Arch. Inst. Razi, 1967, 19, 51 - 56

CYTOLOGY OF MONKEY KIDNEY CELLS INFECTED WITH AFRICAN HORSE-SICKNESS VIRUS (*)

By Y. OZAWA, I. G. HOPKINS, A. HAZRATI,

Near East Animal Health Institute. Razi Serum and Vaccine Institute, Teheran, Iran
and

A. MODJITABAI and P. KAVEH

Cancer Research Institute, Pahlavi Hospital, Teheran, Iran

Successful adaptation of all known types of African horse-sickness virus to monkey kidney stable (MS)[1] cells by Ozawa and Hazrati [2] opened the way for further virus studies. Tissue culture has been found suitable for vaccine production[3], serological studies[4], plaque assay[5], and studies of various properties of the virus. Sequential cellular changes were examined in a separate study[6] by using MS cell cultures and all types of African horse-sickness virus. In this communication, characteristic changes observed in the infected MS cells at the late stage of infection are described.

The virus used was S2 strain[7] of African horse-sickness virus confirmed by neutralization tests using type-specific rabbit antiserum as described in a previous report[4]. After 100 mouse brain passages this strain has been used in Iran as the attenuated 'type 9' vaccine strain. The seventh passage in MS cell cultures was used for this experiment.

The origin of MS cell line, methods of cultivation of the cells and constituents of growth and maintenance media have also been previously described[2]. Coverslip cultures prepared by placing one 22 × 33 mm coverslip into each sealable Petri dish of 50 mm diameter were used for observations under the light microscope. Approximately 1.5×10^5 cells were seeded in each Petri dish. After 3 days incubation at 37° C, the growth medium was discarded, and 1 ml. of virus fluid was placed on each coverslip culture. After 3 h adsorption at 37° C, the virus fluid was removed, and 5 ml. of fresh maintenance medium added to each Petri

(*) Reprinted from Nature, Vol. 206, No. 4991, pp. 1321-1323.

dish. Control MS cell cultures were treated in the same manner, but maintenance medium was used in place of virus fluid. These cultures were incubated at 37° C for 48 h, then washed gently in phosphate-buffered saline at *p*H 7.2.

For haematoxylin—eosin staining, washed coverslip cultures were fixed in 95 per cent ethanol. They were stained with Harris's haematoxylin for 20 min,

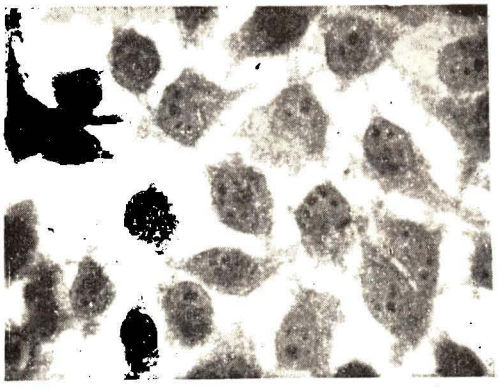

Fig. 1. Normal monkey kidney stable (MS) cell culture. (Haematoxylin and eosin stain, ×560)

