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ULTRASTRUCTURAL LOCALIZATION BY IMMUNOPEROXIDASE TECHNIQUES OF INFLUENZA VIRUS ANTIGENS IN ABORTIVE INFECTION OF L CELLS

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Summary. — An abortive infection was induced in L cells by influenza virus A/Hong Kong/68 (H3N2). With the use of antibody and peroxidase-labelled protein A, the localization of virus protein synthesis but not the maturation of virus particles was demonstrated at the ultrastructural level. Five days after inoculation (p.i.), the synthesis of viral haemagglutinin was localized in the region of the rough endoplasmic reticulum; at later intervals p.i., haemagglutinin accumulated in the plasma membranes, where membrane vesicles, containing haemagglutinin in their membranes, were released from the cell surface. The cytoplasmic viral ribonucleoprotein was localized in the region of free cytoplasmic ribosomes and that of the outer sheet of the nuclear membrane. Viral proteins were detected in the cytoplasm and plasma membranes also after 70 and 390 days of passaging of the cells or of their long-term cultivation with regular change of medium.

Key words: influenza virus; abortive infection; persistent infection; immunoperoxidase labelling; electron microscopy

Introduction

Synthesis of virus-specific structural proteins in reproduction of influenza viruses is host cell dependent. The results concerning polypeptide synthesis in various cells infected by various influenza viruses showed that two groups of abortive infection can be distinguished: (*a*) virus particles are formed, but contain inactive haemagglutinin, so that they are noninfectious; and (*b*) virus-specific structures are synthesized, but no virions are assembled (Bosch *et al.*, 1978).

L cells infected with influenza viruses represent a system in which the latter type of abortive infection occurs. Electron microscopy of L cells infected with influenza virus A/Hong Kong/68 (H3N2) (further on A/HK/68) showed that the virus is adsorbed on to, and penetrates into, these cells; that filamentous structures 14—15 nm in diameter, resembling the viral ribonucleoprotein

(RNP), appear in the cytoplasm; but that no budding and maturation of new virions occurs (Sidorenko and Čiampor, 1974). Studies with the Dobson strain of fowl plague virus (FPV) revealed that, as compared with a productive system, the production in L cells of mRNA 7 (coding for M protein synthesis) is low at all stages of the viral reproduction cycle and the production of mRNAs 4, 6 and 7 (coding for the synthesis, of HA, NA and M polypeptides) is lowered at later stages of the reproduction cycle (Israel, 1980).

Studies on the biosynthesis of influenza virus envelope structures in abortive infections (BHK, HeLa and L cells; FPV strain Rostock) revealed the accumulation of haemagglutinin and neuraminidase in the plasma membranes without assembly and maturation of new virions. It appears, therefore, that M protein, which is not formed during an abortive infection, is required just in the process of virus budding and thus also in the formation of virus particles (Lohmeyer *et al.*, 1979).

We were interested not only in the synthesis and localization of the synthesis of virus-specific structural proteins in influenza virus-infected L cells, but also in the persistence of the synthesised virus proteins after long-term passaging of infected L cells or their long-term clutivation with regular change of medium. Long-term persistence of influenza and in animal organisms has been repeatedlv reported (Henle, 1964; Gavrilov *et al.*, 1966; Zuev *et al.*, 1970; Timakov *et al.*, 1971; Wilkinson and Borland, 1977).

Populations of influenza virus A/HK/68 propagated in chick embryos are not homogenous. Subpopulations obtained by gradual elution from DEAE-Sephadex with phosphate buffers containing different molarities of NaCl differed in their structure and functional properties (Sidorenko *et al.*, 1977). Therefore we also compared the whole population of A/HK/68 virus with the subpopulations thus obtained at the ultrastructural level by the immunoperoxidase technique under conditions of acute and persistent abortive infection.

Materials and Methods

Virus. Influenza virus A/Hong Kong/68 (H3N2) was grown in 10 days old chick embryos (1—10 EID_{50}/0.2 ml). Allantoic fluids were clarified by low speed centrifugation. The virus was purified by chromatography on DEAE-Sephadex A50, concentrated by high speed centrifugation and purified by sucrose density gradient (10—55%) centrifugation. The purified virus contained 50 000—100 000 haemagglutinating (HA) units per ml. By stepwise elution of the purified virus from DEAE-Sephadex A50 by phosphate buffers containing 0.1, 0.5 and 1 M NaCl, we obtained three virus subpopulations designated A/HK/68-0.1, A/HK/68-0.5 and A/HK/68-1.0, respectively (Sidorenko *et al.*, 1979).

