 2, A). These sectors were situated near the nucleus in the form of thickly interwoven membranes of a changed endoplasmic network and Golgi complex. An osmiophilic substances is contained in the reservoirs of the endoplasmic network, the vesicles and vacuoles of the Golgi complex. Thanks to the great density of this substance and the heavy accumulation of membrane structures the zone of inclusion stands out on the background of the surrounding cytoplasm.

The latter is a light hyaloplasm with sections of reservoirs of the endoplasmic network (for the most part agranular) seldom situated in it, mitochondria and lipid bodies. In infected cells the lipid bodies have an irregular form of pitted lobed formations distributed frequently in the vicinity of the inclusions. The nuclear membrane delimits the fine granules and fibers of nuclear substance from the cytoplasm. In some sectors around the nuclear membrane there is a clearly expressed perinuclear reservoir, in others it is absent, and the nuclear membrane has an eroded nature or is completely destroyed.

Viral particles are detected in the netlike zones, which are sectors

of changed and intensively developed membranes of the endoplasmic network of the Golgi complex. The viral particles are distributed in the reservoirs of the endoplasmic network and in the vesicles of the Golgi complex. The individual viral particle in the plane of the section has the form of a round or ground corpuscle with a high electron optical density, and the diameter of a particle is 37 $\pm$ 2 mμ. (figures 3 and 4). In the majority of cases the viral particles are encountered singly, sometimes there are several particles in one reservoir or vesicle, however, their total number usually does not exceed 20--25. The particles are separated from the surrounding cytoplasm by the smooth contoured membrane of the vesicle. On the outer side of the reservoir of the endoplasmic network there are dense corpuscles, identical to ribosomes in external appearance. In some reservoirs of the endoplasmic network the viral particles are distributed in connection with a dense substance filling up these reservoirs. Some reservoirs are filled with substance and do not contain viral particles or the quantity of them is very small. In the peripheral sectors of the cell long, narrow, smooth contoured tubules filled with virus are detected. It is possible to trace the continuous extension of a tubule up to 10 microns into the surface of the section. The width of a tubule is not uniform: In considerable segments it corresponds approximately to the diameter of a viral particle, sometimes widenings are observed along the length of the tubule. The tubules may have a twisting nature, in some sectors they are rectilinear. The virus accumulates both in the widened and in the narrow segments of the tubules. In the narrow sectors of the tubules the viral particles are arranged in the form of beads. The viruses are also detected in the intercellular spaces, where they form narrow rows of regularly arranged particles. In those cases when there are wide spaces between the cells the extracellular accumulations of virus are quite massive and do not have a well regulated nature.

In an individual virus particle it is possible to distinguish a central dense sector with a round or oval form, a nucleoid and a peripheral lighter membrane (see figure 4). The diameter of the nucleoid is around 25 mμ, the thickness of the peripheral membrane around 6 mμ. Two layers of approximately the same thickness are distinguished in the membrane: An inner osmiophobic layer adjoining the nucleoid, in sections it appears lighter, and an outer osmiophilic layer, in preparations it is revealed in the form of a darker ring.

Following a simultaneous determination of the virus content in a cultural fluid the values presented in the table were obtained. It can be seen from the table that the greatest accumulation of extracellular virus was on the 3rd and 4th day after infection.

Discussion

There are no facts in the literature concerning the results of electron microscope investigations devoted to the study of the interaction of the OHF virus with the cells of the infected culture. In our work for the first time the intracellular multiplication of the OHF virus was shown, and the localization and morphology of the virus revealed. The results

of the investigation showed that eosinophilic cytoplasmic inclusions, which also possess osmiophilic properties, are formed in the cells after infection with virus. In stained preparations these inclusions, which also possess osmiophilic properties, are formed in the cells after infection with virus. In stained preparations these inclusions are exposed with the help of a light microscope: After fixation in osmic acid, which at the same time exerts a staining action, the osmiophilic portion of the inclusions is revealed in a phase contrast microscope.

