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PRODUCTION OF HUMAN INFLUENZA VIRUS IN A STABILE LINE OF GUINEA PIG TONGUE CELLS EXPRESSING ENDOGENOUS ONCOVIRUS: AN ELECTRON MICROSCOPIC STUDY.

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Summary. — Guinea pig tongue (GPT) cells represent a highly sensitive host system for influenza A/WSN (H1N1) infection as evidenced by numerous ultrastructural changes, considerable production of NS1 protein and widespread budding of viral particles at the cytoplasmic membrane. Vesicles of smooth endoplasmic reticulum and of the Golgi complex were transported to the apical area of cell membrane, where the budding of virions took place. Numerous microtubules were directed vertically to these portions of plasma membrane. In contrast, maturation of the endogenous oncovirus particles occurred at the lateral cytoplasmic membrane. Beneath the area of oncovirus maturation and release, a network was seen of microfilaments oriented towards the plasma membrane. The cytoplasm of GPT cells contained numerous nonstructural protein inclusions, which evidently accumulated at the periphery of nucleoli and were seen to reach the cytoplasm crossing the pores of nuclear membrane.

Key words: influenza virus; guinea pig oncovirus (retrovirus); guinea pig tongue cells; polarized delivery of viral glycoproteins; electron microscopy

Introduction

Although influenza virus multiplies in different cells (Hoyle, 1968), ultrastructural changes associated with its multiplication were described in detail predominantly in chick embryo cells (CEC) (Compans and Dimmock, 1969; Compans *et al.*, 1970; Čiampor, 1972; Čiampor and Turčan, 1972; Čiampor, 1974; Höglund and Čiampor, 1975; Čiampor, 1978), in Ehrlich ascitic tumour cells (Bächi *et al.*, 1969; Bächi, 1970) and in BHK-21 cells (Anisimová *et al.*, 1973).

Recent interest is focussed to the role of individual cell structures in the synthesis of influenza virus-specific components and virus-coded nonstructural proteins. The participation of cell structures such as endoplasmic reticulum (ER), nucleus, nucleolus, free ribosomes, vesicles of the smooth ER and Golgi

complex in influenza virus replication has been well established (Compans *et al.*, 1973; Čiampor *et al.*, 1974; Klenk *et al.*, 1974). On the other hand, the mechanisms of the transport of virus-specific structural proteins from the site of their synthesis to the site of virion maturation are not so well known. Previous studies have pointed at the crucial role of the membranes of smooth ER (Compans, 1973; Čiampor *et al.*, 1974) and of the Golgi complex vesicles (Rodriguez-Boulan *et al.*, 1984; Rindler *et al.*, 1985). Most recent findings indicate the importance of cytoskeleton for replication and transportation of virus components in general (Dales and Chardonet, 1973; Wang *et al.*, 1978; Huismans and Els, 1979; Genty and Bussereau, 1980; Cervera *et al.*, 1981; Lozatulimowska *et al.*, 1981; Leavitt *et al.*, 1981; Bohn *et al.*, 1986; Križanová *et al.*, 1986).

With the aim to follow the role of cytoskeleton in the synthesis and transport of influenza virus-specific structural proteins we have chosen a cell line derived from tongues of guinea pig embryos revealing well defined cytoskeleton structures by electron microscopy. The influenza virus A/WSN (H1N1) multiplies well in these cells. In addition, GPT cells expressed a guinea pig oncovirus which allowed to compare the replication of influenza virus in the presence of another virus.

Materials and Methods

Viruses and cells. GPT cells were kindly provided by dr. J. Svobodová (Chair of Virology and Microbiology, Fac. Nat. Sci., Bratislava) and propagated as described (Svobodová *et al.*, 1977). The cells were cultured in BEM supplemented with 5% inactivated calf serum (ICS) and were used in their 207th passage. The expression of an endogenous oncovirus has been already demonstrated by electron microscopy in these cells. GPT cells were infected with influenza virus A/WSN (H1N1) at a multiplicity of 5—10 PFU per cell. After 45 min adsorption at room temperature, nonadsorbed virus was removed and the cells were incubated for 24 hr at 37 °C in BEM with 5% ICS.

