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SEQUENTIAL CELLULAR CHANGES PRODUCED BY AFRICAN HORSE-SIKNESS VIRUS IN MONKEY KIDNEY CELLS

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SUMMARY

Monkey kidney stable cells developed characteristic changes when infected with all known types of African horse-sickness virus. First, there was an increase in the size of cell nuclei. Then, basophilic Feulgen-positive granules were seen in the vicinity of nucleoli. The granules increased in number and were scattered throughout the nucleus. As infection progressed, the nucleolus became covered with basophilic Feulgen-positive substance, and nuclear chromatin began to accumulate at the periphery of the nucleus. Nuclei became smaller, and the nuclear chromatin eventually merged to form several Feulgen-positive bodies which had a smooth surface at the last stage of infection.

In electron micrographs, particles of African horse-sickness virus were seen mainly in the nucleus. Clusters of virus particles were observed around the denatured nucleoli. Most particles were ovoid, with diameters between 45 and 75 milli micron. Occasionally, virus particles were seen in denatured cytoplasm that had numerous vacuoles surrounded by endoplasmic reticulum. Dense bodies of various sizes were observed in the cytoplasm.

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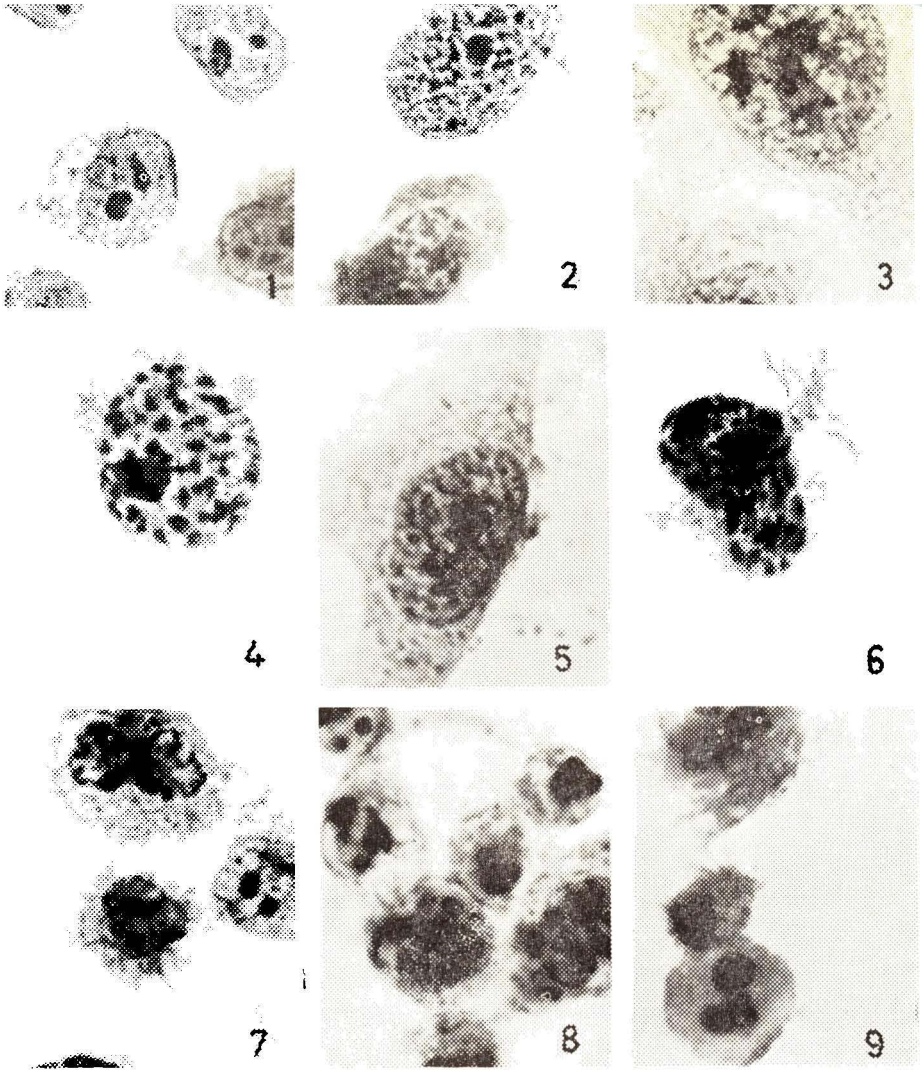

Fig. 1—9—Monkey kidney stable cells infected with S2 strain of African horse-sickness virus. H&E stain; x 1,400.