Infection of L cells. L_{929} mouse fibroblasts, obtained from the Ivanovsly Institute of Virology, Moscow, were grown in medium 199 supplemented with 10% heated bovine serum. After 4 days of growth, the L cells were inoculated with the whole virus population and its three subpopulations at multiplicities of 1 EID_{50} per cell. The whole population (A/HK/68-all.) and the subpopulations (A/HK/68-0.1, A/HK/68-0.5 and A/HK/68-1.0) used for infection had HA titres of 512, 128, 1024 and 256 and infectious titres of 7.9, 6, 8 and 5 log EID_{50}/0.2 ml, respectively. After inoculation, the cells were cultivated for 5, 70 and 390 days, with changes of medium at 8—10-day intervals, or subcultured at the same intervals (50 000 cells per Roux bottle).

Virus-infected cells were harvested at intervals; they were designated L-A/HK/68-all., L-A/HK/68-0.1, L-A/HK/68-0.5 and L-A/HK/68-1.0, respectively.

Antisera. V and S antigens of A/HK/68 virus were prepared according to Lief and Henle (1956). Sera with antibody against S antigen were obtained by immunization of white rates and guinea pigs; the first dose of native virus was administered by introducing one drop into each nostril; further 3 doses of 0.5 ml each were injected intraperitoneally at 5-day intervals. The titres of complement-fixing antibody were from 320—640. Sera with antibody against V antigen were obtained by 4-fold intraperitoneal immunization at weekly intervals with V antigen ($256-512 \times 10^3$ HA units per ml). The antihaemagglutinin titre of these sera was from 2560 to 5120.

Demonstration of virual structures in infected cells. The L-cell cultures were examined at intervals for haemagglutinin in the culture fluids, by haemadsorption, by inoculation of chick embryos with culture fluids or cell homogenates and by indirect immunofluorescence.

Immunoperoxidase electron microscopy. At intervals of 5, 70 and 390 days, the infected L cells were subcultured on therophthalate plates in flat-bottom test-tubes (2×10^5 cells per ml). Four days after seeding, when a continuous monolayer had formed on the plates, the cells were washed several times with phosphate buffered saline and pre-fixed for 5 min at 4 °C with a mixture of 0.05% saponine, 0.05% glutaraldehyde and 1% paraformaldehyde in 0.2 M phosphate buffer, pH 7.3. Then the cells were briefly washed with phosphate buffer and fixed for 45 min at 4 °C with the same solution as used for pre-fixation, but without saponine (Bohn, 1978). The fixed cells were washed for 30 min in phosphate buffer at 4 °C. The washed cells were incubated with antibody against V or S antigen or antibodies against both V and S antigen for 45 min at room temperature. After incubation with serum, the cells were thoroughly washed for 45 min at room temperature in phosphate buffer and incubated with peroxidase-labelled protein A, diluted 1 : 5 before use (Dubois-Dalcq *et al.*, 1977), in the presence of 1% bovine serum albumin for 45 min at room temperature. The cells were then washed again with phosphate buffer for 45 min at room temperature and stained with diamino benzidine according to Graham and Karnovsky (1966) for 10 min at room temperature. The stained cells were washed for 20 min in phosphate buffer and post-fixed with 1% OsO_4 in 0.2 M sodium cacodylate buffer, pH 7.2, for 1 hr at room temperature. Thereafter the cells were dehydrated in a acetone series and embedded into Araldite CY 212 (Serva, Heidelberg). Ultrathin sections cut on an LKB Ultrotome III were stained for 1 hr with a 2% aqueous solution of uranyl acetate and examined in a Philips EM 300 electron microscope at 80 kV.

Uninfected L cells were treated in the same way as infected cells and examined for endogenous peroxidase or nonspecific binding of the peroxidase conjugate on to cell structures.

