EFFECT OF CHRONIC INFECTION WITH INFLUENZA VIRUS AO/WSN ON LIFESPAN OF GUINEA PIG EMBRYONIC LUNG CELL CULTURES IN VITRO

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Chronic infection of primary trypsinized cultures of guinea pig embryonic lungs with influenza virus AO/WSN was studied. In the course of 119 days, the infected culture went through nine passages. Control uninfected cultures degenerated on the 17th day after only three passages.

Chronic influenzal infection in vitro has been studied mainly with the use of diploid strains [1-3] and transplanted line RES [4]. Increased proliferative activity and increased duration of survival in vitro of cultures of diploid cells infected with influenza virus AO/PR8 have been demonstrated [1-3]. In some investigations, the appearance of transformed cells of epithelioid type has been observed in chronically infected cultures [3, 10]. Morphological transformation of mouse kidney cell cultures was accompanied by acquisition of neoplastic properties by the cells for the isologous strain of mice [10]. These findings demonstrate that the model of chronic influenzal infection in vitro is suitable for the study of the effect of influenza viruses on the proliferative function of cells and tissues. The importance of such investigations is obvious in the light of data showing the role of influenzal infection in the pathogenesis of chronic diseases of the respiratory organs (bronchitis, emphysema) associated with pathological tissue proliferation [7, 9].

The object of the investigation described below was to study chronically infected cultures of the lungs of guinea pig embryos (GPL_{WSN}) with the aid of several indices including changes in proliferative activity and lifespan in vitro compared with those of control, uninfected cultures, and to observe the possible appearance of transformed cells with the capability of unlimited growth.

EXPERIMENTAL METHOD

A neurovirulent variant of influenza virus type AO/WSN was used in the investigation [8]. Pieces of lungs from guinea pig embryos were trypsinized by the usual method. Into each of a series of 50-ml flasks was added 10 ml of medium No. 199 with 10% bovine serum (seeding dose 3 million cells). For reinoculation of the cell cultures, a mixture consisting of two-thirds of 0.2% versene solution and one-third of 0.25% Difco trypsin solution was used. Isolation and titration of influenza virus from samples of chronically infected cultures were carried out as described previously [1]. The neutralization reaction on chick embryos and the inhibition of hemagglutination test (IHT), which were carried out in the usual manner, were used for serologic identification of the virus. Diagnostic antisera against influenza viruses AO, A1, A2, B, and C (Leningrad Institute of Vaccines and Sera) and rat sera prepared by the writers against influenza viruses AO/WSN and the variants isolated from chronically infected cultures of RES cells [4] and GPL_{WSN} cells, were used for this purpose.

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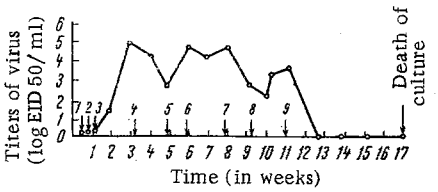

Fig. 1. Dynamics of production of influenza virus AO/WSN and chief parameters of survival of chronically infected GPL_{WSN} cultures. Titers of WSN/GPL virus in culture medium. Arrows indicate days of subculture.

EXPERIMENTAL RESULTS

A layer of GPL cells, washed off into a 50-ml flask, was inoculated with an allantoic culture of influenza virus AO/WSN (multiplicity of inoculation 0.02 EID_{50}/cell). After contact for 1 h at room temperature, the cell layer was washed off by Hanks's solution, introduced into a flask of growth medium, and incubated at 37°. The medium was changed after 48 h, and starting from the 3rd day of incubation, the GPL_{WSN} cultures were reinoculated.

Nine such passages were carried out during 77 days. Before the third passage, the cultures were reinoculated after the formation of a continuous monolayer at intervals of 2-3 days in the ratio of 1 : 2, and later, after the slowing of cell proliferation, at intervals of 10-12 days in the ratio of 1 : 1. In the period from the 36th to the 56th days, a transient increase in proliferative activity of the cells was again observed, and for this reason the sixth subculture (42nd day) was carried out in the ratio of 1 : 2. Throughout the experiment, cytopathic changes were absent in the GPL_{WSN} culture. Subculture of normal GPL cells was carried out in parallel tests (three passages in the course of 17 days). From the 1st to the 9th day in the control culture, active cell proliferation also was observed, so that the subcultures could be made at intervals of 2 days. By the 10th day, however, cell proliferation slowed considerably, and this coincided with the appearance of signs of nonspecific degeneration (granulation of the cytoplasm, rounding and detachment of some cells, formation of defects in the monolayer). On the 17th day, despite a change of medium, the control culture died.