differentiated in 1 per cent acid alcohol, washed in tap-water, counter-stained with 1 per cent eosin for 1 min, then dehydrated and mounted on glass slides. For Feulgen staining, coverslip cultures were fixed in 10 per cent formalin for 1 h, and washed overnight in running tap-water. They were rinsed in 70 per cent alcohol, then placed in 1 N HCl at 60° for 12 min. Feulgen reagent was applied for 1 h. They were then washed three times in sulphurous acid, 5 min each wash, washed in water, dehydrated and mounted. For electron microscopy, ultra-thin sections and electron micrographs were made at the Cancer Research Institute, Pahlavi Hospital. MS cell cultures were prepared in 4-oz. medical flat bottles. Drained monolayers of MS cells were inoculated with 0.2 ml. of virus fluid containing 2×10^6 TCID50. After 3 h adsorption at 37°. C, 15 ml. of maintenance medium was added to each bottle. After 42 h incubation at 37° C, the maintenance medium was discarded, cultures were washed with phosphate-buffered saline (*p*H 7.2) then fixed undisturbed for 1 h at 4° C with 1 per cent osmium tetroxide buffered at *p*H 7.4. The fixed cells were scraped from the bottles and dehydrated by passing through 35, 50, 70, 95 and 100 per cent ethanol, 15 min each. Pellets formed by low-speed centrifugation of the cells were embedded in methacrylate[8]. Ultra-thin sections were contrast-stained with uranyl acetate for 1 h, then washed 4 times with CO2-free distilled water, and dried. Ultra-thin sections of uninfected MS cells were prepared in the same manner, and the preparations were examined in a Philips 'EM100' microscope.

In the infected MS cells, major changes appeared in the nuclei. An accumulation of basophilic Feulgen-positive material around the nucleoli of slightly enlarged nuclei was observed under an ordinary light microscope (Figs. 2 and 3). As

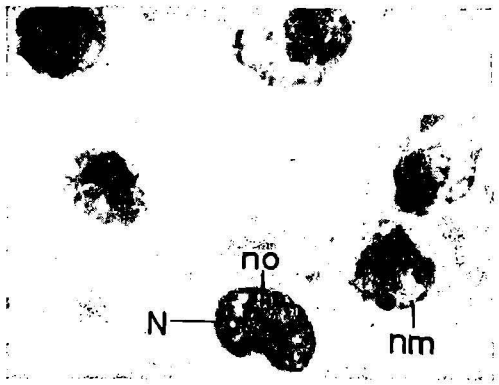

Fig. 2. MS cells 40 h after infection with *Vryheid* strain of African horse-sickness virus. Note a large basophilic mass around the nucleolus (*no*). Spherical basophilic bodies are seen at the periphery of nuclei (*N*), usually attached to the nuclear membrane (*nm*). Many vacuoles are seen in the cytoplasm. (Haematoxylin and eosin stain, ×1,120)

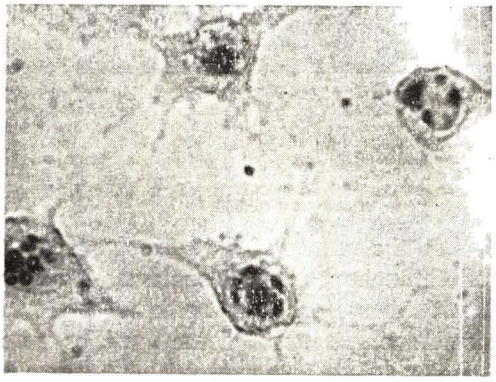

Fig. 3. MS cells 40 h after infection with S2 strain of African horse-sickness virus. Feulgen-positive bodies are seen both at the periphery and in the centre of nuclei. (Feulgen stain, ×1,120)

infection progressed, margination of basophilic Feulgen-positive substances occurred, and several Feulgen-positive oval bodies were formed on the interior surface of nuclear membranes. At this stage the nuclei were pyknotic and, apart from these oval bodies, appeared empty. During infection an increase in the number of vacuoles in the cytoplasm was observed. Cytolysis occurred leaving radiant forms of cytoplasmic branches. Occasionally very minute Feulgen-positive bodies were seen in the cytoplasm. At the last stage of infection, only fragments of cytoplasm remained surrounding the pyknotic nucleus.