Fig. 1—Normal MS cells at the time of infection.

Fig. 2—Sixteen hours after infection. Notice enlarged nucleus containing a number of basophilic granules.

Fig. 3—Twenty-four hours after infection. An accumulation of basophilic substance near dark nucleoli is seen.

Fig. 4—Thirty-one hours after infection. The nucleolus is covered or mixed with basophilic substance. There is flocculation of chromatin and many dark basophilic aggregates have been formed at the periphery of the nucleus.

Fig. 5—Thirty-one hours after infection. An enlarged dark nucleolus and basophilic aggregates attached to the nuclear membrance are evident.

Fig. 6—Forty hours after infection. Notice basophilic aggregates increased in size and density, and irregular outiine of the nucleus.

Fig. 7—Forty hours after infection. There are a few large spherical bodies at the membranes of irregularly shaped nuclei.

Fig. 8—Forty-eight hours after infection. Pyknotic nuclei contain dark basophilic inclusions with a smooth surface. A few dense bodies are seen in the cytoplasm.

Fig. 9—Forty-eight hours after infection. Oval bodies are in the nucelus, which has a clear glassy background.

Introduction

African horse-sickness virus was successfully adapted to monkey kidney stable (MS) cells by Ozawa and Hazrati.[11] This tissue-culture system was found suitable for vaccine production, serologic studies, diagnostic work, and studies of properties of the virus.

In preliminary observations on changes in MS cells infected with African horse-sickness virus,[11] a marked change was observed only in nuclei, and cytoplasmic inclusions were not found. In this study, a detailed investigation was made by examining stained MS cells infected with all known types of African horse-sickness virus and by examining electron micrographs.

Materials and Methods

Virus.—Nine antigenically different types of African horse-sickness virus were used. Types 1 to 7—designated A501, OD, L, Vryheid, VH, 114, and Karen respectively—were obtained from Onderstepoort Veterinary Laboratory. They were adapted to MS cells after 102 mouse passages. Type 8, designated 18/60, was also obtained from Onderstepoort Veterinary Laboratory, and was adapted to MS cells after 15 mouse brain passages. Type 9 neurotropic Iranian strain, designated S2,[4] was adapted to MS cells after 102 mouse brain passages. Type 9 viscerotropic Iranian strain, designated 10/60,[6] was transferred to MS cells after 4 cerebral passages in suckling mice.

All strains were used after 5 to 7 passages in MS cells. Their type specificity was confirmed by neutralization tests with type-specific rabbit antiserums as described in previous reports.[5,11]

Cell Cultures.—The origin of MS cells and the methods of cultivation were previously described.[11] The growth medium consisted of Earl's balanced salt solution with 0.5% of lactalbumin hydrolysate, 0.005% of yeast extract, 0.0015% of phenol red, 10.0% of calf serum (heated at 56 C. for 30 minutes), 100 units/ml. of penicillin, 100 micro g./ml. of streptomycin, and sodium bicarbonate to adjust pH to 7.2. The maintenance medium contained the same constituents except that calf serum was reduced to 2.0%.

Coverslip Cultures.—One 22- by 33-mm. coverslip was placed ino each sealable petri dish of 50-mm. diameter. Five milliliters of MS cell suspension in the growth medium containing approximately 7.5×10^5 cells was placed on the coverslip in each petri dish and then incubated 3 days at 37 C. The growth medium was removed and discarded, and 1 ml. of viral fluid was placed on each coverslip culture.

After 3 hours' adsorption at 37 C., the viral inoculum was removed, and 5 ml. of fresh maintenance medium was added to each petri dish. Control MS cell cultures were handled in the same manner, but maintenance medium was used in place of viral fluid. At specified intervals, coverslip cultures of infected and noninfected MS cells were removed for staining.