Hence, the total life span of GPL_{WSN} cultures in vitro was longer than that of the control cultures by 112 days.

To study the dynamics of infective virus production, samples of medium of GPL_{WSN} cell cultures were taken (3rd-9th day at intervals of 2-3 days, 10th-119th day at intervals of 10-12 days). In the first three samples (Fig. 1), virus was present only in minimal quantities (hemagglutinins were found in single embryos inoculated with whole culture fluid). From the 10th to the 19th day, the output of virus into the culture medium rose sharply, and on the 19th day the titer of virus in the medium reached $10^{4.7}$ EID_{50}/ml. Approximately the same values were obtained for titers of virus from the 28th to the 56th day, although on the 34th day a marked decrease in output of infective virus was observed (Fig. 1). The subsequent increase in the titers of virus coincided with the period of stimulation of proliferative activity of the cells. Starting from the 63rd day, despite a transient and slight increase in the concentration of virus (samples taken on the 71st and 77th days), a tendency was observed for the titers to fall.

In the period from the 10th to the 77th days, the titers of hemagglutinins in the culture fluid varied between 1 : 160 and 1 : 640.

No virus was detected in any of the samples taken from the 91st to the 119th day, the time of death of the GPL_{WSN} culture. It was accordingly decided to test for its presence in the medium by the method of successive subcultures in chick embryos. However, after four successive subcultures no hemagglutinins could be found in the allantoic fluid.

Virus isolated from medium of the 1st, 3rd, and 9th passages of GPL_{WSN} cultures was identified in the neutralization test and IHT as influenza virus AO. The crossed IHT using the original AO/WSN virus, its variant obtained previously [4], and the variant isolated from the GPL_{WSN} culture on the 77th day, and the corresponding antisera failed to detect any antigenic differences between these viruses.

The results obtained thus indicate that chronic influenzal infection considerably (by 7×) increased the lifespan of cultures of GPL_{WSN} cells compared with the control. It is noteworthy that a transient increase in proliferative activity of cultures of GPL_{WSN} cells (36-56th day) coincided with increased reproduction of virus. On the other hand, after the cultures had lost their ability to maintain reproduction of virus, their proliferative activity declined sharply, and this coincided with rapid development of nonspecific degeneration and death of the cells by the 119th day.

The causes of the gradual increase in titers of influenza virus in the medium in the period from the 3rd to the 19th day are not fully clear. They are perhaps connected with one or several factors: a) selec-

tion of cells more sensitive to AO/WSN virus during subculture of a mixed population of GPL_{WSN} cultures; b) selection of preexisting variants of influenza virus, reproducing more actively in cultures of GPL cells; c) the appearance of a mutant of the virus with increased reproductive activity.

Factors such as the frequency of change of medium, intervals between subcultures, number of cells, and so on also have some effect on the fluctuation in levels of virus in the medium.

It is difficult to say what lay at the base of the rapid and sudden loss in sensitivity of the cultures to virus. Nevertheless, it is obvious that chronic infection of GPL_{WSN} cultures with influenza virus AO/WSN affected some features of metabolism of the cells, determining their ability to proliferate for long periods in vitro.

A similar phenomenon of increased proliferative activity of cultures of chronically infected cells and an increase in their lifespan in vitro as a result of infection with influenza virus AO was observed by Gavrilov et al. [1-3] and Scholtissek [11]. In this connection, it is curious to note that Scholtissek [11] observed an increase in proliferative activity of HeLa cells by 100% after inoculation of cultures with fowl plague virus, belonging to the same antigenic type as group AO influenza virus, inactivated with ethylenimino-quinone. This indicates that the phenomenon described above is connected with particular cistrons of RNA of the virus and does not therefore require integrity of the whole virus genome. It is important to note that Scholtissek concluded from his observations that influenza viruses possess oncogenic properties which, however, are not realized because of the cytopathic activity which is characteristic of these agents.

In the present investigation, chronic infection of the cultures did not cause transformation of the cells. In other systems of cultures chronically infected with AO influenza viruses, the appearance of transformed cells with the capacity for unrestricted growth in vitro was observed [3, 10], while in the experiments of Leuchtenberger et al. [11], the cells acquired neoplastic properties for mice.

The results obtained can help to explain some aspects of the pathogenesis of chronic diseases of the respiratory organs such as chronic bronchitis and emphysema, an essential element in which is pathological cell proliferation [5, 6]. In many clinical virological investigations undertaken in recent years it has been shown that repeated infections caused by myxoviruses, especially influenza viruses, play an important role in the pathogenesis of these diseases [7, 9]. It can be considered that chronic influenzal infection in vitro will provide a model for the subsequent explanation of other important aspects of the pathogenesis of chronic diseases of the respiratory organs.

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