Results

L cells infected with influenza virus A/HK/68-all. and its three subpopulations synthesized virus-specific proteins (V and S antigen) which were detected by the immunoperoxidase technique at the ultrastructural level. The synthesis of virus-specific proteins was the most marked at 5 days p.i., but they could be detected also after regular passaging of the cells at 70 and 390 days p.i. The localization of the synthesis of V and S antigen in infected L cells, visualized by the immunoperoxidase technique, did not differ from the localization of these antigens in productively infected cells. We observed differences in the distribution of the synthesized antigens — diffusely throughout the cytoplasm or in smaller or greater aggregates — in those regions of the cytoplasm that contain organelles responsible for the synthesis and transport of the antigens. The individual A/HK/68 virus populations differed from one another only little, mainly in the mode of distribution of the synthesized virus-specific proteins in the cytoplasm.

Infected and uninfected L cells showed no endogenous peroxidase activity, nor did peroxidase-labelled conjugate-treated infected cells show nonspecific staining.

L cells infected with A/HK/68 virus and its subpopulations were examined by the immunoperoxidase technique in 3 parallel experiments with similar results.

Intracellular localization of virus-specific proteins 5 days p.i.

In L cells infected with A/HK/68-all. virus and its three subpopulations, S antigen was present at 5 days p.i. almost in the whole cytoplasm in the area of endoplasmic reticulum (Er) and free cytoplasmic ribosomes as well as in the region of the outer sheet of the nuclear membrane. At this interval, V antigen occurred in the region of rough and smooth Er and as short fragments also in the plasma membrane.

The most marked was the diffusely distributed product of the peroxidase reaction in L-A/HK/68-0.5 cells treated with antiserum to S antigen. This product was localized mainly on the slightly dilated Er membranes and free cytoplasmic ribosomes. It represented cytoplasmic S antigen and RNP transported from the cell nucleus (Fig. 1). The immunoperoxidase complex does not penetrate into the nuclei of infected cells so that any relationship between nuclear and cytoplasmic RNP of influenza virus could not be investigated by the method used.

In L-A/HK/68-0.1 cells treated with antibodies against S and V antigen, positive peroxidase reactions occurred in small areas of the plasma membrane and on the ribosomes of rough Er, or formed small agregates adjacent to the nucleus (Fig. 2).

Intracellular localization of virus-specific proteins 70 days p.i.

In L cells regularly subpassaged for 70 days p.i. with A/HK/68-all. virus and its subpopulations, V antigen was distributed almost on the whole plasma membrane. The S antigen persisted in the cytoplasm in smaller or greater aggregates in the region of free cytoplasmic ribosomes and Er membranes. The characteristic localization of S-antigen aggregates in the cytoplasm was observed mainly in L cells infected with the three virus subpopulations, while in L-A/HK/68-all. cells the S antigen was diffusely distributed in the cytoplasm close to the nuclear membrane, the localization of V antigen on the plasma membrane having been less marked.

At 70 days p.i., in L-A/HK/68-0.1 cells treated with antibody against V antigen, the immunoperoxidase reaction product was bound to the plasma membrane (Fig. 3). In the same cells treated in parallel with antibody to S antigen, the reaction product formed large aggregates localized mainly in the vicinity of the nucleus (Fig. 4).

In L-A/HK/68-0.5 cells treated with antibody against V antigen, the peroxidase reaction product occurred both at the plasma membrane and on the Er membranes in the cytoplasm (Fig. 5). In the same cells treated with antiserum to S antigen, small aggregates of the reaction product were

observed close to the nucleus and in places of accumulations of free cytoplasmic ribosomes also under the plasma membrane (Fig. 6).

In the cytoplasm of L-A/HK/68-1.0 cells treated with antibody against S antigen, a positive reaction was shown by aggregates in the region of free cytoplasmic ribosomes and rough Er membranes (Fig. 7).

In L-A/HK/68-all. cells treated with antibody against V antigen, a positive reaction product occurred mainly on the membranes of rough Er and on the outer sheets of the nuclear membranes, the labelling of the plasma membranes having been hardly obvious (Fig. 8). In the same cells treated with antibody against S antigen, a positive reaction produst was localized mainly in the region of free cytoplasmic ribosomes in the vicinity of the nuclear membrane (Fig. 9)

Intracellular localization of virus-specific proteins 390 days p.i.

At this interval, exocytosis could be observed on the infected cells. Membrane vesicles containing V antigen within their membranes were released from the cell surface. No budding of mature virions was seen, but exocytosis induced the formation of a certain kind of extracellular pleomorphic particles which, in view of the presence of V antigen in their membranes, were responsible for haemagglutinating and neuraminidase activities occurring in the abortively infected cells at late intervals p.i. No deposits of influenza virus RNP were found in the V antigen-containing released vesicles. The latter were devoid of an internal structure and appeared electron-transparent.