In the electron micrographs, several characteristic changes within the infected MS cells were observed. The nucleoli were in the form of dense oval bodies with clear margins. Clusters of dense particles that were considered to be virus par-

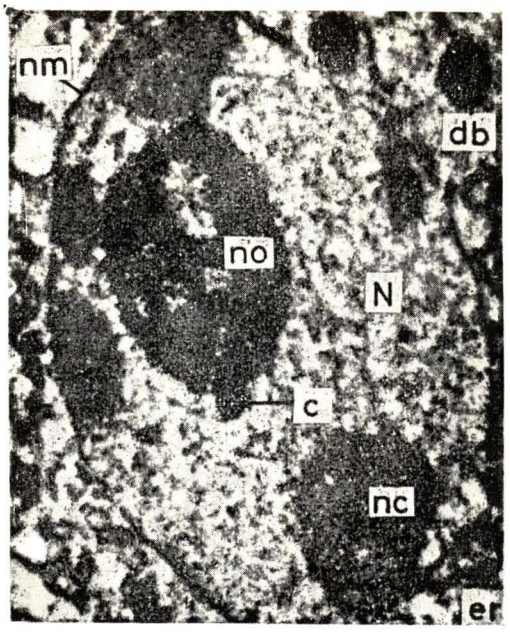

Fig 4. Electron micrograph of MS cells 48 h after infection with *S2* strain of African horse-sickness virus. Margination of nuclear chromatin (*nc*) and nucleolus (*no*) has occurred. Bodies of nuclear chromatin are attached to the nuclear membrane (*nm*). The denatured nucleolus is darkly stained and clusters (*c*) of virus particles are seen within and on the surface of the body. Two dense bodies (*db*) and many vacuoles are seen in the cytoplasm. *N*, nucleus; *er*, endoplasmic reticulum (×6,800)

ticles were seen within and on the surface of the nucleolus (Fig. 4). The dense particles were also scattered throughout the nucleoplasm. Margination of nuclear chromatin was observed with several of these bodies occurring alongside and attached to the nuclear membrane. Scattered dense particles were often seen even within the denatured nucleolus (Fig. 5). As infection progressed, the nucleoplasm cleared, and many tears were often seen in the nuclear membrane. When examined under high magnification, aggregates of dense particles as well as dispersed particles were observed. Most of these particles were oval in shape, with an average diameter of 75 milli micron on the long axis, the shorter axis being 45 milli micron (Fig. 6).

In the cytoplasm of infected MS cells, electron micrographs revealed a number of vacuoles surrounded by endoplasmic reticulum (Fig. 4). Lipid inculsions were occasionally seen. Dense bodies (Fig. 4) of various sizes were often seen in the cytoplasm. The exact origin and their functions could not be clarified, but no dense particles were detected in or around the dense bodies.

From these observations and the previous report made by Mirchamsy and Taslimi[10] using fluorescent antibody technique applied on the same system, it became evident that replication of virus particles occurs in the nucleus. The accumulation of DNA substance around the nucleoli of infected MS cells and frequent

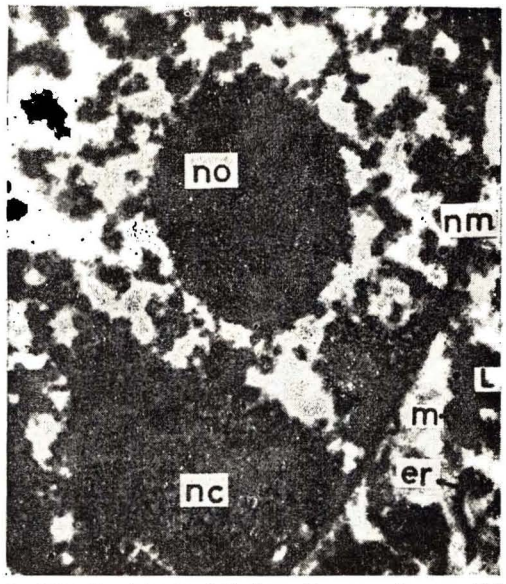

Fig. 5. MS cells 48 h after infection. Numerous dense particles are scattered throughout nucleoplasm and in the denatured nucleolus (*no*). Note irregular outer layer of nuclear membrane (*nm*). *L*, lipid inclusion; *m*, mitochondrion; *er*, endoplasmic reticulum; *nc*, nuclear chromatin (× 16,800)