Titration of Virus.—Viruses were titrated in MS cell tube cultures. Each was prepared in a 14-by 150-mm. tube to which was added 1.5 ml. of MS cell suspension containing approximately 2.25×10^5 cells. These were held for 3 days' incubation at 37 C.; then the growth medium was removed, and the tube cultures were infected with 0.1 ml. of virus dilutions prepared in maintenance medium. Four tube cultures were used for each dilution. After 30 minutes' adsorpiton at 37 C., 15 ml. of fresh maintenance medium was added to each tube. The results were read 10 days after infection. Tissue culture infective doses (TCID50) were calculated by the method of Reed and Muench. [14]

Hematoxylin and Eosin Staining.—Coverslip cultures were washed with phosphate buffer (pH 7.2), fixed in 95% alcohol for 5 minutes, and then washed in 70% alcohol. They were stained with Harris' hematoxylin for 20 minutes, differentiated in 1% acid alcohol, washed in tap water, counter-stained with 1% eosin for 1 minute, dehydrated, cleared, and mounted on glass slides.

Feulgen Staining.—Washed coverslip cultures were fixed in 10% formalin for 1 hour, then washed in running tap water overnight. They were rinsed in 70% alcohol, placed in 1 N HCl at 60 C. for 12 minutes, stained with Feulgen reagent for 1 hour, and then washed 3 times in sulfurous acid for 5 minutes each. The stained coverslips were washed in water, dehydrated, cleared, and mounted on glass slides.

Electron Microscope Examination of Ultrathin Sections.—Preparations of ultrathin sections and electron micrographs were made at the Cancer Research Institute, Pahlavi Hospital.

Drained monolayers of MS cells in 4-oz. bottles containing approximately 4 million cells were inoculated with 0.2 ml. of type 9 (S2) virus containing $2 \times 10^{6.0}$ TCID50 and then incubated 3 hours at 37 C. Fifteen milliliters of maintenance medium was added to each bottle, and incubation was continued for 48 hours at 37 C. Then, the maintenance medium was decanted, and the remaining cells were washed once in chilled phosphate buffer (at pH 7.2.). The cells were fixed for 60 minuts at 4 C. in 1% osmium tetraoxide (at pH 7.4). The fixed cells were scraped from the bottles with a rubber scraper and dehydrated by passing through 35, 50, 70, 95, and 100% alcohol for 15 minutes each. As required, the cells were cent-