L-A/HK/68-0.1 cells treated with antibodies against V antigen yielded a positive immunoperoxidase reaction on the plasma membrane and on the budding and released membrane vesicles (Fig. 10).

The immunoperoxidase reaction was more marked in L-A/HK/68-0.5 cells treated with antibodies against V and S antigen. The positive reaction was localized in the region adjacent to the nuclear membrane, in membranes of cytoplasmic vesicles and Er, in the plasma membrane and membrane vesicles released from the cell surface (Figs 11 and 12).

In L-A/HK/68-all. and L-A/HK/68-1.0 cells treated with antibody to S antigen, the positive reaction product was localized on the membranes of cytoplasmic vesicles and Er membranes (Figs 13 and 14).

Discussion

The present results showed that V and S antigens of influenza virus in abortively infected L cells were synthesized in regions of the cytoplasm in which the synthesis of these antigens occurs also in productively infected cells. These regions are those of the Er, the outer sheet of the nuclear membrane, smooth Er membranes and free cytoplasmic ribosomes, the sites of V antigen accumulation being the plasma membranes (Compans, 1973; Čiampor *et al.*, 1974; Klenk *et al.*, 1974; Čiampor, 1978).

Lohmeyer *et al.* (1979) showed that the majority of influenza virus proteins in abortively infected cells are synthesized in amounts similar to those in

productively infected cells. They observed, however, that the synthesis of M protein was significantly reduced in abortive infection.

These results were confirmed by immunoperoxidase electron microscopy. Haemagglutinin is synthesized like in productive cells (Čiampor *et al.*, 1974; Čiampor, 1978) in the region of rough Er diffusely throughout the cytoplasm and, after transport through smooth Er membranes, is accumulated in the plasma membranes. There is no budding of mature virus particles from the cell surface, but a certain kind of shedding of plasma membrane vesicles containing V antigen of influenza virus in their membranes.

Cytoplasmic RNP of influenza virus, localized in the region of free cytoplasmic ribosomes and in the region of the outer sheet of the nuclear membrane, is not accumulated in these membrane vesicles shed from the cell surface. The morphological findings confirmed the fact that due to insufficient synthesis of M protein in influenza virus-infected L cells assembly of complete virions cannot take place and the regulation of processes connected with the formation of complete infectious virus particles is unsatisfactory.

Another situation was observed in abortive FPV infection of BHK-21 cells, where assembly of virus particles on the cell surface with typical budding of virions had occurred, but this process was discontinued and no virions were released from the cell surface into the intercellular space (Čiampor and Turčan, 1972). It appears, therefore, that the effects of insufficient synthesis of influenza virus M protein in abortively infected cells on the assembly and maturation of virus particles differ in different cultured cells.

Long-term persistence of influenza virus in man, animals and cell cultures under natural and experimental conditions has been reported (Franklin and Breitenfeld, 1959; Fraser, 1967; Kornyushenko *et all.*, 1968; Wilkinson and Borland, 1972; Zuev, 1973). In our experiments we demonstrated that the synthesized viral antigens can persist for a long time also in abortively infected cells. Long-term passaging of the latter showed that the synthesized viral proteins persisted in these cells in spite of that the diffuse localization of V-antigen synthesis in the region of rough Er was no more observed; instead, aggregates of virus proteins were formed at one or more sites in the cytoplasm. We carried out no quantitative assay of viral proteins in the abortively infected cells at various intervals p.i., but the possibility cannot be excluded that synthesis of viral proteins proceeded continuously, although at a lower level, and no mature virions were released.

Electron microscopy of A/HK/68 influenza virus-infected L cells showed that no intracytoplasmic inclusions, characterizing the formation of virus-coded nonstructural (NS) protein in productively infected cells, were formed. Synthesis of NS protein under conditions of abortive FPV infection was reported (Lohmeyer *et al.*, 1979). But even in productive infection systems FPV does not induce the formation of morphologically clear-cut intracytoplasmic inclusions, as distinct from productive infection of chick embryo cells with influenza virus A/WSN (H0N1) (Čiampor, 1972).

