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CHARACTERIZATION OF THE REPRODUCTION OF INFLUENZA A EPIDEMIC VIRUSES IN CELL CULTURES

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Summary. — Two influenza A epidemic viruses with different indices of virulence for humans have been compared with respect to their reproduction in human embryo kidney (HEK), human embryo lung (HEL), and chick embryo kidney (CEK) cell cultures. It has been shown that the highly virulent for humans A/Victoria/35/72 (H3N2) strain reproduced intensively in HEK and HEL cells irrespective of the inoculated dose (multiplicity of infection = 1 EID_{50} per cell and of 0.001 EID_{50} per cell, respectively). Efficient infection of a moderately virulent virus A/Bangkok/1/79 (H3N2) was registered in these cell cultures only after addition of trypsin to the maintenance medium. The viruses tested exhibited essentially no difference as to the intensity of their reproduction in CEK cell culture whose sensitivity remained unchanged after addition of trypsin to the maintenance medium.

Key words: influenza virus; virulence; reproduction in cell culture

Introduction

Bosh *et al.* (1979) observed a distinct correlation between in vitro pathogenicity of fowl influenza viruses and their ability to yield virus with cleaved haemagglutinin in a wide variety of homologous cell cultures. The purpose of this paper was to study the reproducibility of influenza A viruses with different degree of virulence for humans in cell cultures of human origin. These studies have been carried out with low-passaged epidemic strains of influenza virus A/Victoria/35/72 and A/Bangkok/1/79 of different reactogenicity for humans which was 80% and 30%, respectively (Smorodintsev and Zhilova, 1978; Zhilova *et al.*, 1979).

Materials and Methods

Viruses. Two reference influenza virus epidemic strains have been used: A/Victoria/35/72 and A/Bangkok/1/79 which underwent 5 or 6 and 4 or 5 passages, respectively, in developing chick embryos at 32 °C.

Cell cultures. HEK, HEL and CEK cell cultures have been prepared using conventional techniques. Cells were grown in Eagle's medium supplemented with 10% bovine serum in penicillin

flasks at concentration of 750,000 cells per 1 ml for CEK and HEK cultures and 1 million per 1 ml for HEL culture. By day 2 in culture, fresh growth medium was added. As a rule, continuous monolayer formation was observed after 72 hr incubation (at 37 °C) of CEK cells and/or after 96 hr for HEK and HEL cells.

The course of virus reproduction in HEK, HEL and CEK cells. After removal of the growth medium, HEK, HEL, and CEK cell monolayers were washed three times with large vol of Dulbecco buffer (B) pH 7.2 stored at room temperature. Then 0.1 ml of the tested virus-containing material at multiplicity of infection (MOI) of 1 EID_{50} and/or 0.001 EID_{50} per cell was introduced into 25 ml penicillin flasks. The MOI expressed in EID_{50}/cell was determined according to the formula: $MOI = \frac{T \times V}{N}$, where T was the virus titre (EID_{50}), V the volume of the introduced virus containing material (ml), N the number of cells. Virus dilutions were prepared in maintenance medium (MM) consisting of equal portions of medium 199 and Earle's solution. The infected cells were incubated for one hr at room temperature and then washed once with large vol of buffer B. To remove the non-penetrated virus, infected cells were treated with specific anti-influenza serum, containing 16 to 32 antihaemagglutinin antibody units which completely inhibited the infectious virus. After 30 min absorption at 37 °C, the cells were washed 3 times with large vol of buffer B and then MM containing or lacking trypsin (10 μg/ml) was added. By 1, 6, 18, 24, 48, 72, and 96 hr cultivation at 34 °C the monolayer from 2 penicillin flasks was frozen at −20 °C. The cells were disrupted by rapid melting and shaking with beads for 5 min, then centrifuged at 2000 rev/min for 10 min. At various intervals of incubation, the haemagglutinin (HA) in the supernatant was measured and the amount of infectious virus determined by titration of a single batch of 10 to 11-day-old chick embryos. The infectious titres were calculated according to Reed and Muench. The degree of removal of nonadsorbed virus from infected cell cultures was controlled by titration (in developing chick embryo) of the samples collected 1 hr after the addition of MM.