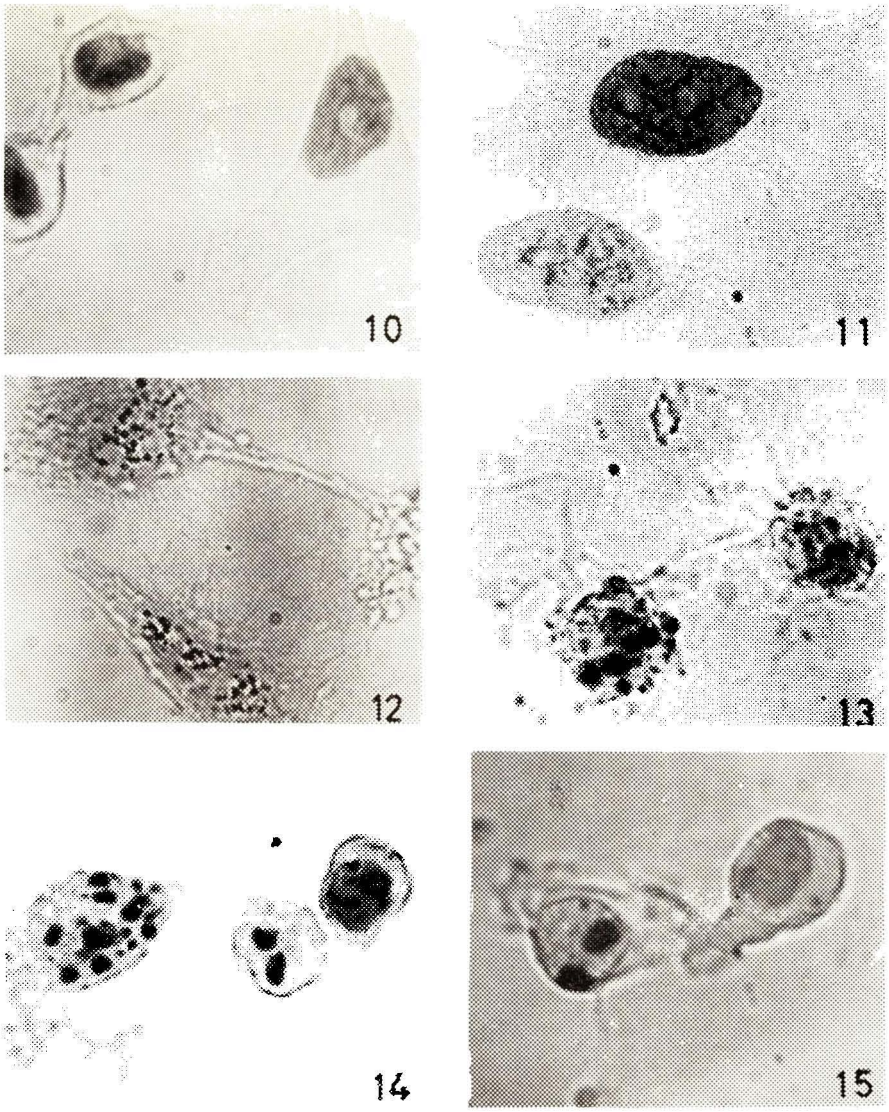

Fig. 10—Normal MS cells at the time of infection. Notice dividing cells at the left.

Fig. 10-15—Monkey kidney stable cells infected with Vryheid strain of African horse-sickness virus. Feulgen's stain; x 1,400.

Fig. 11—Sixteen hours after infection. An increase in DNA concentration in one cell is noticed. The concentration was higher around the nucleoli.

Fig. 12—Twenty-four hours after infection. Notice clumps of Feulgen-positive substance around the nucleoli.

Fig. 13—Thirty-one hours after infection. Formation of Feulgen-positive bodies is noticed both at periphery and around the center of the nucleus. Notice threadlike, radiated cytoplasm.

Fig. 14—Forty hours after infection. Oval Feulgen-positive bodies having a clear margin are at periphery of the nucleoli.

Fig. 15—Forty-eight hours after infection. One or 2 large Feulgen-positive bodies remain in the pyknotic nucleus which has a clear nuclear back ground. Very little cytoplasm is around the nucleus.

rifuged at 800 r.p.m. for 5 minutes between changes of fluid. The dehydrated cells were embedded in methacrylate plastic.[10]

Ultrathin sections of uninfected MS cells were prepared at the same time in the same manner.

Ultrathin sections of both infected and uninfected MS cells were stained with uranyl acetate and examined with an electron microscope. (Philips EM 100 electron microscope, Philips, Eindhoven, The Netherlands)*

Results

All virus inoculums contained approximately 10^6 to 10^7 TCID50/ml. of virus. Complete cytopathic changes were observed on the 3rd day after infection.

At 16, 24, 31, 40, and 48 hours after infection, coverslip cultures of MS cells infected with types 4 and 9 viruses were stained with hematoxylin and eosin and by Feulgens' method and examined with an ordinary microscope.

The 1st changes in infected cells were visible approximately 16 hours after infection. The nuclei of infected cells became larger (Fig. 2, and there was an increase in Feulgen positive substance (Fig. 11). In the preparation stained with hematoxylin and eosin, basophilic granules filled the enlarged nuclei, and the nucleoli were eosinophilic. Between 16 and 24 hours after infection, accumulations of basophilic Feulgen-positive substance in and around the nucleoli were noticed (Fig. 3, 12). At 31 hours after infection, flocculation of the rest of basophilic substance became evident, and most of the aggregates attached to the nuclear membrane (Fig. 4, 5). Between 31 and 40 hours after infection, the basophilic aggregates attached to the nuclear membrane increased in size and eventually formed a few large basophilic Feulgen-positive bodies (Fig. 6, 7, 13, 14). There were one or a few large basophilic bodies remaining unattached to the nuclear membrane. They were considered denatured nucleoli. Between 40 and 48 hours after infection, nuclei became pyknotic (Fig. 8) and clearing of nucleoplasm was observed. The Feulgen-positive bodies with smooth surfaces were seen in the clear glassy or empty nuclear background (Fig. 9, 15).