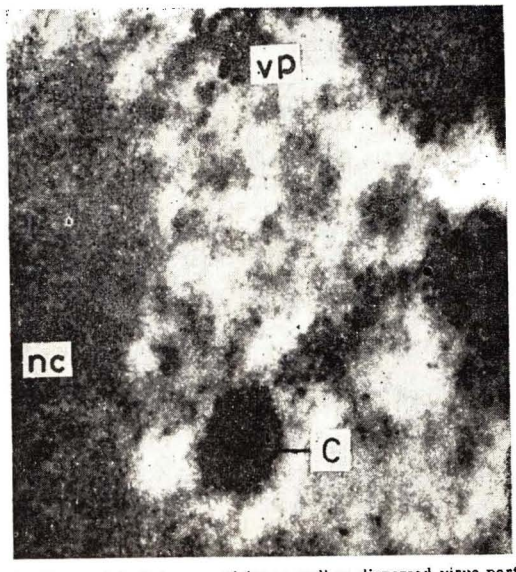

Fig. 6. Cluster (*c*) of virus particles as well as dispersed virus particles (*vp*) are seen in the nucleoplasm. Many of them are ov alparticles having average diameters of 45 × 75 mμ. *nc*, nuclear chromatin (× 16,800)

observation of clusters of virus particles in and around the nucleoli indicate that virus replication occurs there, and that the virus particle may possess a DNA moiety. Many of these characteristic findings are similar to the recent observations using monkey kidney cells infected with adenovirus[11] and simian papova virus, *SV*40 (ref. 12). Both of these viruses are known to be DNA viruses and are nearly the same size as African horse-sickness virus.

Growth curves of African horse-sickness virus in MS cell cultures have been previously reported[2,3]. The excretion of the virus into the culture medium was first observed approximately 7-8 h after infection and maximum titres with type 9 virus were obtained between 48 and 60 h after infection depending on the inoculum used.

When using electron microscopy virus particles were occasionally detected in the cytoplasm during the advanced stage of infection. Tears in the nuclear membrane and the excretion of nuclear contents were also observed. Further experiments are required to determine how the virus reaches and penetrates the nucleus. the mode of replication and excretion, and the function of the dense bodies in the cytoplasm.

We thank Dr. A. Rafyi of Razi Institute, Dr. H. Rahmatian of the Cancer Research Institute of Pahlavi Hospital, Dr. W. M. Moulton and Dr. E. Traub of the Near East Animal Health Institute for their advice.

REFERENCES

1 Kanda, Y., and Melnick, J. L., *J. Exp. Med.*, **109**, 9 (1959).
2 Ozawa, Y., and Hazrati, A., *Amer. J. Vet. Res.*, **25**, 505 (1964).
3 Ozawa, Y., Hazrati, A., and Erol, N., *Amer. J. Vet. Res.* (in the press).
4 Hazrati, A., and Ozawa, Y., *Amer. J. Vet. Res.* (in the press).
5 Hopkins, I. G., Hazrati, A., and Ozawa, Y., (unpublished results).
6 Ozawa, Y., Modjtabai, A., Hopkins, I. G., Hazrati, A., and Kaveh, P. (in preparation).
7 Hazrati, A., and Taslimi, H., *Proc. XVII Wld. Vet. Congr.*, **1**, 535 (1963).
8 Newmann, S. B., Borysko, E., and Swerdlow, M., *Science*, **110**, 66 (1949).
9 Watson, M. L., *J. Biophys. Biochem. Cytol.*, **4**, 475 (1958).
10 Mirchamsy, H., and Taslimi, H., *Immunology*, **7**, 213 (1964).
11 Mayor, H. D., *J. Exp. Med.*, **119**, 433 (1964).
12 Melnick, J. L., Stinebaugh, S. E., and Rapp, F., *J. Exp. Med.*, **119**, 313 (1964).