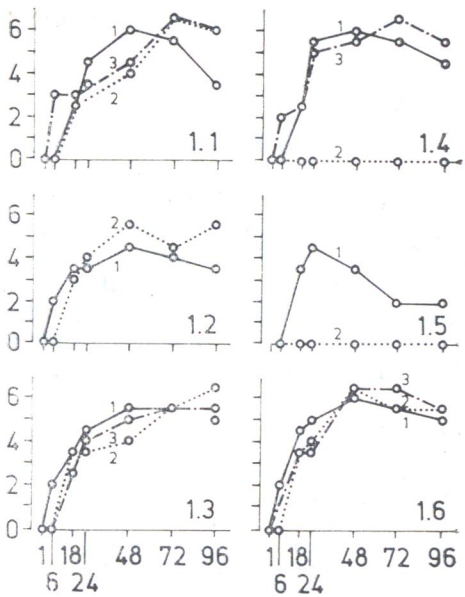

Fig. 1.

Infectious titres of A/Victoria/35/72 and A/Bangkok/1/79 viruses during their reproduction in HEK, HEL and CEK cell cultures

1.1, 1.2, 1.3 and 1.4, 1.5, 1.6 titres of A/Victoria/35/72 and A/Bangkok/1/79 viruses, respectively, during reproduction in HEK, HEL and CEK cells. 1 — reproduction of the viruses at MOI = 1 EID_{50} cell; 2 — reproduction of the viruses at MOI = 0.001 EID_{50}/cell without trypsin; 3 — reproduction of the viruses at MOI = 0.001 EID_{50}/cell after addition of trypsin (10 μg/ml) to MM. Abscissa: time p.i. (hr); ordinate: virus titres (log_{10} EID_{50}/ml)

Results

Characterization of reproduction of A/Victoria/35/72 and A/Bangkok/1/79 viruses in HEK, HEL and CEK cells at high MOI

As shown in Fig. 1 by 18 hr postinfection (p.i.), the A/Victoria /35/72 virus was detected in HEK cells in a titre of 3.2×10^2 EID_{50}/ml. Maximum concentration of the virus (10^6 EID_{50}/ml) was registered after 48 hr. After 92 hr, the virus yield was reduced to 3.2×10^3 EID_{50}/ml. In HEL and CEK cells the reproduction of the A/Victoria/35/72 virus was noted as early as 6 hr p.i. (in a titre 10^2 EID_{50}/ml). The peak of virus reproduction (3.2×10^4 EID_{50}/ml) in HEL cells was observed at 48 hr p.i. At later intervals the amount of infectious virus in HEL cells gradually decreased, e.g. at 96 hr its titre was lower than 3.2×10^3 EID_{50}/ml. In CEK tissue culture the maximum concentration of A/Victoria/35/72 virus (3.2×10^5 EID_{50}/ml) was registered 48 and 72 hr p.i.

With increasing infectious virus titre in the inoculated cell cultures, the concentration of haemagglutinin rised progressively. The HA titre of A/Victoria/35/72 virus has increased from 2—4 HAU (18 hr p.i.) up to 16 HAU (48 to 72 hr p.i.) in HEK and HEL cells or to 64 HAU in CEK cells (Fig. 2), respectively.

The accumulation of A/Bangkok/1/79 virus in HEK, HEL, and CEK cells proceeded generally in a similar way. Thus, for instance, in HEK cells the virus was detected after 18 hr in a titre of 3.2×10^2 EID_{50}/ml, reaching a maximum concentration of 10^6 EID_{50}/ml at 48 p.i. Then the virus yield

