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ROLE OF THE HETEROGENEITY OF A/HONG KONG/1/68 (H3N2) INFLUENZA VIRUS POPULATIONS IN ESTABLISHMENT OF PERSISTENT INFECTION OF L CELLS

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Summary. — Three subpopulations of A/Hong Kong/1/68 (H3N2) influenza virus differing from one another in biological properties obtained by elution from DEAE-Sephadex with phosphate buffer containing increasing concentrations (0.1, 0.5 and 1 mol/l) of NaCl, were used to induce persistent infection of L_{929} mouse fibroblast cells. In the course of 25 passages, cell destruction occurred only at low passage levels, especially in the $L_{A-68/0.5}$ and $L_{A-68/all}$ sublines. The proliferating activities of $L_{A-68/0.1}$ and $L_{A-68/1.0}$ were higher than those of the other two sublines. The size of cell nuclei in all infected sublines was increased. Influenza virus antigen was demonstrated by immunofluorescence in cells of all sublines. The virus was recovered irregularly and only at some passage levels: after 2, 2 and 5 chick embryo passages from the $L_{A-68/0.5}$, $L_{A-68/0.1}$ and $L_{A-68/1.0}$ cells, respectively. The viruses recovered from the $L_{A-68/1.0}$ subline possessed the lowest haemagglutinating activity against various animal erythrocytes.

Key words: *influenza virus*; *population heterogeneity*; *persistent infection*; *L cells*

Introduction

The establishment of latent and persistent infection of cell cultures with influenza virus depends on the kind of cells (permissive or nonpermissive) and on the properties of the virus population. Defective virus particles may play a certain role in this process (Huang and Baltimore, 1970; Prokudina-Kantorovich *et al.*, 1975; Timyakov and Zuev, 1976; Tsilinsky and Lvov, 1977). Persistent infections were induced with allantoic fluids containing standard virus (Henle, 1964; Gavrilov *et al.*, 1969, 1970; Zuev *et al.*, 1971; Timakov *et al.*, 1972) or infectious allantoic fluids enriched with defective particles obtained according to von Magnus (1954). Denisov *et al.* (1975) found that either a productive or latent infection of L cells may be induced, depending on the prevailing type of particle in the virus populations.

We separated an A (H3N2) influenza virus by ion exchange chromatography into three subpopulations differing from one another in their biological

properties (Kornyushenko *et al.*, 1972). The structurally-functional properties of certain subpopulations led to the establishment of latent infection in animals, from which the virus could have been reisolated up to 180 days after inoculation (Sidorenko *et al.*, 1977). In the present work we studied the properties of the virus subpopulations in cell cultures in vitro.

Materials and Methods

Viruses. We used influenza virus A/Hong Kong/1/68 (H3N2) passed in 10-day chick embryos (A-68/all.) and its three subpopulations (A-68/0.1, A-68/0.5 and A-68/1.0) obtained by elution from DEAE-Sephadex A-50 with 0.067 mol/l phosphate buffer containing 0.1, 0.5 and 1.0 mol/l NaCl, respectively (Taikova *et al.*, 1971). The haemagglutinin and infectious titres of the A-68/all., A-68/0.1, A-68/0.5 and A-68/1.0 viruses were 1024, 64, 1024 and 256 and 7.9, 6, 8 and 5 log EID_{50}/ /0.2 ml, respectively.

Cell cultures. L_{929} mouse fibroblasts, obtained from the D. I. Ivanovsky Institute of Virology, U.S.S.R. Academy of Medical Sciences, Moscow, were grown in 50-ml bottles in medium 199 supplemented with 10% heated bovine serum. After 4 days of growth, the cell culltures were inoculated with A-68/all. virus and its three subpopulations at a multiplicity of 1 EID_{50} per cell. After an adsorption period of 30 min, the cells were twice washed with Hanks' solution and supplied with fresh growth medium. Starting with 10 days after inoculation (p. i.), the cells were subpassaged at intervals of 8-10 days. For subculturing, the cells were scraped off from the glass and used at a concentration of 10^5 cells per ml. From 3 to 7 days after subculturing, the cells grown on coverslips were fixed in Bouin's solution and stained with Mayer's haematoxylin and eosin. We determined the cell morpohology, the size of nuclei (Khesin, 1967), the mitotic activity and the degree of cell proliferation based on the proliferation index (McAllister and Coriell, 1956).

Detection of virus in cell cultures. Both the culture fluids and cell homogenates were assayed for virus by haemagglutination (HA), haemadsorption and complement-fixation (CF) tests as well as by inoculation of 10-day chick embryos (up to 5 chick embryo passages were carried out). The cells were also examined by direct immunofluorescence with labelled antiserum to A (H3N2) influenza virus. To abolish nonspecific fluorescence, the serum was absorbed with activated charcoal and L_{929}-cell powder. The cells grown on coverslips were fixed in ethanol-picric acid and stained in a humid chamber for 30 min at 37 °C. The specificity controls included uninfected cells stained with the same serum; and cells infected with the influenza virus subpopulations, treated first with immune serum against A (H3N2) virus and then with labelled specific antiserum. The isolated viruses were typed with type antigens and antisera produced in our laboratory or with reference preparations obtained from the Pasteur Institute of Sera and Vaccines, U.S.S.R. Ministry of Public Health. Before used in haemagglutination inhibition (HI) tests, the sera were treated with potassium periodate to remove nonspecific inhibitors. For electron microscopy, the isolated viruses were contrasted with a 1% aqueous solution of phosphotungstic acid, pH 7.2, and examined in a JEM-100B electron microscope at an instrumental magnification of × 50 000.