The investigation of one and the same cell in a phase contrast microscope, and then of ultrathin sections of the same cell in the electron microscope, made it possible to expose the submicroscopic structure of the inclusions. It was shown that the area of the inclusions is a netlike zone of the hypertrophic endoplasmic network and the Golgi complex. The substance in which the virus particles are disposed is accumulated in reservoirs of the former. An anlysis of the interrelation of this substance and the virus particles gave us a basis to conclude that it has a direct connection with the formation of virus particles and we have a right to view this substance as the virus seed.

Solitary virus particles are revealed in narrow reservoirs of the endoplasmic network in the paranuclear area, in widened reservoirs small accumulations of them are detected. Narrow long tubules with intermittent convolutions and rectilinear sectors are observed in the peripheral zones of the cell. The configuration of the tubules and the amount of virus formed are conditioned by the beaded or irregular disposition of virus particles in them. In the majority of cases in the peripheral tubules, filled with virus, the substance which represents the seed is absent, in rare cases it is contained in small quantities.

On the basis of the data obtained the conclusion can be made that the multiplication of the OHF virus takes place in the zone of inclusions, located in the cytoplasm of the paranuclear area. These zones are condensed sectors of the endoplasmic network and the Golgi complex. Hypertrophy and hyperplasia of the structural components of the endoplasmic network and the Golgi complex are observed during the process of virus multiplication. It can be assumed that as part of the formation of a virus in the endoplasmic network it enters the vesicles and Golgi vacuoles, where it matures. However, further investigations are required for defining the role of the components of the Golgi complex in the process of virus multiplication. The mature virus escapes from it by way of the intracellular tubules. As part of the development of the pathological process the destruction of the cell takes place, accompanied by the liberation of the virus.

The role of the nucleus in the process of virus formation remains unclear.

In the cultural fluid the virus accumulates in maximum quantities on the 3rd and 4th day after infection (see the table). These quantitative data conform fully with the results of electron microscope observations of the virus within the cell, where it is detected in the greatest quantities by the end of the 2nd day after infection. During this period cells with a various

degree of structural changes are exposed in preparations of an infected culture. Together with the large flattened cells, containing oxyphilic inclusions of a fibrous nature, there are individual rounded small sized cells with dense compact oxyphilic masses. A larger number of similar cells is encountered in preparations from later periods, correspondingly there are less large cells with inclusions with a fibrous structure. In ultrathin sections of the dense rounded cells structures are detected which testify to the profound process of degeneration setting in. In such cells the virus particles either are not revealed or are detected in very small quantities.

The virus particles in sections have a rounded or restrained form and consequently in space represent spherical or restrained bodies. The nucleoid has a similar form but of a correspondingly smaller size. A double layer membrane forms the peripheral zone of the virus particle. Taking into consideration that the OHF virus is ether sensitive and during the study of ultrathin sections of infected cells containing virus particles a change in the lipids is observed, which are apparently also involved in the process of virus multiplication, we propose that the outer layer of the membrane of the virus particle is a lipid cover. The inner layer, separating the nucleoid and the lipid membrane, may be considered the capsid of the virus.

Conclusions

During an investigation of a transplanted tissue culture of a swine embryo kidney, infected with the OHF virus, cytoplasmic oxyphilic inclusions were detected in the infected cells and their submicroscopic structure was revealed.

Hypertrophy and hyperplasia of the endoplasmic network and the Golgi complex are noted during the process of virus multiplication.

The Omsk hemorrhagic fever virus has a spherical or polygonal form. Its diameter equals 37 $\pm$ 2 mμ. In the center of the virus particle is a dense nucleoid with a diameter of about 25 mμ ; the peripheral zone is formed by a membrane consisting of 2 layers, apparently corresponding to the capsid and the lipid cover.