Electron microscopy. Infected and noninfected cells were fixed in 2.5% glutaraldehyde in 0.2 mol/l sodium cacodylate buffer pH 7.2 for 30 min at 4 °C, washed in the same buffer and postfixed with 1% OsO_4 prepared in the same buffer for 60 min at room temperature. The cells were then dehydrated at increasing acetone concentrations and embedded into Araldite CY 212 (Serva, Heidelberg). Ultrathin sections were prepared on Ultramicrotome III LKB and stained with 2% water solution of uranyl acetate and lead citrate (Venable and Coggeshall, 1965). The samples were examined in electron microscope Philips EM 300 at 80 kV.

Results

Ultrastructural changes after influenza virus infection are characterized by changes of nucleoli and formation of cytoplasmic inclusions represented by virus coded nonstructural protein NS1. GPT cells have been found highly sensitive to influenza A/WSN (H1N1) infection. During virus replication the ultrastructural changes were much more extensive than in other cells studied so far.

Ultrastructural changes in the nuclei of GPT cells

By 24 hr post-infection (p.i.) with influenza virus, the nuclei of GPT cells showed widespread alterations of their morphology. In noninfected cells the

nuclei had an ovoid or elongated shape comprising about 1/3 of the cell volume. Nucleoli revealed an equal distribution of their granular and fibrilar components. The cells revealed the morphologic signs of fibroblasts. At 24 hr the nuclei encountered about 2/3 of cell volume. Frequently the cytoplasm formed a narrow band around the enlarged nuclei. Such nuclei showed a lobular appearance. In their protrusions chromatin condensation and accumulation were seen. Numerous ribosomal structures formed in the nucleus were transported to cytoplasm in areas revealing a peculiar arrangement of nuclear protrusions. The outer leaflet of the nuclear membrane covered with ribosomes folded inside to the nucleoplasm (Fig. 5).

Many nuclei contained fine vacuoles of irregular size in their matrix. These originated probably from the leaflets of nuclear membrane during invagination and after disruption of the membrane continuity (Fig. 3). Nucleolus became the site of the deposition of NS1 virus-coded protein in the form of electron-dense structures. Nonstructural protein accumulated also at the nucleolar margin where it was not surrounded with ribosomal granules (Fig. 1). In small aggregates it spread across nucleoplasm towards the nuclear membrane. Through the nuclear pores NS1 protein reached the cytoplasm (Fig. 4), where together with ribosomes it formed larger aggregates — intracytoplasmic inclusions surrounded with ribosomes. Complexes of NS1 protein and ribosomes were occasionally present at different sites in the cytoplasm, but mainly formed large bulks in the vicinity of the nucleus (Fig. 2).

Ultrastructural changes in the cytoplasm of GPT cells and maturation of virus particles at the plasma membrane.

No virus-coded proteins other than NS1 could be seen by means of standard electron microscopy in the cytoplasm of GPT cells. In addition to above mentioned dense intracytoplasmic inclusions the cytoplasm of these cells occasionally contained concentric channels of rough ER with very few ribosomes on their surface. Nevertheless, the majority of ER was formed by shorter slightly widened channels.

The vesicles of smooth ER and Golgi complex in the cytoplasm were numerous. In GPT cells expressing the endogenous oncovirus, maturation of both viruses showed characteristic polarity. Many influenza virus particles budded from the apical plasma membrane. To these areas vesicles were directed originating from the Golgi complex and from the smooth ER (Fig. 6). After maturation and release of influenza virus particles, the amount of ER and Golgi complex vesicles decreased. Contemporarily, the microtubules aggregated in this area and were situated vertically to the plasma membrane accompanying the release of virus particles (Fig. 7).

Guinea pig oncovirus particles produced in GPT cells matured at lateral sites of the plasma membrane separated from the influenza virus maturation sites. Their maturation and release were followed with the formation of a dense network of microfilaments, from which some were directed to the plasma membrane at the sites of the release of particles to extracellular space (Fig. 8). Less frequently portions of plasma membrane were observed where

influenza virus maturation was in close vicinity to the maturation of oncovirus particles (Fig. 9).