Results

On inoculation of L cells with the original virus population and its 3 subpopulations and in the course of subpassages we observed certain differences in the growth characteristics, cytopathic changes and behaviour of the virus.

In L cells infected with A-68/all. virus ($L_{A-68/all.}$ cells), as early as 12—24 hr p. i., but especially 3-5 days p. i., there occurred a cytopathic effect (CPE): the cells in certain areas of the monolayer were destroyed. Subsequently, foci of cell proliferation appeared and cell multilayers developed in these areas. Similar destructive changes, also followed by the appearance of multilayers, appeared in L cells infected with A-68/0.5 virus (Figs 1—3). Cell destruction was less marked in $L_{A-68/1.0}$ and $L_{A-68/0.1}$ cells. At the 6th—9th

day p. i., the cultures consisted of sheets of cells with granular cytoplasm, each containing from 2 to 9 eosinophilic inclusions.

Starting with 2 days p. i., all cell sublines showed hypertrophy of nuclei and nucleoli, disturbances in cell division and appearance of multilayer cell growth. The differences between the influenza virus subpopulations were manifested in different functional reactions of the cells which themselves consisted of disintegrative swelling of nuclei of different intensity. The nuclei were the most enlarged by day 2—3 p. i. in $L_{A-68/0.5}$ cells, by day 6 in $L_{A-68/0.1}$ cells and by day 8 in $L_{A-68/1.0}$ cells. By day 9 p. i., the nuclei in control and infected cultures had essentially the same size. Some differences in the proportion of modal class nuclei continued to persist; they were the most pronounced in the $L_{A-68/1.0}$ subline.

The infected cells showed a different character of the foci of cell proliferation and differences between the cell sublines could also be established based on the mitotic activity. On day 2, the mitotic activities were the highest in $L_{A-68/1.0}$ and $L_{A-68/0.1}$ cells (3.4 and 2.7 per cent, respectively) as compared with $L_{A-68/0.5}$, $L_{A-68/all.}$ and control L cells (1.8, 1.6 and 2.2 per cent, respectively). At later intervals, the mitotic activities of infected cells decreased but remained higher than in the uninfected control. On the 5th-7th day, the mitotic index was in $L_{A-68/0.1}$ and $L_{A-68/1.0}$ cells considerably (2-—3-fold) higher than in $L_{A-68/0.5}$ and $L_{A-68/all.}$ cells. On the 9th day, the mitotic activities were 1.7, 2.1, 0.8—0.9, 1.2 and 0.6 per cent in $L_{A-68/0.1}$, $L_{A-68/1.0}$, $L_{A-68/0.5}$, $L_{A-68/all.}$ and control L cells, respectively. The considerably lower mitotic activity of control L cells on the 5th day is a characteristic feature of the cell growth under usual conditions when the medium is not changed. The proliferative activity of the infected cultures was higher than that of the control, having been the highest in the $L_{A-68/1.0}$ subline.

Differences in the character of growth and cell morphology were also observed after 1—4 passages. Destructive changes were the most marked in the $L_{A-68/all.}$, $L_{A-68/0.5}$ and partially also in the $L_{A-68/0.1}$ subline. These cells, usually mononucleate, were rounded, heavily vacuolated and contained eosinophilic and basophilic inclusions. The monolayers of $L_{A-68/1.0}$ cells showed less destructive changes; multinucleate cells were frequent. In this period, the cell growth was very poor, the highest indices were found in $L_{A-68/0.1}$ and $L_{A-68/1.0}$ cells. In all infected sublines, the nuclei were larger than in the control, i. e. there occurred disintegrative swelling of the nuclei which was also observed in subsequent passages.

At passages 5—7, the sublines showed no manifest morphological changes. The mitotic and proliferative activities of $L_{A-68/0.1}$ and $L_{A-68/1.0}$ were higher than of the other sublines. In subsequent passages, the destructive cell changes disappeared, but the differences in proliferative activities were preserved. In the 11th-14th passage, the proliferative activities were the highest in the $L_{A-68/0.1}$ and $L_{A-68/1.0}$ sublines. The indices of proliferative activity in these sublines in the course of 16 passages were by 20% higher than in the other sublines and control L cells.

Influenza virus antigen was detected by various methods after inoculation

of L cells with the original virus population and its subpopulations. Haemadsorption was positive in all cell sublines from the 4th to the 9th day p. i. HA activity in the culture fluids was only detected in the $L_{A-68/all.}$ and $L_{A-68/1.0}$ sublines. Influenza antigen was demonstrated by immunofluorescence in all sublines in the form of single fluorescent granules in the perinuclear zone.