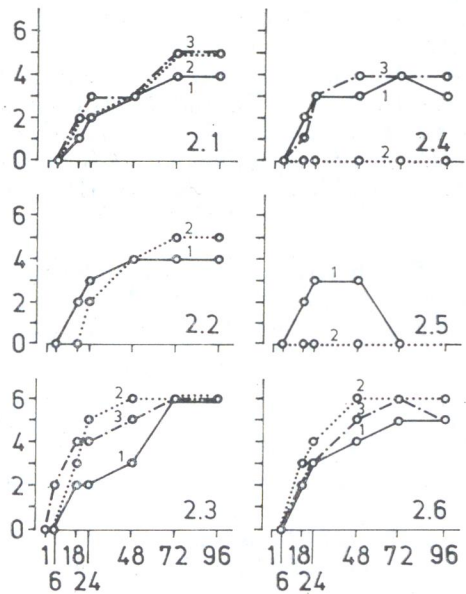

Fig. 2.

Titres of A/Victoria/35/72 and A/Bangkok/1/79 virus haemagglutinins produced in HEK, HEL and CEK cell cultures

2.1, 2.2, 2.3 and 2.4, 2.5, 2.6 titres of A/Victoria/35/72 and A/Bangkok/1/79 virus haemagglutinins, respectively, during reproduction in HEK, HEL and CEK cells. 1 — HA titres at MOI — 1 EID_{50}/cell; 2 — HA titres at MOI = 0.001 EID_{50}/cell without trypsin; 3 — HA titres at MOI = 0.001 EID_{50}/cell after addition of trypsin (10 μg/ml) to MM. Abscissa: time p.i. (hr); ordinate: HA activity (log_2 HAU)

was decreased reaching 3.2×10^4 EID_{50}/ml after 96 hr. In HEL cells A/Bangkok/1/79 virus was first detected after 18 hr (3.2×10^3 EID_{50}/ml), by 24 hr its concentration increased up to 3.2×10^4 and at 72 hr p.i. it decreased noticeably (to 10^2 EID_{50}/ml).

The infection of CEK cells by A/Bangkok/1/79 virus resulted in a more rapid virus accumulation. The virus was first detected at 6 hr p.i. (10^2 EID_{50}/ml) and reached its maximum at 48 hr p.i. (10^6 EID_{50}/ml), which was followed by gradual decrease of its concentration. The amount of A/Bangkok/1/79 virus haemagglutinin progressively increased in all the cell cultures used from 4 HAU after 18 hr up to 8 — 16 HAU and 32 HAU for HEK, HEL and CEK cells, respectively (see Fig. 2).

Thus, at high MOI (1 EID_{50}/cell) the viruses tested had an essentially similar ability to reproduce in HEK, HEL and CEK cells. The time course and intensity of accumulation of the viruses varied depending on the particular cellular system. HEK, and especially CEK cultures, appeared to be the most sensitive.

Characterization of the reproduction of A/Victoria/35/72 and A/Bangkok/1/79 viruses in HEK, HEL and CEK cultures at low MOI

In further experiments the ability of the viruses A/Victoria/35/72 and A/Bangkok/1/79 to reproduce in the same cell cultures at low MOI (0.001) has been studied (Fig. 1). It has been found that CEK cell culture ensured an equally high reproduction of either virus. The viruses were detected in the infected cells at 18 hr p.i. in a titre of 3.2×10^3 EID_{50}/ml. Then the virus yield increased reaching its maximum (3.2×10^6/ml) after 48 and 96 hr for viruses A/Bangkok/1/79 and A/Victoria/35/72, respectively. An increase in the yield was accompanied by a parallel raise of the amount of haemagglutinin from 8 HAU at 18 hr p.i. to 64 HAU at 48 hr p.i. (Fig. 2).

The reproduction in HEK and HEL cells was registered only for A/Victoria/35/72 virus. At 18 hr p.i. the virus titre in HEK and HEL cells was 3.2×10^2 EID_{50}/ml and 10^3 EID_{50}/ml, respectively. The maximum concentrations of the virus (3.2×10^6 or 3.2×10^5 EID_{50}/ml) in HEK or HEL cells were reached after 48 to 72 hr. Meanwhile, in HEK and HEL cells inoculated by A/Bangkok/1/79 strain no production of the infectious virus was observed.