The MS cells infected with the other strains of virus types 1, 2, 3, 5, 6, 7, and 8 were similarly examined and all of them had these characteristic nuclear changes.

The MS cells infected 48 hours with African horse-sickness (S2) virus were observed by electron microscopy to have characteristic changes in the nucleus and

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* Philips EM 100 electron microscope, Philips, Eindhoven, The Netherlands.

cytoplasm. The nucleoli of infected cells were dense, and the margin was regular and clear (Fig. 16, 17). Clusters of dense particles were seen on and around the denatured nucleolus. In magnified electron-micrographs of the clusters (Fig. 18, 19), aggregates of spehrical or avoid particles were seen. The particles ranged from 45 45 to 75 milli-micron. Dense particles were often seen in and on the surface of the denatured nucleolus (Fig. 17, 20).

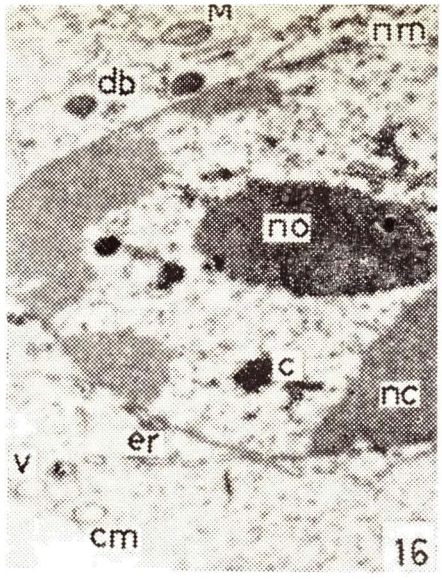
As infection progressed, nuclear chromatin formed large Feulgen-positive bodies attached to the nuclear membrane, and clearing of nucleoplasm occurred (Fig. 17, 20). Irregular clusters of dense osmiophilic granules were seen about the nucleolus. The nuclear membrane became irregular, and several breaks in the outer layer of the membrane were observed.

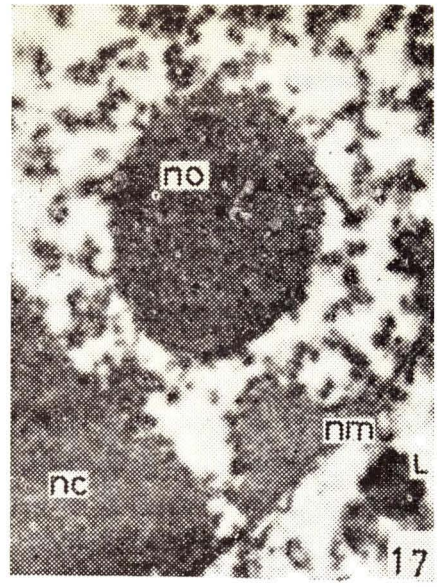
Occasionally there were virus-like particles in the cytoplasm (Fig. 22). Conspicuous changes in the cytoplasm were the formation of numerous vacuoles surrounded by endoplasmic reticulum and the occasional appearance of dense bodies around the denatured nucleus (Fig. 16, 21, 23). The oval, dense bodies varied from 0.4 to 2.0 micron in diameter. No membrane was seen around the dense bodies. Their surface was covered with drak osmiophilic granules, but there was no evidence that virus particles were reproduced in or around these bodies. At a late stage of infection, the surface of cells became very irregular, and vesicular bodies were observed at the periphery of the cytoplasm (Fig. 21).

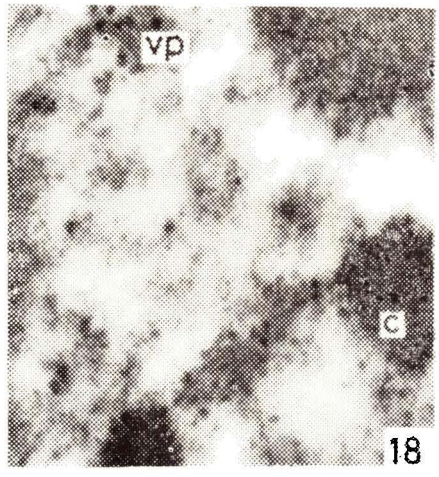
Discussion

The size of African horse-sickness virus was previously investigated (in infected mouse brains), both by ultracentrifugation and electron micrography. Polson and Madsen [13] originally reported infective particles having at least 2 sedimentation constants. Assuming density of 1.33 Gm./ml., it was determined that the 2 particles were 31.2 and 50.8 milli-micron, respectively. In electron micrographs, Bakaya and Guturk [1] observed particles between 40 and 80 milli-micron. Polson and Deeks [12] prepared purified specimens of the virus and found that virus particles have diameters of 70 to 80 milli-micron and have 92 rod-shaped subunits.