In the course of passaging of the sublines, HA activity in the culture fluids was never demonstrated. Haemadsorption was positive only in sublines $L_{A-68/0.5}$ (passages 2 and 3) and $L_{A-68/all.}$ (passages 1—7). No infectious virus was demonstrated by inoculation of chick embryos with the culture fluids, with the exception of the $L_{A-68/all.}$ subline in passages 1—4. Immunofluorescence revealed viral antigen in all passages of all sublines in the form of small granules or aggregates in the perinuclear zone; fluorescence was also displayed by the nucleoplasm around the nucleoli, as was diffuse fluorescence by isolated areas of the cytoplasm (Figs 4—8). The CF test with cell homogenates was positive in all sublines at all passage levels, the titres ranging from 2 to 16. Virus was isolated from the cell homogenates by inoculation of chick embryos only at some passages of the sublines. In the $L_{A-68/0.1}$ and $L_{A-68/0.5}$ sublines the virus was detected only after repeated passaging in chick embryos, in the $L_{A-68/1.0}$ subline this was achieved only after 4—5 blind passages. Persistence of virus was demonstrated in the sublines after up to 25 passages, i. e. throughout the whole experiment. The viruses recovered after 5—10 passages of the sublines were able to agglutinate chicken, guinea pig, mouse and rat erythrocytes, with a decreasing tendency in viruses recovered from the $L_{A-68/0.1}$ to the $L_{A-68/1.0}$ subline (Table 1).

All viruses recovered from the subline passages were identified as the A/Hong Kong/1/68 strain in HI tests with reference antisera to various types of influenza virus (A/PR8; A1/Pan; A2/Hong Kong/1/68; and B). Electron microscopy of the isolated viruses revealed thread-like particles from 2—5 μm long, showing swellings and branching, that resembled particles observed after long-term passaging in animals with a low susceptibility to influenza virus.

Discussion

An allantoic culture of influenza virus A/Hong Kong/1/68 (H3N2) was separated into three subpopulations by elution from DEAE-Sephadex A-50

Table 1. HA activities of influenza viruses after prolonged passaging in L-cell sublines

Virus	HA titre with erythrocytes			
	chick	guinea pig	mouse	rat
A/Hong Kong/1/68, original	1024	512	No HA	No HA
$L_{A-68/0-1}$, 10th passage	64	4	128	1024
$L_{A-68/0-5}$, 10th passage	4	4	32	256
$L_{A-68/1-0}$, 10th passage	2	2	16	128

with phosphate buffer containing increasing concentrations (0.1, 0.5 and 1.0 mol/l) of NaCl (Taikova *et al.*, 1971). The eluates (subpopulations) had HA and infectious titres different from those of the original allantoic virus and manifested a different degreee of pathogenicity for mice (Sidorenko *et al.*, 1977).

The character of infection of stable murine L_{929} cells, which represent a nonpermissive system for influenza virus, varied depending on the virus subpopulation used: from a manifest to an absent CPE and by various degrees of proliferative and mitotic activities as compared with control unifected cells. The replication in L cells of the individual virus subpopulations was proved by immunofluorescence detection of viral antigen in the cells and reisolation of the virus from persistently infected cells in chick embryos. Virus in individual passages behaved differently: in some passages reisolation was easy, in others 5 blind passages were necessary. An indirect evidence of viral infection was the hypertrophy of nuclei, and increased mitotic and proliferative activities of the cells. There was no evident regularity of the changes as compared with the controls and the changes could not be definitely related with any of the virus subpopulations. A certain role in this respect could have been played by the host L cells themselves, which represent morphologically heterogeneous transformed cells (see also Fig. 1).

The $A_{-68/0.5}$ virus subpopulation behaved in L cells as the original allantoic virus, i. e. it produced a CPE and stimulated proliferation of the cells. The $A_{-68}/1.0$ subpopulation behaved as an inoculum containing defective particles and had a high proliferative activity. This virus could be reisolated only with difficulty, i. e. only after 5 blind passages of a homogenate of $L_{A-68/1.0}$ cells.

The demonstration of viral antigen for 25 consecutive passages of L cells may be considered evidence of a persistent infection of L cells with influenza virus. Reisolation of virus from the cell cultures confirmed that the replication cycle of virus was complete. An incomplete cycle, i. e. the synthesis of only some structural virus proteins, would be detectable by immunofluorescence without the possibility of recovering infectious virus from the cell culture.

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Explanation of Micrographs (Plates XIV—XVII):

Fig. 1. Uninfected L cells (control).
Figs 2 and 3. $L_{A-68/0,5}$ cells, 10 days p. i.; zones of monolayer (*2*) and multilayer (*3*) cell growth. Figs 1—3: hematoxylin and eosin, × 320.
Figs 4—8. Localization of influenza antigen in various L-cell sublines. × 1350.
Left: direct immunofluorescence; right: phase contrast.
4 — $L_{A-68/0,1}$ subline, 10th passage.
5 and *6* — $L_{A-68/0,5}$ subline, 2nd (*5*) and 10th (*6*) passage.
7 and *8* — $L_{A-68/1,0}$ subline, 2nd (*7*) and 10th (*8*) passage.