Thus, the intensity of reproduction of both virus strains was independent of MOI in CEK cells. The same was observed in HEK and HEL cells only with the A/Victoria/35/72 strain, whereas A/Bangkok/1/79 strain lost its reproducibility in human cell cultures at a low MOI (0.001).

Effect of trypsin on reproducibility of A/Victoria/35/72 and A/Bangkok/1/79 viruses in HEK, HEL and CEK cells at MOI = 0.001

Addition of trypsin to the maintenance medium failed to produce a significant effect on reproduction of A/Victoria/35/72 virus in HEK or CEK cell cultures. An earlier appearance of the infectious virus (after 6 hr at a titre of 10^3 EID_{50}/ml) turned out to be a specific feature of A/Victoria/35/72

virus behaviour in the presence of trypsin in HEK cells. However, maximum accumulation of this virus was not higher than that without trypsin and amounted to 3.2×10^6 EID_{50}/ml at 72 hr p.i. In CEK cells the production of A/Victoria/35/72 virus was also essentially independent of the presence of trypsin. Maximum concentration of the infectious virus grown in the presence of trypsin was 3.2×10^5 EID_{50}/ml 72 hr p.i. Similar indices were also recorded during the virus growth without trypsin. In contrast to this, addition of trypsin to the maintenance medium stimulated active reproduction of the virus A/Bangkok/1/79 in HEK tissue culture, which was insensitive to the virus at MOI = 0.001 in the absence of the enzyme.

Addition of trypsin failed to change the reproduction of A/Bangkok/1/79 virus in CEK cells. The virus was detected in the cells at 18 hr p.i. in a titre of 3.2×10^3 EID_{50}/ml. The highest indices of the virus reproduction observed at 48 to 72 hr p.i. (3.2×10^6 EID_{50}/ml) were only 3.2 times higher than the concentration of the virus grown without trypsin.

Thus, a distinct correlation exists between the effect of trypsin on the reproduction of influenza virus strains and their ability to reproduce in homologous cell cultures. The enhancing effect of trypsin applied to the A/Bangkok/1/79 virus only, which was insensitive to the proteolysis in HEK and HEL cells.

Discussion

We studied the reproduction in CEK, HEK, and HEL cell cultures of 2 epidemic strains of influenza virus of different virulence for humans. Moreover, we followed the effect of trypsin on the character and extent of virus reproduction. Since the degree of virus reproduction at moderate and low input doses is an important indicator of the sensitivity of cells, we have compared the replication of the tested influenza virus strains at MOI = 1 and MOI = 0.001 EID_{50} per cell.

It has been demonstrated that high MOI (1) led to the accumulation of 3.2×10^4 to 1.0×10^6 EID_{50}/ml of infectious virus in all cell cultures infected with either A/Victoria/35/72 or A/Bangkok/1/79 viruses. The rate and intensity of reproduction of either virus under conditions employed depended on the particular cellular system rather than on the virus strain.

The use of low MOI (0.001 EID_{50} per cell) of the tested virus yielded different results. In this case the viruses behaved differently in the cell cultures and the addition of trypsin to MM changed the character of their reproduction. The highly virulent A/Victoria/35/72 virus was able to reproduce in all the 3 tissue cultures and this ability remained unchanged in the presence of trypsin. The similar result was observed with the less virulent A/Bangkok/1/79 strain in CEK cells. On the other hand, HEK and HEL tissue cultures failed to support the reproduction of A/Bangkok/1/79 virus, while an attempt to grow this virus in the presence of trypsin led to its accumulation in HEK cells.

These results indicate that human cell cultures (HEK and HEL) are not equally sensitive to influenza A viruses of the same serotype (H3N2)

showing non-identical antigenic structure and different degree of virulence for humans. These data are also in agreement with the observations of several investigators (Klenk *et al.*, 1982; Bosch and Von Hongingen-Huence, 1980) indicating to different sensitivity of virulent and avirulent strains to proteolytic cleavage.

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