The extensive maturation of both viruses at the cytoplasmic membranes testifies the ability of GPT cells to replicate these two viruses. Influenza virus replication was accompanied with manifest ultrastructural changes in the nucleus as well as in the cytoplasm.

Discussion

GPT cells were permissive for the influenza A/WSN (H1N1) replication. In addition, they produced an endogenous oncovirus which maturation did not interfere with influenza virus replication. Influenza virions were budding from the apical plasma membrane separately from the oncovirus maturation areas. Only rarely these maturation areas occured in a close vicinity to each other.

Structural changes of GPT cells elicited by influenza virus infection were extensive. They encountered the changes described in CEC (Compans and Dimmock, 1969; Čiampor, 1972), in HeLa cells (Caliguiri and Holmes, 1979), in MDCK cells (Alonso *et al.*, 1982), in HEF cells (Anisimová *et al.*, 1974), in MDBK and BHK-21 cells (Shaw and Compans, 1978; Anisimová *et al.*, 1973). Rindler *et al.* (1985) described the polarized transport of virus glycoproteins to the apical and laterobasal membrane of MDCK cells double-infected with ts mutants of vesicular stomatitis and influenza viruses. A similar effect was found in GPT cells infected with influenza virus. Due to the increasing knowledge of the role of cytoskeleton constituants (microtubules, microfilaments) in virus replication one may assume that the polarized delivery of virion components was mediated by cytoskeleton elements. Our results showed the presence of microtubular structures at the sites of influenza virus release, while at the site of oncovirus maturation a microfilamentary network had formed.

The accumulation of NS1 protein is a hallmark of the majority of influenza virus-infected cells. They are rarely seen in L-cells abortively infected with influenza virus A/Hong Kong/68 (Čiampor *et al.*, 1981). In BHK-21 cells infected with fowl plaque virus NS1 protein deposits occur mainly in the nucleus and only occasionally in the cytoplasm (Čiampor, 1974). In GPT cells the accumulation of protein was of considerable extent in the nucleus; it was accompanied with its transfer to the cytoplasm where NS1 protein accumulated. Taylor *et al.* (1970) showed that NS1 protein molecules are first found by radioactive labelling in the ribosomes in cytoplasm and then within 5 min they migrate to the nucleus and accumulate in the nucleolus (Becht, 1971). The role of the latter is not clear from morphological studies, but it seems rather improbable that the nucleolus were the site of a passive accumulation only preceding the migration of NS1 protein to cytoplasm where it forms larger aggregates with ribosomes. Possibly, the nuclear matrix components are involved as well as the structures of cytoskeleton in these processes, and many questions remain to be answered so far.

One may assume that GPT cells expressing an endogenous retrovirus manifested a great variety of morphologic alterations in the course of replication of influenza virus which testify the necessity of further investigation especially of the role of nuclear matrix and cytoplasmic cytoskeleton. The polarized delivery of influenza virus glycoproteins has been confirmed too along with separated maturation of the endogenous oncovirus, the one being associated with aggregation of microtubules while the other with microfilaments. Leavitt *et al.* (1981) showed that haemagglutinin and other influenza virus polypeptides not only became inserted into the cytoplasmic membranes but also bind to cytoskeleton proteins. Križanová *et al.* (1986) pointed at the changes in the organization of cytoskeleton during influenza virus penetration. Studies of the role of cytoskeleton and nuclear matrix during influenza virus replication may give further new hints for future.

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References

Alonso, F. V., Roth, M. G., Melsen, L. R., Srinivas, R. V., and Compans, R. W. (1982): Inhibition of viral glycoprotein transport in MDCK cells pp. 34—37. In Bailey, G. W. (Ed.): Proceedings Electron Microscopy Society of America.

Anisimová, E., Tučková, E., and Vonka, V. (1973): Morphological changes in BHK-21 cells infected with S/N (H3N1) influenza virus. *Arch. ges. Virusforsch.* **43**, 221—234.

Anisimová, E., Tučková, E., and Vonka V. (1974): Penetration of nuclear inclusions into the cytoplasm in influenza-virus infected cells. *Intervirology* **3**, 121—124.