It appears, therefore, that abortive infection of cells with influenza viruses does not represent a uniform model, but that it depends first of all on cells which affect the reproduction and synthesis of influenza viruses at various steps of the reproduction and maturation process. This has been repeatedly confirmed (Bosch *et al.*, 1978; Bukrinskaya *et al.*, 1978; Caliguiri and Gerstein, 1978; Valcavi *et al.*, 1978).

Our experiments showed that the intensity of immunoperoxidase reaction was higher in L cells infected with the individual virus subpopulations than with the whole A/HK/68-all. population, which can be ascribed to a more intensive synthesis of influenza virus protein (Sidorenko *et al.*, 1980). It is possible that the whole virus population contain in addition to complete infectious also defective virions. Following inoculation, interference would thus result, leading to lowered reproduction of individual structural components of influenza virus. By contrast, the individual subpopulations contain virions of approxinately the same morphology and infectivity, which becomes manifested by an enhanced synthesis of structural virus proteins on the cellular structures. But this problem requires further studies under conditions of both productive and abortive infection.

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Explanations of Electron Micrographs (Plates XLIX–LV):

Fig. 1. L-A/HK/68-0.5 cell, 5 days p.i., treated with antibody to S antigen. Diffuse positive product of the peroxidase reaction in the region of free cytoplasmic ribosomes, on the outer sheet of the nuclear membrane and on rough Er membranes. ×11 200.

Fig. 2. L-A/HK/68-0.1 cell, 5 days p.i., treated with antibodies to S and V antigens. Positive product of the peroxidase reaction in small areas of the plasma membrane and in the region of free cytoplasmic ribosomes close to the nuclear membrane. ×25 000.

Fig. 3. L-A/HK/68–0.1 cell, 70 days p.i., treated with antibody to V antigen. Positive product of the peroxidase reaction in the area of the plasma membrane. ×20 000.

Fig. 4. L-A/HK/68-0.1 cell, 70 days p.i., treated with antibody to S antigen. Positive product of the peroxidase reaction in the region of free cytoplasmic ribosomes close to the nucleus. ×40 000.

Fig. 5. L-A/HK/68-0.5 cell, 70 days p.i., treated with antibody to V antigen. Positive product of the peroxidase reaction in the area of the surface plasma and on the smooth vesicles in the cytoplasm. × 32 000.

Fig. 6. L-A/HK/68-0.5 cell, 70 days p.i., treated with antibody to S antigen. Positive product of the peroxidase reaction in small aggregates in the cytoplasm in the region of free cytoplasmic ribosomes. × 25 000.

Fig. 7. L-A/HK/68-1.0 cell, 70 days p.i., treated with antibody to S antigen. Positive product of the peroxidase reaction in the area of free cytoplasmic ribosomes and rough Er. × 40 000.

Fig. 8. L-A/HK/68-all. cell, 70 days p.i., treated with antibody to V antigen. Positive product of the peroxidase reaction in the region of the outer sheet of the nuclear membrane and on ribosomes of dilated Er. Labelling of plasma membrane hardly visible. × 40 000.

Fig. 9. L-A/HK/68-all. cell, 70 days p.i., treated with antibody to S antigen. Positive product of the peroxidase reaction in the region of free cytoplasmic ribosomes close to the nucleus. × 32 000.

Fig. 10. L-A/HK/68-0.1 cell, 390 days p.i., treated with antibody to V antigen. Positive product of the peroxidase reaction in the area of the plasma membrane and in membranes of vesicles released from the cell surface. × 30 000.

Fig. 11. L-A/HK/68-0.5 cell, 390 days p.i., treated with antibodies to S and V antigen. Positive product of the peroxidase reaction on the outer sheet of the nuclear membrane, on dilated membranes of Er and on the plasma membranes with released membranes vesicles. × 20 000.

Fig. 12. L-A/HK/68-0.5 cell, 390 days p.i., treated with antibody to V antigen. Positive product of the peroxidase reaction on the plasma membrane with released membrane vesicles. × 40 000.

Fig. 13. L-A/HK/68-1.0 cell, 390 days p.i., treated with antibody to S antigen. Positive product of the peroxidase reaction in the region of smooth membrane vesicles. × 40 000.

Fig. 14. L-A/HK/68-all. cell, 390 days p.i., treated with antibody to S antigen. Positive product of the peroxidase reaction in the region of cytoplasmic membrane structures. × 32 000.

N — nucleus; n — nucleolus.