In this investigation, dense particles of various size were detected mainly in the nucleus of infected cells. Most particles were ovoid, approximately 45 by 75 milli-micron in diameter. Generally the particles of other animal viruses [7,8] of similar size are also ovoid when detected in infected cells. Since such particles were not seen in the nucleus of normal MS cells, the dense particles detected in the nucleus of infected MS cells are considered virus particles.







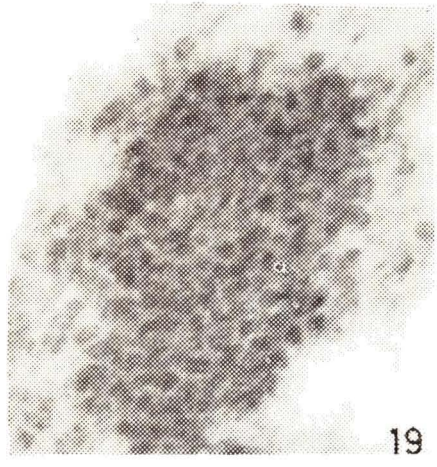


Fig. 16—Electron micrograph of monkey kidney stable cells infected with S2 strain of African horse-sickness virus. Clumps of nuclear chromatin (nc) are along the nuclear membranes (nm). An oval dense body in the center of the nucleus is a denatured nucleolus (no). Clusters (c) of dense particles are seen on and around the nucleolus. Two small dense bodies (db) are in the cytoplasm at the upper corner. Other features are (M) mitochondrion, (er) endoplasmic reticulum, (v) vacuole, (cm) cytoplasmic membrane. x 8,500.

Fig. 17—Denaturation of nuclear structures progressed. Dense particles are within and on the denatured nucleolus (no). Notice irregular outer layer of nuclear membrane (nm). A lipid inclusion (L) is in the cytoplasm; (nc) nuclear chromatin. x 21,000.

Fig. 18—Higher magnification of clusters (c) of particles. Virus particles (vp) are scattered throughout nucleoplasm. x 21,000.

Fig. 19—A magnified cluster of virus particles. Most of them are oval particles, 45 X 75 milli micron.