Bächi, T., Gerhard, W., Lindenmann, J., and Mühlethaler, K. (1969): Morphogenesis of influneza A virus in Ehrich ascites tumor cells as revealed by thin-sectioning and freeze-etching. *J. Virol.* **4**, 769—776.

Bächi, T. (1970): Elektronenmikroskopische Untersuchungen über den Infektionszyklus von Influenza A-Viren in Ehrlich-Aszites-Tumor-Zellen. *Path. Microbiol.* **36**, 81—107.

Becht, H. (1971): Cytoplasmic synthesis of an arginine rich nuclear component during infection with an influenza virus. *J. Virol.* **7**, 204—207.

Bohn, W., Rutter, G., Hohenberg, H., Mannweiler, K., and Nobis, P. (1986): Involvement of actin filaments in budding of measles virus: Studies on cytoskeletons of infected cells. *Virology* **149**, 91—106.

Caliguiri, L. A., and Holmes, K. V. (1979): Host-dependent restriction of influenza virus maturation. *Virology* **92**, 15—30.

Cervera, M., Dreyfuss, G., and Penman, S. (1981): Messenger RNA is tranlated when associated with cytoskeletal framework in normal and VSV-infected HeLa cells. *Cell* **23**, 113—120.

Čiampor, F. (1972): Electron microscopy of tissue culture cells infected with myxoviruses. I. Nucleo-cytoplasmic changes in A/WSN influenza virus-infected chick embryo cells. *Acta virol.* **16**, 9—16.

Čiampor, F., and Turčan, P. (1972): Electron microscopy of tissue culture cells infected with myxoviruses. II. Nucleo-cytoplasmic changes in fowl plague virus-infected cells. *Acta virol.* **16**, 177—182.

Čiampor, F. (1974): An electron microscopic study of influenza virus reproductive cycle in chick embryo cells with selective inhibition of host cell macromolecular synthesis. *Acta virol.* **18**, 449—456.

Čiampor, F., Bystrická, M., and Rajčáni, J. (1974): Ultrastructural localization of influenza virus antigens in infected chick embryo cells as revealed by a peroxidase-labelled antibody method. *Arch. ges. Virusforsch.* **46**, 341—352.

Čiampor, F. (1978): Electron microscopy of the influenza virus infected tissue cultures (in Slovak). *Lekárske práce* XV, Nr. 2, Vyd. Veda, SAV, Bratislava, 121 p.

Čiampor, F., Sidorenko, E. V., Taikova, N. V., and Bystrická, M. (1981): Ultrastructural localization by immunoperoxidase techniques of influenza virus antigens in abortive infection of L cells. *Acta virol.* **25**, 381—389.

Compans, R. W., and Dimmock, N. J. (1969): An electron microscopic study of single-cycle infection of chick embryo fibroblasts by influenza virus. *Virology* **39**, 499—515.

Compans, R. W., Dimmock, N. J., and Meier-Ewert, H. (1970): An electron microscopic study of the influenza virus infected cells. In: Barry, R. D., Mahy, B. W. J. (Eds.): *The Biology of Large RNA Viruses.* London—New York, Acad. Press, 87—108.

Compans, R. W. (1973): Influenza virus proteins. II. Association with components of the cytoplasm. *Virology* **51**, 56—70.

Dales, S., and Chardonnet, Y. (1973): Early events in the interaction of adenoviruses with HeLa cells. IV. Association with microtubules and the nuclear pore complex during vectorial movement of the inoculum. *Virology* **56**, 465—483.

Genty, N., and Bussereau, F. (1980): Is cytoskeleton involved in vesicular stomatitis virus reproduction? *Virology* **34**, 777—781.

Höglund, S., and Čiampor, F. (1973): Morphology of A/WSN influenza virus-infected chick embryo cells. *Acta virol.* **19**, 155—157.

Hoyle, L. (1968): The influenza viruses. *Virology Monographs* 4, S. Gard, C. Hallauer, K. F. Meyer (Eds.), Springer Verlag, Wien—New York, 375 pp.

Huismans, H., and Els, H. J. (1979): Characterization of the tubules associated with the replication of three different orbiviruses. *Virology* **92**, 397—406.