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Titers of the Omsk hemorrhagic fever virus in cultural fluid

Period after infection (in hours)	Titer of virus (in lg TCD_{50}/ml)
2	2.0
24	2.67
48	3.1
72	3.5
96	3.47
120	2.6
144	2.0
168	1.5
192	0

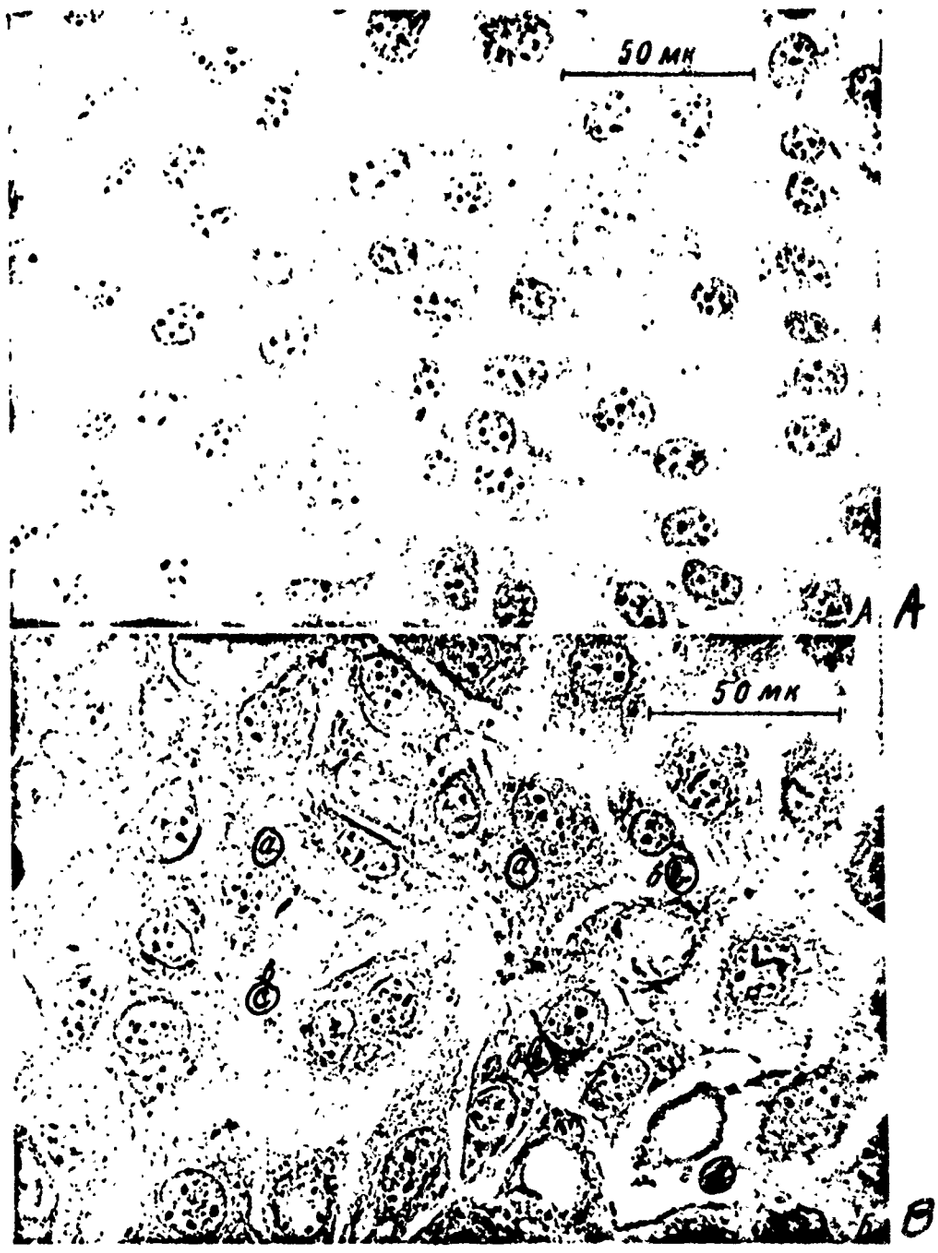

Figure 1. Transplanted tissue culture of swine embryo kidney.
A - control; B - 2 days after infection with Omsk hemorrhagic fever virus.
C - in the infected culture cells are seen with oxyphilic inclusions in various phases of development: a - inclusion of the fibrous type; b - fibrous inclusion with compact dense masses; c - dense massive inclusion; d - degenerating cells.