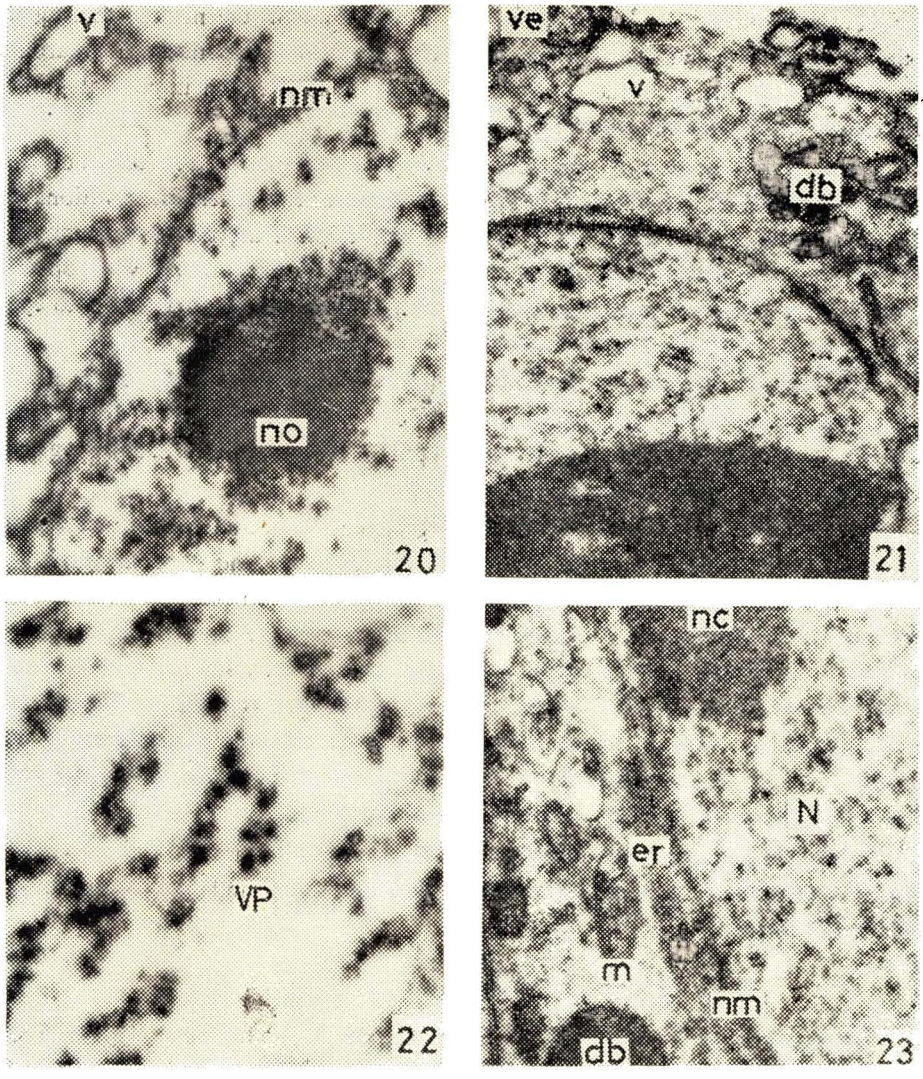


Fig. 20—The last stage of infected monkey kidney stable cells. Notice clearing of nucleoplasm, aggregates of dense particles around the denatured nucleolus (no), transudation of nuclear contents through broken nuclear membrane (nm), and many vacuoles (v) surrounded by endoplasmic reticulum. x 28,000.

Fig. 21—In the cytoplasm, there are several vacuoles (v), dense bodies (db), and vesicular bodies (ve). x 8,500.

Fig. 22—Oval virus particles (vp) are occasionally detected in the cytoplasm. x 50,000.

Fig. 23—Virus particles are scattered throughout the nucleoplasm. Dense bodies (db) of 2 different sizes are in the cytoplasm. Other features are (N) nucleus, (nm) nuclear membrane, (nc) nuclear chromatin, (er) endoplasmic reticulum, and (m) mitochondria. x 17,100.

The accumulation of Feulgen-positive substance about the nucleoli during the course of infection and the appearance of clusters of virus particles in or around the nucleoli indicate that nucleoli of infected MS cells play an important part in the replication of African horse-sickness virus.

Virus paricles were not often seen in cytoplasm of the infected cells but were occasionally seen in cytoplasm at a late stage of infection. These observations are similar to those of Mirchamsy and Taslimi [9] who applied the fluorescent-antibody technique to MS cells infected with African horse-sickness virus. Virus-specific antigens were only in the nucleus, and diffusion of the antigenic material into cytoplasm was noticed as the infection progressed.

The origin of the dense bodies in cytoplasm of the infected cells could not be determined. They may play a part in the production of some of the antigens. They possibly represent denatured cytoplasmic particles or microbodies. Further investigations are needed to determine their eaxct role.

Among other animal viruses reproducing in the nuclei and causing the formation of characteristic intranuclear inclusion bodies, adeno- and papova-viruses appear to produce characteristic changes similar to African horse-sickness virus. Observations of HeLa cells infected with adenoviruses have been made by Boyer *et al.*[2] and by Mayor,[7] using infected monkey kidney cells stained with acridine orange. Simian papova virus SV40 was studied by Melnick *et al.*[8] using kidney cells of African green monkey. Some adenoviruses form crystalline structures in the nucleus at a late stage of infection. This was, however, not observed in the cells infected with papova virus or African horse-sickness virus.

Both adeno- and papova-viruses are known to be DNA viruses,[7,8] their particle sizes [3,8] approximate that of African horse-sickness virus, and they also grow in monkey kidney cells.

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