Klenk, H. D., Wöllert, W., Rott, R., and Scholtissek, C. (1974): Association of influenza virus proteins with cytoplasmic fractions. *Virology* **57**, 28—41.

Križanová, O., Čiampor, F., Závodská, E., Matis, J., and Stanček, D. (1986): Effect of influenza virus on the interaction of cytoskeleton with cell membranes. *Acta virol.* **30** 273—280.

Leavitt, J., Bushar, G., Mahanty, N., Mayner, R., Kagunaga, T., and Ennis, F. A. (1981): Interaction of the structural polypeptides of influenza virus with the cellular cytoskeleton during productive infection of human fibroblasts. In: D. H. L. Bishop, R. W. Compans (Eds.), The Replication of Negative Strand Viruses, *Developments in Cell Biology*, Vol. 7, 353—362, Elsevier, North-Holland, 990 pp.

Lozatulimowska, M., Michalak, T., and Semkow, R. (1981): Attempts at detection of actomyosin associated with influenza virus. *Acta virol.* **25**, 251—253.

Rinder, M. J., Ivanov, I. E., Plesken, H., and Sabatini, D. D. (1985): Polarized delivery of viral glycoproteins to the apical and basolateral plasma membranes of Madin-Darby canine kidney cells infected with temperature-sensitive viruses. *J. Cell Biol.* **100**, 136—151.

Rodriguez-Boulan, E., Paskiet, K. T., Salas, P. J. I., and Bard, E. (1984): Intracellular transport of influenza virus haemagglutinin to the apical surface of Madin-Darby canine kidney cells. *J. Cell Biol.* **98**, 308—319.

Shaw, M. W., and Compans, R. W. (1978): Isolation and characterization of cytoplasmic inclusions from influenza A virus-infected cells. *J. Virol.* **25**, 605—615.

Svobodová, J., Blaškovič, D., and Stančeková, M. (1977): Long-term cultivation of cells derived from guinea pig tongue. *Acta virol.* **21**, 349.

Taylor, J. M., Hampson, A. W., Layton, J. E., and White, D. O. (1970): The polypeptides of influenza virus. V. An analysis of nuclear accumulation. *Virology* **42**, 744—752.

Venable, J. H., and Coggeshall, R. (1965): A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25**, 407—408.

Wang, E., Cross, R. K., and Choppin, R. W. (1978): Possible roles of microtubules and 10 nm filaments in intracellular movement in cells infected with a paramyxovirus (SV 5). *J. Cell Biol.* **79**, 303a.

Explanations to Figures (Plates LXIII—LXVIII).

GPT cells 24 hr p.i. with influenza virus A/WSN (H1N1) in addition expressing an endogenous oncovirus.

Fig. 1. Enlarged nucleus with a narrow band of cytoplasm and numerous inclusions of NS1 dispersed in nucleoplasm and surrounding the nucleolus; magn. ×22 000.

Fig. 2. Invaginations and protrusions of the nucleus with migration of NS1 to cytoplasm; magn. ×44 000.

Fig. 3. Fine membrane bound vacuoles in the nucleus; magn. ×44 000.

Fig. 4. Detail of nuclear pores showing subsequent stages of NS1 protein transport (arrows); magn. ×34 000.

Fig. 5. Chromatin condensation in the nuclear protrusions, escape of ribosomal granules into cytoplasm; the outer leaflet of nuclear membrane folds into nucleoplasm; magn. ×34 000.

Fig. 6. Apical conus of GPT cell with numerous budding influenza virions; magn. ×34 000.

Fig. 7. Microtubules directed vertically to the plasma membrane at the budding site of influenza virus; magn. ×44 000.

Fig. 8. Microfilaments directed to plasma membrane in the area of oncovirus maturation; magn. ×44 000.

Fig. 9. Budding of influenza virions in the vicinity of oncovirus maturation at the plasma membrane; magn. ×55 100.

N — nucleus; n — nucleolus; Vi — influenza virions; Vr — oncovirus (retrovirus) virions; NS — nonstructural influenza virus-coded protein; r — ribosomes; G — Golgi complex; t — microtubules; f — microfilaments.