GRAPHIC NOT REPRODUCIBLE

GRAPHIC NOT REPRODUCIBLE

Figure 2. Cell fragment from a tissue culture of swine embryo kidney, infected with the Omsk hemorrhagic fever virus.
zv - zone of inclusion; m - mitochondria; g - hyaloplasm; yam - nuclear membrane; pr - perinuclear reservoir; ya - nucleus; km - cellular membrane; c - aggregate of OHF virus in intercellular space.
A - detail of sector c , taken at a larger electron microscope magnification.

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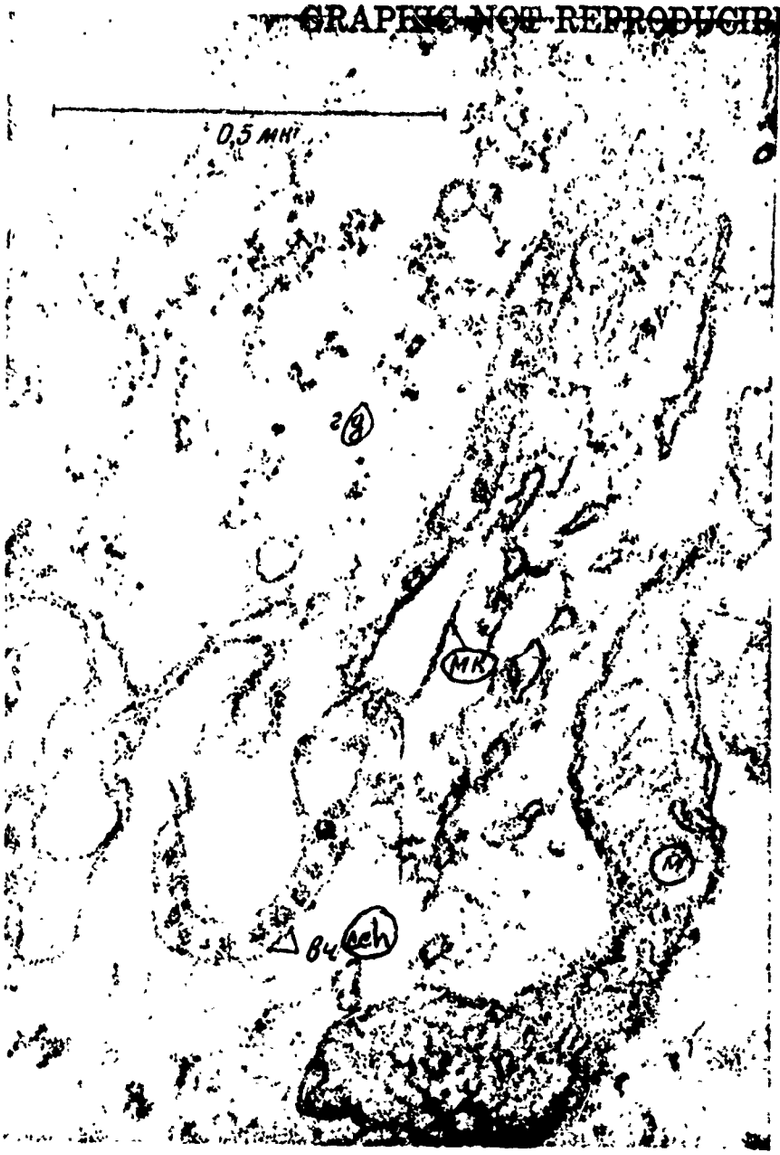

Figure 3. Fragment of an infected cell from a tissue culture of swine embryo kidney. Intracellular tubule of the peripheral zone of the cytoplasm, filled with virus.
mk - tubule membrane; cch - virus particles; g - hyaloplasm; m - mitochondria.

GRAPHIC NOT REPRODUCIBLE

Figure 4. Extracellular accumulation of virus.
n - nucleoid; o - peripheral membrane; cs - inner layer; ns - outer layer.